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Cholesterol Lowering Effects of Milk with Added Phytosterols

Laura Kells Cusack

B.S., University of Connecticut, 2010

A Thesis

Submitted in partial fulfillment of the

Requirements of the Degree of Masters of Science

at the

University of Connecticut

2012

APPROVAL PAGE

Master of Science Thesis

Cholesterol Lowering Effects of Milk with Added Phytosterols

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Acknowledgements

I would like to thank Dr. Volek and the other members of my committee Dr. Maresh and Dr. Kraemer as well as all those in the Kinesiology department at UCONN for providing me with the opportunity to learn and grow as a student, person and professional.

List of Tables

Table 1. Food Matrices: The design, methodology, matrix, and LDL-c percent change, as well as, subject and PS characteristics of references that utilized foods with added PS as a cholesterol lowering mono-therapy.

Table 2. The percent range breakdown of the specific PS composition relative to the plant source.

Table 3. The average LDL-c percent reduction from baseline for each plant origin (a) and type of PS (b).

Table 4. Subject baseline characteristics.

Table 5. Baseline nutrient analysis.

Table 6. Average plasma lipid values after each phase.

Table 7. Average Apolipoprotein values after each phase.

Table 8. Average plasma lipid values after each phase for responders only (n=17).

List of Figures

Figure 1. LDL-c % change per gram/day of PS for individual food matrices.

Figure 2. Total LDL-c concentration after PS intervention based on baseline LDL-c concentrations.

Figure 3. Individual LDL-c response from free PS milk to TRP milk. Percent change from the P1 control (2% milk) (n = 20).

Figure 4. LDL-c responses to the milk products. Stratified according to the U.S. Department of Health and human Services LDL-c classifications

Figure 5. Correlation between baseline LDL-c values and the LDL-c response to TRP, (n=20).

Table of Contents

ACKNOWLEDGEMENTS II
LIST OF TABLES III
LIST OF FIGURES IV

ABSTRACT VII

CHAPTER 1 1

INTRODUCTION 1

CHAPTER 2 1

2.0.0 LITERATURE REVIEW 1

2.1.0 INTRODUCTION: 1
2.1.1 MECHANISM: 2
2.2.0 METHODS: 4
2.2.1 LITERATURE SEARCH: 4
2.2.2 SELECTION CRITERIA: 4
2.2.3 DATA ABSTRACTION: 5
2.2.4 DATA CATEGORIZATION: 5
2.3.0 RESULTS AND DISCUSSION: 5
2.3.1 FOOD MATRIX: 6
2.3.1.A MARGARINE: 10
2.3.1.B MAYONNAISE: 12
2.3.1.C DAIRY: YOGURT: 13
2.3.1D DAIRY: MILK: 14
2.3.1.F OTHER: 16
2.3.1.G COMBINATIONS: 18
2.3.2 ORIGIN OF PS: 19
2.3.3 PS STRUCTURE: 21
2.3.4 BASELINE LDL-C AND PS THERAPY: 22
2.4.0 CONCLUSION: 24

CHAPTER 3 24

3.0.0 METHODS 24

3.1.0 EXPERIMENTAL DESIGN 24
3.2.0 SUBJECTS 25
3.3.0 MILK SUPPLEMENT 26
3.4.0 TESTING PROTOCOL AND WEEKLY VISITS 26
3.5.0 ANALYSIS 28
3.6.0 STATISTICS 28

CHAPTER 4 29

4.0.0 RESULTS 29

CHAPTER 5 ..... **34**
5.0.0 DISCUSSION **34**
CHAPTER 6 ..... **37**
6.0.0 CONCLUSION: **37**
REFERENCES **38**
APPENDIX A. INFORMED CONSENT DOCUMENT 44

Abstract

Foods incorporating plant sterols (PS) reduce Low-density lipoprotein (LDL) on average approximately 10%. PS with a higher lipid solubility may promote greater reductions. We examined the cholesterol lowering effect of a novel triglyceride recrystallized phystosterol (TRP). Twenty subjects (mean ± SD; age, 56 ± 10 years; BMI, 27 ± 5) with elevated LDL (>100 mg/dL) participated in three 4-week phases; Phase I, 2% milk; Phase II, milk with 2.0 grams (g) free PS; Phase III, milk with 2.0 g TRP. Before and after each phase two fasting blood draws were obtained for determination of serum cholesterol. Between the 2% and TRP milk phases total, LDL and High-density lipoprotein (HDL) cholesterol changed by -9.0% ($p = .001$), -11.0% ($p = .002$) and -8.0% ($p = .029$) respectively: between the free PS and TRP milk phases total, LDL and HDL cholesterol changed by -3.3% ($p = .136$), -2.3% ($p = 1.00$), -6.4% ($p = .023$) respectively. Apo B concentrations resulted in a decrease of -6.9% ($p = .048$) between the free PS and TRP milk phases and did not significantly change between the 2% and TRP milk phases. Triglycerides and Apo A1 concentrations did not significantly change between any of the phases. In conclusion, TRP can be effectively incorporated into a skim milk resulting in a significant cholesterol lowering effect. (218 words)

Chapter 1

Introduction

Lowering plasma low-density lipoprotein cholesterol (LDL-C) has been shown to lower the risk of cardiovascular events. Typically lifestyle modifications including the consumption of 2.0 grams (g) phytosterol(s)/phytostanol(s) (PS) per day are recommended by the National Cholesterol Education Program Adult Treatment Panel III and health care professionals to facilitate the reduction in LDL-c (1). PS block the intestinal absorption of dietary and biliary cholesterols (2), which result in reductions in total and LDL cholesterol (3) concentrations on average 10%. Conventionally, consuming functional foods incorporating PS are a good way to reach the recommended goal.

Although not all PS reduce LDL-c, and recommendations do not offer further guidance, it is known that soy and wood PS with structural manipulations that increase lipid solubility and incorporation into a food's matrix. Both of these characteristics may promote further reductions in cholesterol.

We examined the cholesterol lowering effect of a novel aided triglyceride recrystallized phystosterol (TRP) in a matrix of skim milk. The primary hypothesis being that, as a mono-therapy, the TRP milk matrix would lower cholesterol more effectively than an unaided free PS milk matrix.

Chapter 2

2.0.0 Literature Review

2.1.0 Introduction:

High plasma cholesterol, predominantly plasma low density lipoprotein-cholesterol (LDL-c), is associated with increased heart disease and stroke, the leading

causes of death in the United States. Therefore, lowering LDL-c is an important health priority for individuals at risk for these conditions. Health care professionals recommend that lifestyle changes (i.e., diet and exercise) be the first line of therapy for cholesterol conscious individuals. To facilitate the lifestyle change process The National Cholesterol Education Program Adult Treatment Panel III recommends a combination diet therapy consisting of low saturated fat (<7%), low to moderate total fat (25-35%), low cholesterol (<200 mg), 10-25 grams of soluble fiber and 2.0 grams of phytosterols/phytostanols (PS) (4). Over the past 60 years a large number of studies have consistently shown that foods with added PS, even as a mono-therapy, safely lower serum total and LDL cholesterol without significantly affecting high density lipoprotein cholesterol (HDL-c) and triglyceride concentrations (5).

Manufacturers have fortified many types of foods with PS providing individuals who are trying to lower their cholesterol the ability to choose foods they prefer (6). Recent reviews on foods with added PS address the incorporation of PS into a non-fat or fat food matrix and whether PS characteristics can modulate their effect, (7, 8). The main purpose of this review is to assess the cholesterol-lowering effect of PS incorporated into specific foods with focus on the fatty acid composition of the food's matrix. In addition, assess the efficiency of PS based on the plant source/specific combination of PS and 3) the PS' structural form, and the subjects' baseline LDL-c concentrations.

2.1.1 Mechanism:

PS lower plasma total and LDL cholesterol through a cycle that starts with the inhibition of dietary and biliary cholesterol absorption in the intestine (3, 9-11). PS displace

cholesterols first in the micelles (12) and second on the Niemann-Pick C1-like 1 transport protein (13, 14). Resulting in less cholesterol transported into the enterocyte and subsequently by the chylomicron (11, 13). All as evidenced by increased cholesterols in the feces (15-17).

The cycle continues with hepatic adaptions, initiated to maintain cholesterol homeostasis in response to the impaired cholesterol absorption. First, enzymatic adaptions replace the bile acid and increase the hepatic cholesterol pools. Cholesterol 7alpha-hydroxylase (CYP7A), the rate-limiting enzyme responsible for bile biosynthesis, is up-regulated in response to a reduced expression of farnesoid x receptor (FXR), a known suppressor of the enzyme (18-21). Concurrently, hepatic 3-hydroxy-3-methylglutaryl-coenzyme A (HMG CoA reductase), the rate-limiting enzyme responsible for cholesterol biosynthesis is also up-regulated (22, 23). Second, to preserve and increase the hepatic cholesterol pool, very low density lipoprotein (VLDL) output is reduced (17, 24, 25), as evidenced by significant decreases in plasma apolipoprotein B (26-29), and hepatic LDL receptors expression is increased (23, 24, 30).

Thus, if PS are consumed the cycle continues; biliary and dietary cholesterol reabsorption/absorption is blocked, and they are discarded in the feces. Plasma concentration of total and LDL cholesterol continue to be reduced as the cholesterol, accumulated in the liver, is continuously shunted to the bile acid pathway. The final outcome of this cycle is a more favorable lipid profile: the plasma total and LDL cholesterol concentration is decreased and high-density lipoprotein cholesterol (HDL-c)

and triglyceride (TG) concentrations are unaffected, leading to a higher HDL-c/LDL-c ratio.

2.2.0 Methods:

2.2.1 Literature search:

To identify studies that examined the effects of PS on plasma cholesterol in humans, PubMed was searched using the search criteria ((Plant sterol*[Title/Abstract]) OR (Plant stanol*[Title/Abstract]) OR phytost*[Title/Abstract]) AND (cholesterol*[Title/Abstract]), and limited to humans, clinical trials, randomized controlled trials published in English. Additional articles were identified in reviews of PS (31).

2.2.2 Selection criteria:

Trials that incorporated PS into foods to create functional foods were identified. A study was eliminated if it: 1) did not describe the source or specific combination of the PS used; 2) did not have a calculated LDL-c change or percentage change; 3) combined dietary modification with foods containing added PS; 4) had subjects with non-lipid disease states (i.e. diabetes); or 5) had >10% of subjects on lipid lowering drugs or agents.

Studies were reported and evaluated as separate strata and labeled with a lower-case letter after the publication year if they reported LDL-c changes for multiple time points, functional foods or dosages. LDL-c percentage change was calculated by dividing the delta change (post minus pre intervention) by the baseline LDL-c concentration. All articles reporting the stated criteria were used in order to obtain a comprehensive literature review.

2.2.3 Data abstraction:

Data was abstracted from either the original articles or the article referenced for a specific topic (i.e., PS used). The parameters extracted were (a) duration in weeks and design (crossover or parallel); (b) at which meal the PS were consumed and frequency of consumption; (c) dose of PS in grams per day; (d) reported percentage change in LDL-c; (e) characteristics of the study population; (f) type of PS; (g) source and specific combination of PS; (h) the reference (Table 1). If data was not reported in the article or reference article the term "not reported" (NR) was used as a placeholder.

2.2.4 Data categorization:

Table 1 was primarily separated by food matrix. The strata within each matrix category were separated further by the PS dosage. A total of 33 studies were identified between the years 1998 and 2011 and 66 strata were isolated for evaluation.

2.3.0 Results and Discussion:

Based on the results presented in Table 1, PS consistently decreased serum LDL-c. However, a few of the foods with added PS showed no effect on LDL-c, and when PS were formulated into a pill (not reported in this review) minimal effects were reported (32, 33). Although there is a fair amount of variability, studies generally show a dose dependent LDL-c lowering effect with PS doses above 1 gram/day for a given food (Figure 1). Some of this variability is likely due to differences in the food matrix, especially the fatty acid composition. A number of other factors may also contribute to variability in the LDL-lowering effect of PS such as source of PS, timing of PS ingestion, duration of treatment, baseline LDL-c concentrations, background macronutrient composition, and genetic differences among individuals. In this paper we specifically

address the LDL lowering effects of specific foods with added PS and discuss the importance of the nutrient composition of the food matrix. Followed by a brief assessment of how the PS plant origin and structure, as well as, how the subjects' baseline LDL-c concentration may affect PS LDL-c lowering effectiveness.

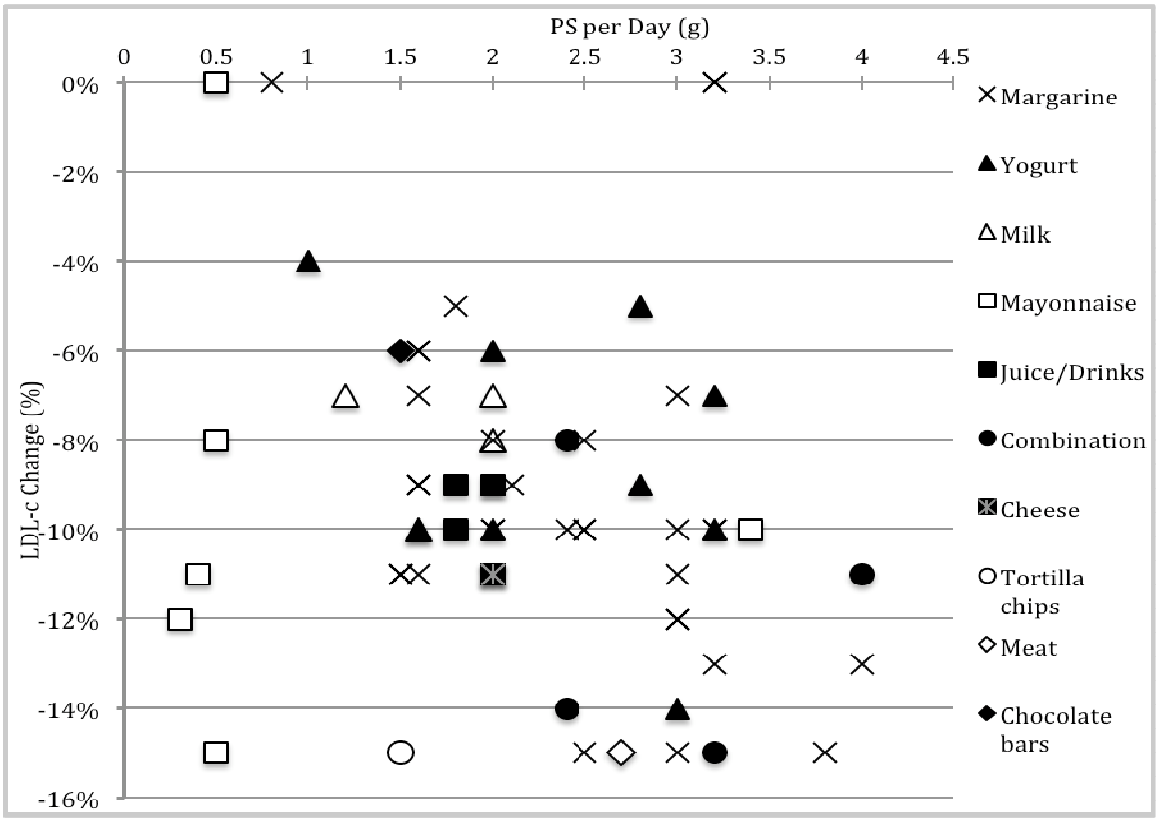

Figure 1. LDL-c % change per gram/day of PS for individual food matrices. To avoid a gap between 5.0 grams and 9.0 grams of PS/day one combination matrix point was removed – (34), found a 17% decrease in LDL-c with 8.9 grams of PS.

2.3.1 Food Matrix:

The most appropriate matrix for PS is thought to be one high in fat to enhance PS solubility (35), however, low fat products may also be effective carriers (36). This may be especially true with the addition of emulsifiers, such as lecithin, used to solubilize the PS for dispersion throughout the matrix (37).

In addition to carrying the PS, the food's matrix also has the ability to enhance or hinder the LDL-c lowering capacity through its fatty acid composition. Certain fatty acids are known to lower cholesterol independent of PS thereby aiding in the PS's ability to lower LDL-c. Poly and monounsaturated fatty acids such as linoleic and oleic acids found in soy oil and rapeseed oil generally lower cholesterol (4), whereas saturated fatty acids on average increase LDL-c, with the exception of stearic acid which has a neutral effect on LDL-c (38).

Just as fats known to decrease LDL-c may aid in overall ability of PS to lower LDL-c, fats known to increase cholesterol concentrations, may hinder the hypocholesterolemic effects of PS. For example, saturated animal fats, and trans fatty acids, acquired through hydrogenation manufacturing processes, are known to independently increase LDL-c concentrations (39).

It is understood that PS functionality is not solely impacted by the matrix of the functional food itself. For instance, if the food is consumed with a meal or snack then the interaction between the meal and the food with added PS becomes the "new" matrix affecting functionality. In theory, a meal might provide additional cholesterol and fat leading to greater bile release. Pairing foods with added PS with a meal should therefore allow the PS to increase elimination of both cholesterol and bile in the feces, thereby promoting greater LDL-c lowering.

Cholesterol lowering effects from studies published between the years 1998 to 2011 evaluating nine food matrices incorporating PS are discussed. These matrices include margarine, mayonnaise, yogurt, milk, cheese, meat, grains, juices and

chocolates.

Table 1. Food Matrices: The design, methodology, matrix, and LDL-c percent change, as well as, subject and PS characteristics of references that utilized foods with added PS as a cholesterol lowering mono-therapy.

Design	X-day	B/L/D	Dose g/day	Reported LDL-c% Δ	Bsl LDL-c	Sex	Type PS	PS plant source	Reference
					Margarine				
4 wk. crossover	2/3	w/ meal	0.8	NS	High	>50% M	Stanol esters	Sitostanol 77 Campestanol 23	(28) a
13 wk. parallel	2	NR	1.5	11%	High	NR	Free sterol	Wood	(40) a
26 wk. parallel	2	NR	1.5	11%	High	NR	Free sterol	Wood	(40) b
3 wk. parallel	NR	w/ meals	1.6	7%	High	<50% M	Sterol esters	Tall oil Rapeseed oil	(41) e
3 wk. parallel	NR	w/ meal	1.6	11%	High	<50% M	Sterol esters	Tall oil	(41) b
3 wk. parallel	NR	w/ meal	1.6	9%	High	>50% M	Sterol esters	Soybean oil	(41) a
3.5 wk. crossover	2	L+D	1.6	9%	Near optimal	<50% M	Sterol esters	Soybean oil	(42) a
4 wk. crossover	2/3	w/ meal	1.6	6%	High	>50% M	Stanol ester	Sitostanol 76 Campestanol 23	(28) b
52 wk. parallel	2	L+D	1.6	6%	Bdln high	<50% M	Sterol esters	Soybean oil	(43)
3 wk. crossover	2	B+D	1.8	5%	Bdln high	<50% M	Sterol esters	Soybean oil	(44)
3 wk. crossover	2	NR	2	10%	High	<50% M	Sterol esters	Vegetable oil	(45) b
4 wk. crossover	NR	NR	2	9%	High	50% M	Sterol esters	Tall oil	(46) b
4 wk. parallel	2	B+D	2	10%	Bdln high	<50% M	Stanol esters	Sitostanol 60 Campestanol 40	(29) a
4 wk. crossover	NR	NR	2	8%	High	50% M	Sterol ester	Rapeseed oil	(46) a
3 wk. crossover	NR	NR	2.1	9%	Optimal	<50% M	Free sterols	Rice bran oil	(47)
4 wk. crossover	2/3	w/ meal	2.4	10%	High	>50% M	Stanol Ester	Sitostanol 76 Campestanol 23	(28) c
4 wk. crossover	NR	NR	2.5	15%	Very high	<50% M	Sterol esters	Soybean 60 Rapeseed 30 Sunflower 5	(27) a
6 wk. crossover	NR	NR	2.5	8%	Bdln high	0% M	Stanol esters	Tall oil	(48) a
6 wk. crossover	NR	NR	2.5	10%	Bdln high	0% M	Stanol esters	Vegetable oil	(48) b
8 wk. crossover	NR	NR	2.5	10%	Very high	<50% M	Sterol esters	Soybean 60 Rapeseed 30 Sunflower 5	(27) b
3 wk. parallel	NR	w/ meal	3	15%	High	<50% M	Sterol esters	Tall oil	(41) d
3 wk. parallel	NR	w/ meals	3	12%	High	<50% M	Sterol esters	Tall oil Rapeseed oil	(41) f

3wk. parallel	NR	w/ meal	3	10%	High	>50% M	Sterol esters	Soybean oil	(41) c
4 wk. parallel	3	B+L+D	3	7%	Bdln high	<50% M	Stanol esters	Sitostanol 60 Campestanol 40	(29) b
12 wk. parallel	2	NR	3	12%	High	NR	Free sterol	Wood	(40) c
26 wk. parallel	2	NR	3	11%	High	NR	Free sterol	Wood	(40) d
3.5 wk. crossover	2	L+D	3.2	NS	Bdln high	50% M	Sterol esters	Rice bran oil	(49) b
3.5 wk. crossover	2	L+D	3.2	NS	Bdln high	50% M	Sterol esters	Sheanut oil	(49) c
3.5 wk. crossover	2	L+D	3.2	13%	Bdln high	50% M	Sterol esters	Soybean oil	(49) a
3.5 wk. crossover	2	L+D	3.2	10%	Near optimal	<50% M	Sterol esters	Soybean oil	(42) b
4 wk. crossover	2/3	w/ meal	3.2	10%	High	>50% M	Stanol esters	Sitostanol 76 Campestanol 23	(28) d
8 wk. parallel	3	B+L+D	3.8	15%	Near optimal	<50% M	Stanol esters	Vegetable oil	(26) a
8 wk. parallel	3	B+L+D	4	13%	Near optimal	<50% M	Stanol esters	Wood	(26) b
Mayonnaise									
4 wk. parallel	1	NR	0.3	12%[SC]	Bdln high	100% M	Sterol esters	Soybean oil	(50) a
4 wk. parallel	1	NR	0.4	11%[SC]	Bdln high	100% M	Sterol esters	Soybean oil	(50) b
2 wk. crossover	1	D	0.5	8%	Bdln high	100% M	Free sterols	Soybean oil	(51) a
2 wk. crossover	1	D	0.5	NS	Bdln high	100% M	Free sterols	Soybean oil	(51) b
4 wk. parallel	1	NR	0.5	15%[SC]	Bdln high	100% M	Sterol esters	Soybean oil	(50) c
6 wk. parallel	NR	NR	3.4	10%	Bdln high	>50% M	Free sterols	Sitostanol 100	(52)
Dairy									
Yogurt									
4 wk. crossover	1	w/ meal	1	4%	Bdln high	>50% M	Free sterols	Soybean oil	(2008a) (53)
6 wk. parallel	1	L	1.6	10%	Bdln high	NR	Free sterols	Tall oil	(54) a
4 wk. parallel	1	B	2	10%	Near optimal	>50% M	Stanol esters	Soybean oil	(55)
8 wk. parallel	2	w/ meal	2	6%	High	>50% M	Free sterols	Soybean oil	(53) b
6 wk. parallel	NR	NR	< 2	9%	Bdln high	<50% M	Free sterol	Pine tree oil	(56) a
4 wk. parallel	1	L	2.8	9%	Bdln high	<50% M	Sterol esters	Tall oil	(57) c
4 wk. parallel	1	w/o meal	2.8	5%	Bdln high	<50% M	Sterol esters	Tall oil	(57) d
4 wk. parallel	2/3	B+L+D	3	14%	Near optimal	<50% M	Stanol esters	Vegetable oils	(58)
4 wk. parallel	1	L	3.2	10%	Bdln high	<50% M	Sterol esters	Tall oil	(57) a
4 wk. parallel	1	w/o meal	3.2	7%	Bdln high	<50% M	Sterol esters	Tall oil	(57) b
Milk									

4 wk. crossover	2	B+L	1.2	7%	High	<50% M	Free sterols	Vegetable oil	(36) a
4 wk. crossover	2	B+L	1.6	10%	High	<50% M	Free sterols	Vegetable oil	(36) b
3 wk. crossover	2	NR	2	8%	High	<50% M	Sterol esters	Vegetable oil	(45) a
12 wk. parallel	2	w/ meals	2	7%	High	>50% M	Sterol esters	Vegetable oil	(59) a
Cheese									
6 wk. parallel	NR	NR	2	11%	Bdln high	<50% M	Free sterols	Pine tree oil	(2006b) (56)
6 wk. parallel	NR	NR	2	11%	Bdln high	<50% M	Free sterols	Pine tree oil	(2006c) (56)
Other									
Tortilla chips									
4 wk. crossover	2	L+D	1.5	15%	High	>50% M	Free sterols	Soybean oil	(60)
Meat									
4 wk. parallel	1	L	2.7	15%	Bdln high	100% M	Sterol esters	Soybean oil	(61)
Chocolate bars									
6 wk. parallel	2	w/o meal	1.5	6%	High	<50% M	Sterol esters	Soybean oil	(62)
Juice/Drinks									
3 wk. crossover	3	B+L+D	1.8	10%	High	>50% M	Free sterols	Tall oil	(63) a
3 wk. crossover	3	B+L+D	1.8	9%	Bdln high	>50% M	Free sterols	Tall oil	(63) b
8 wk. parallel	2	B+D	2	9%	Bdln high	<50% M	Free sterols	Vegetable oils	(64)
Combination									
Croissants + muffins									
8 wk. parallel	2	NR	3.2	15%	Optimal	<50% M	Sterol esters	Soybean oil	(65)
Cereal + bread + margarine									
4 wk. crossover	NR	w/as a meal	2.4	14%	High	>50% M	Sterol esters	Soybean oil	(66) a
Milk + margarine									
3 wk. crossover	2	NR	4	11%	High	<50% M	Sterol esters	Vegetable oil	(45) c
Cereal + bread + margarine									
4 wk. crossover	NR	w/as a meal	2.4	8%	High	>50% M	Stanols	Soybean oil	(66) b
Margarine + oat drink									
10 wk. parallel	3	B+L+D	8.9	17%	Bdln high	<50%M	Free sterols + stanols	Vegetable oil	(34)

NR, not recorded; B+L+D, breakfast lunch dinner; w/, with; w/o, without; wk., week(s); X-day, times per day; PS, phytosterol/phytostanol; Δ, change; /, or; NS, no statistical significance; [SC], simple % change calculation.

2.3.1.a Margarine:

Over half the studies incorporating PS into foods used margarine. It is one of the most effective food matrix; 19 strata showed a ≥10% decrease in LDL-c, and three strata

showed a ≥15% decrease. Margarine has a high fat content supporting the PS solubility and concentration in the matrix. Margarine is versatile, used to prepare and to supplement foods, allowing for consumption with almost any meal or snack, which also facilitates functionality. For these reasons margarine is a well-suited delivery matrix (35, 37).

Two of the three margarine products that decreased LDL-c by 15% were higher in fat (70% and 74%) (26, 27) compared to the other (47%) (41). The 15% decrease in LDL-c in response to low fat margarine is an anomaly considering that other low fat margarine matrices (60% and 35%) resulted in significant LDL-c reductions of only 5% and 6% respectively (43, 44). This discrepancy may be explained by the margarine's fatty acid composition. The successful low fat (47%) margarine matrix added 3.0 grams of PS to a highly polyunsaturated fat matrix. The other two studies that reported 15% decreases in LDL-c, either added 2.5 grams of PS to a 70% soy oil margarine matrix (27) or added 3.8 grams of PS to a 74% rapeseed oil matrix (26). Other slightly less effective products, resulting in LDL-c reductions of 13% and 9-10%, used linoleic and oleic fatty acids in a 73% and 70% fat matrix respectively (42, 49) and LDL-c reductions of 11-12% resulted when PS were added to an unreported percent rapeseed oil matrix (40).

A high proportion of saturated fats on the other hand may hinder a PS's LDL-c lowering capacity. Saturated fat made up 20% of the total fat in the 60% fat matrix mentioned above to lower LDL-c 5%(44). In addition when 2.5 grams of PS were added to a high saturated fat butter matrix, the resultant LDL-c concentrations were not significantly lower than baseline although they were lower then butter alone (48). These

results indicate that the specific fat make up of the high-fat matrix is an important characteristic of the functional food.

Margarines can also contain trans fatty acids which may have negative effects on serum lipid profiles by promoting a rise in plasma total and LDL cholesterol, as well as a decrease in HDL-c (67). To date no PS margarine matrix, with a measureable amount of trans fatty acids, has been manufactured and tested. This is most likely because manufacturers have developed techniques to either filter or inhibit the formation of trans fatty acids.

In summary, margarine is a common carrier of PS that consistently lowers LDL-c, especially when it is comprised of a higher percentage of fat that includes a high proportion of linoleic acid and/or oleic acid.

2.3.1.b Mayonnaise:

Only three studies and six strata have examined mayonnaise as a PS carrying matrix, all with either rapeseed, soybean or safflower oil based matrices. Mayonnaise, a fat-based spread like margarine, consistently contains 60% fat and shows significant LDL-c lowering capabilities.

Two of the three studies used <1.0 gram of PS per day. Daily intake of 0.3, 0.4, and 0.5 grams of PS resulted in 12, 11, and 15% decreases in LDL-c respectively (50). The second study, which used only 0.5 grams of PS, lowered LDL-c by 8% (51).

Mayonnaise food matrices can be created with two varieties of fat, a diacylglycerol (DAG) and triglyceride (TG), a characteristic that may affect PS functionality. The two studies mentioned above were the only to report the use of a

DAG PS matrix, which had a base of rapeseed, soybean and safflower oil. These two studies were also the only (from the studies in this review) to have significant LDL-c decreases with <1.0 gram of PS. The reports speculated first that a DAG matrix increases PS solubility allowing for even distribution throughout the matrix, facilitating maximal cholesterol displacement in the intestine. It was also speculated, that a DAG matrix may hinder the rise in postprandial plasma triglycerides, decreasing Very Low Density Lipoprotein Cholesterol synthesis and subsequently LDL-c concentration (68). Although postprandial TG concentrations were not reported it should be noted when a low dosage (0.5 grams) of PS was combined in a TG based rapeseed, soybean and safflower oil mayonnaise matrix, LDL-c did not significantly decrease (51). To the contrary, when a moderate dosage (3.4 grams) of PS was added to a TG based rapeseed oil matrix a 9% decrease in LDL-c was achieved (52).

These results indicate that a DAG mayonnaise matrix may be more effective than a TG matrix. Considering mayonnaise is the only functional food to report a matrix of DAG, perhaps a DAG matrix would be equally as effective in other types of functional foods, a consideration that warrants further investigation.

2.3.1.c Dairy: yogurt:

Yogurt products are a relatively new and effective PS delivery matrix. Five of six studies and eight of ten strata, resulted in a significant decrease in LDL-c, with four resulting in ≥10% decrease.

Similar to margarine and mayonnaise which are consumed with other foods, when yogurt was consumed with either a breakfast or a lunch, 10% reductions in LDL-c

were seen (54, 55), and when consumed three times a day with breakfast, lunch and dinner there was a 14% reduction (58). However, not all yogurt products resulted in significant LDL-c reductions when consumed with a meal. Two low fat (2%) yogurts, with 1.0 gram of PS, reduced LDL-c concentrations by an insignificant 4 and 6% (53). Even though an emulsifier (which was not identified) was used, it was speculated that these results were the product of poor PS solubility in the matrix.

Yogurt also has an advantage over margarine and mayonnaise in that it can be consumed independently as a snack. Snack foods are not consumed with excess cholesterol and fat from a meal, however, they are more versatile to the consumer. One of the more successful reduced fat (3.3%) yogurt products, when consumed with a meal, resulted in an LDL-c reduction of 9%. When this same product was consumed without a meal, it resulted in a reduction of 5%. In the same investigation a low fat (2.2%) yogurt product, when consumed with a meal, resulted in an LDL-c reduction of 10%. When this same product was consumed without a meal, it resulted in a reduction of 7% (57). It is interesting to note that the reductions in LDL-c when yogurts were consumed independently as a snack were only slightly less significant then when consumed with a meal. It can be speculated that the composition of fat in the yogurt caused bile acid to be released and excreted in significant amounts (69). Additional snack foods such as hummus and cottage cheese could maintain similar characteristics, and promote successful variety and versatility to PS containing snack foods.

2.3.1d Dairy: milk:

Cows' milk PS matrices with PS have not been as prominently studied as other

matrices, with only four strata and three studies. These milk products, although showing ability to lower LDL-c, have not shown reductions of more than 10%.

All four milk matrix strata consisted of low fat products containing <2% fat (1.2% (36), 1.4% (45) and 1.8% (59)). Three of these milk products were consumed with a meal, and one was consumed independently which did not seem to affect the magnitude of the LDL-c reductions. All four of the milk matrices contained added poly and monounsaturated fatty acids from vegetable oils rather than saturated milk fat alone which may have enhanced the LDL-c lowering effect. A 7% decrease in LDL-c was seen when 0.5 grams of monounsaturated fatty acids and 1.05 grams of polyunsaturated fatty acids were added to create a 1.8% fat milk (59), and an 8% decrease in LDL-c was seen when sunflower oil was added to create a 1.4% fat milk (45).

Even though these milk matrices contained added fatty acids known to enhance LDL-c reductions, milks with added PS have yet to reduce LDL-c concentrations more than 10%. This might be caused by an insufficient percentage of fat (<2%) in the matrix. Although speculative, if the percentage of fat of the matrix was increased to 2 or 3%, by increasing unsaturated fatty acids, then the potential increase in PS solubility could lead to greater decreases in LDL-c.

2.3.1.e Dairy: cheese:

Only one study and two strata have reported a cheese matrix with added PS. LDL-c reductions of 11% were achieved with the incorporation of 2.0 grams of PS into a hard and a soft cheese (56). Both were considered low fat cheeses yet they contained a higher percentage of fat (10-15%) compared to other dairy PS foods. The cheeses had a

predominantly saturated fatty acid composition yet the magnitude of LDL-c lowering was similar to PS containing foods with a higher proportion of unsaturated fats. The cheese matrices were likely able to effectively lower LDL-c because the amount of total fat was enough to successfully incorporate the PS into the matrix, while the amount of saturated fat was not enough to counteract the PS function. It is difficult to conclude on the functionality of a matrix with only two reports, however, it seems that the slightly higher percentage fat, even though predominantly saturated fat, promoted the LDL-c lowering potential of the PS.

2.3.1.f Other:

Four additional foods have been studied: tortilla chips; ground meat; chocolates and nonfat drinks. The PS enriched chips and meats both showed a 15% decrease in LDL-c. The chips were fried in a 12% PS safflower oil which resulted in 1.5 gram of PS per serving (60). Safflower oil is high in oleic acid and, as mentioned above, may independently lower LDL-c. In order to maximize PS incorporation into the fatty acids the PS were superheated with the TG from safflower oil. When this superheated PS TG mixture cooled, the PS were recrystallized into the TG to from a TG recrystallized PS matrix, which may enhance the LDL-c lowering capacity of the PS.

The meat was prepared by adding 2.7 grams of PS to an 11% fat ground meat. It was consumed everyday for lunch. Generally ground beef has a moderate fat content conducive to PS incorporation, but this specific ground beef had been manufactured to contain less saturated animal fat myristic and palmitic acids and more polyunsaturated vegetable fat linoleic acid. (61). The addition of PS to oil, used to fry foods, and to

ground meat results in a relatively high decrease in LDL-c.

Stearic acid is found in chocolate and may have a neutral effect on LDL-c despite its saturated nature (38). When a chocolate snack bar with added PS was consumed between meals, LDL-c was only reduced by 6% (62). Although the methods used to incorporate the PS were not reported, the result may be explained by the timing of consumption, within 30 minutes of a meal instead of at a meal, and the small serving size of chocolate consumed. This may have resulted in low amounts of fat and cholesterol ingestion, reducing the PS ability to discard dietary and biliary cholesterol in the feces. Considering these results and the potential presence of chocolate in the American diet further investigations may increase the serving size to take better advantage of the natural characteristics of chocolate and support the LDL-c lowering capacity of this food.

When nonfat drinks were consumed three times a day with each meal or twice a day with breakfast and dinner, 10 and 9% reductions in LDL-c were seen respectively (63, 64). When a 1% fat drink was consumed with breakfast, lunch and dinner a 9% reduction was seen (63). Although these results are significant, they continue the trend seen with low fat PS milk in that the LDL-c reduction could be greater. If these drink matrices were fortified with lecithin or poly and monounsaturated fatty acids to increase the percentage fat then perhaps these added characteristics would first promote LDL-c reduction and second disperse the PS efficiently through the matrix.

As the results indicate, PS can be added to almost any food matrix with or without a high fatty acid composition. Modifications, however, can be made to increase

LDL-c lowering efficiency. For instance the fatty acid composition of the matrix, the consumption with a meal or as a snack and the timing of consumption all impact the LDL-c lowering capacity. Therefore, the nature of the food and its potential matrix should be considered in the development of PS enriched food products to maximize functionality.

2.3.1.g Combinations:

Based on the literature, a variety of matrices provided cholesterol lowering benefits. This variety is beneficial to consumers who have been advised to change their lifestyle in order to reduce cholesterol and avoid medications. As more food products are shown to efficiently reduce cholesterol, more options will come to market giving consumers flexibility and a better chance for success.

Four articles and five strata examined LDL-c response when more than one type of food with added PS was consumed per day. Two of these five strata provided ≤2.4 grams of PS per day, a daily dosage typical for one PS food. The LDL-c response for these two trials was between 8-14% with matrices of whole-meal based starches paired with an oleic canola oil based margarine (66). The three remaining strata had moderate to high dosages of PS. The first strata was a combination of baked goods comprised of 20% fat and added oleic acid with a PS dose of 3.2 grams per day, the second strata was a combination of low fat milk and vegetable oil based margarine with a PS dose of 4.0 grams per day, and the third strata was a combination of a vegetable oil based margarine and a low fat oat based drink with 8.8 grams of PS per day. These reports found LDL-c decreases of 15, 11 and 17% respectively (34, 45, 65).

These results indicate that as foods with added PS are combined over the course of a day, and as the PS dosage increases, the LDL-c response is more favorable (37, 70). However, continued investigations should verify the efficiency and safety of combining these products in attempts to control hypercholesterolemia.

2.3.2 Origin of PS:

In addition to the lipid composition of the food's matrix, the PS plant source, or origin, and any synthetic structural additions may impact the PS LDL-c lowering effectives. Turning first to PS origin, different PS make-up a plants unique PS composition. Origin and specific PS are presented in (Table 2). PS from one plant source may be more effective over another (26, 47), however, the most common plants used to obtain PS are soy and wood. Many studies also used a unique ratio of β. sitosterol and campesterol, which are though to have increased LDL-c lowering capacity (28, 29)

Table 2. The percent range breakdown of the specific PS composition relative to the plant source.

	Soy	Wood	Rapeseed	Ricebran[c]	Sheanut[c]
Specific PS	% range				
β. Sitosterol	45-55	75-85	50-55		
24meth cycloartenol				35-40	
α. Amyrin					30-35
Campesterol	20-30	1-5	30-35	10-15	
Sitostanol		10-20		5-10	
Cycloartenol				25-30	
Lupeol					15-20
Stigmasterol	15-25		1-5		
β. Sitostanol		10-20			
Brassicasterol			5-10		
Butyrospermol					10-15

β. Amyrin					5-10

PS, phytosterols/phytostanols; α., alpha; β. beta.

[c] ratios do not add to 100% due to trace PS that are not reported in the literature.

Soy PS and wood PS were studied alone in 20 and 19 strata respectively, and combined with rapeseed PS in 22 and 21 strata respectively. The part soy or wood, part rapeseed PS combinations ultimately had the same PS composition as soy or wood alone and therefore were comparable (see Table 2). The soy and wood PS average LDL-c responses were separated out for comparison and are presented in Table 3a. In comparing the LDL-c responses there seems to be no striking difference between the two PS ratios per subject's baseline LDL-c level. The findings also show vegetable oils, which may have atypical ratios of B. sitosterol and campesterol/(B. sitostanol and campestanol), seem to lower LDL-c more effectively than the soy and wood PS. When strata that used only B. sitosterol and campesterol / (B. sitostanol and campestanol) were separated out the percent change in LDL-c was even greater then when soy, wood or vegetable oil PS were used. These results support that a higher ratio of B. sitosterol to campesterol / (B. sitostanol and campestanol) may enhance the reduction in LDL-c (26, 71).

β. sitosterol and campesterol are not, however, present in all plant sources, like rice bran oil and sheanut oil (see Table 2). One of three strata, that used rice bran oil PS, noted it also contained 1.0 gram per day of β. sitosterol and campesterol, and resulted in a 9% reduction in LDL-c. The two remaining strata used a rice bran PS and a sheanut PS and resulted in insignificant LDL-c reductions. These results and the literature (47, 49,

72) suggest that not all plants contain a PS combination that will facilitate a decrease in cholesterol. In addition to the lack of B. sitosterol and campesterol, these results may also be linked to synthetic structural modification. All but one of the seven strata which tested B. sitosterol and campesterol / (B. sitostanol and campestanol) used the free sterol structure, the other six modified the PS to their stanol ester structure. Indicating that the structural modification may have influenced the greater average reduction in LDL-c.

2.3.3 PS structure:

Structural changes to PS can be manufactured to create, what are commonly thought to be more efficient PS. For example, stanols the saturated counterparts of the free sterols, and PS esters the esterified counterpart of free sterols are both thought to be more fat soluble and less absorbable, characteristics which increase cholesterol displacement in the intestine. Overall, based on the results in Table 1, there are only three main PS structures used in functional foods, free sterols, sterol esters, and stanol esters, with 19, 33 and 13 strata reported in this review respectively. There are no cases of free stanols. This is likely because free stanols have restricted fat solubility, inhibiting the stanols incorporation into the matrix of the food, resulting in a decreased ability to interfere with cholesterol and bile acid absorption. Once esterified however, the stanol ester takes on even greater fat-soluble qualities than the sterol counterpart (73).

Table 3. The average LDL-c percent reduction from baseline for each plant origin (a) and type of PS (b).

g, gram; LDL-c, low density lipoprotein cholesterol; PS, phytosterol/phytostanol.

	% change	Range g PS
a.		
Soy (n=22)	-9.3 ± 4.6%	0.3 - 4.0
Wood (n=21)	-9.1 ± 3.2%	1.5 - 4.0
Vegetable oil (n=12)	-10.8 ± 3.8%	1.2 - 8.9
B. sitosterol and Campesterol PS (n=7)	-12.7 ± 4.6%	0.8 - 3.4
b.		
Free sterols (n=19)	-9.8 ± 3.8%	0.5 - 3.0
Sterol esters (n=33)	-9.3 ± 4.1%	0.3 - 4.0
Stanol esters (n=13)	-11.8 ± 3.8%	0.8 - 4.0

Numbers are averages ± standard deviations
Rice bran PS, and Sheanut PS were excluded from the plant origin table due to an n size of thee and one respectively.

To evaluate the LDL-c lowering efficiency of the PS structural differences, each structure was averaged and presented in Table 3b. These results indicate that structurally modified

stanol esters may have an advantage over both free sterols and sterol esters. These results most likely occurred because not only are the sterol esters unable to be absorbed but they also have a higher lipid solubility (17, 71, 74). However, it should be noted that half the strata making up the stanol ester category used the PS B. sitostanol and campestanol, which may have also played a roll in the LDL-c reductions. Often it is thought that sterol esters also lower LDL-c more efficiently than free sterols. This conclusion was not supported by the studies in this review.

2.3.4 Baseline LDL-c and PS therapy:

In addition to the two factors discussed in this review, it is thought that the subject's baseline LDL-c concentration may also play a role in the effectiveness of a PS

therapy (75) (figure #2.). To evaluate the matter, the strata were separated by subjects' baseline LDL-c level. A total of 2, 6, 28, 28 and 2 studies were classified into an LDL-c baseline of optimal <100 mg/dL (<2.60 mmol/L), near optimal 100-129 mg/dL (2.60-3.34 mmol/L), borderline high 130-159 mg/dL (3.35-4.11 mmol/L), high 160-189 mg/dL (4.12-4.84 mmol/L) and very high >190 mg/dL (>4.85 mmol/L) (76) respectively. The average decease in LDL-c for each classification was 9.5, 9.5, 8.8, 10.9 and 12.5% respectively. The magnitude in LDL-c reduction, was also greater in subjects with a high or very high baseline LDL-c and is shown in Figure 2. These findings support the use of PS as a mono therapy for individuals with near optimal or borderline high LDL-c concentrations, because the PS will be able to lower the individual's LDL-c to an optimal range. Where LDL-c, in individuals with high or very high baseline concentrations will likely be reduced to the borderline high range thus necessitating other therapeutic methods, like fiber and exercise, to lower LDL-c to an optimal range.

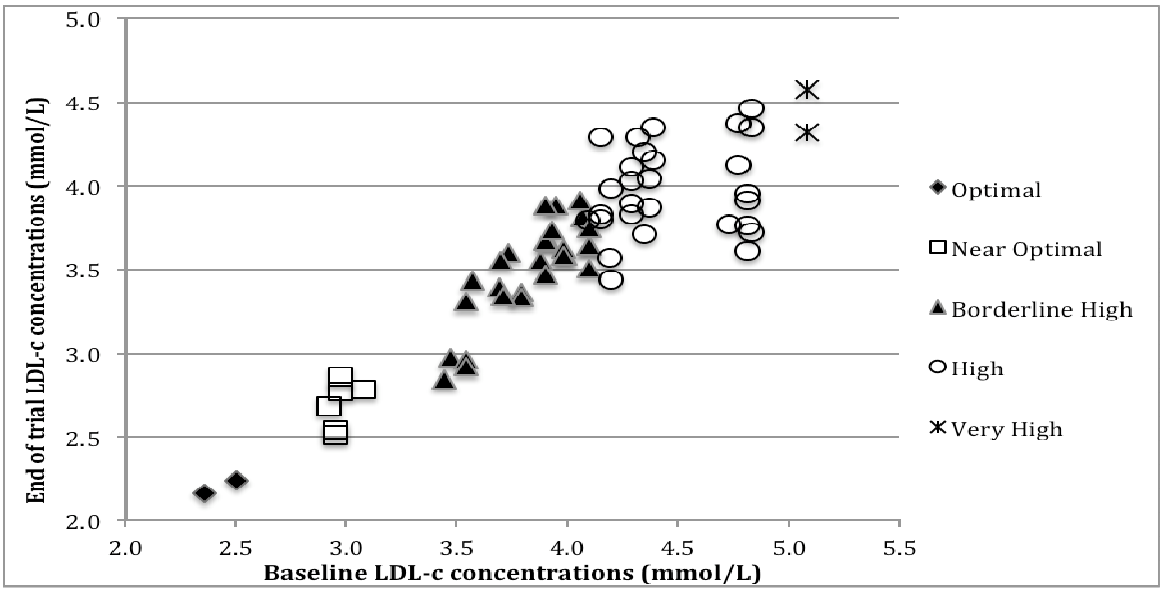

Figure 2. Total LDL-c concentration after PS intervention based on baseline LDL-c concentrations.

2.4.0 Conclusion:

It is clear that foods with added PS are an effective strategy to moderately lower LDL-c. Many types of food matrices resulted in significant decreases in LDL-c, especially when the nutrient composition of the matrix consisted of either poly or monounsaturated fatty acids (i.e., linoleic and oleic acids), which may independently aid in the reduction of LDL-c. Also B. sitostanol and campestanol, as well as, stanol esters may have the potential to enhance the LDL-c lowering capacity. Milk, nonfat beverages and chocolate bars are yet to show LDL-c decreases greater than 10% and therefore, additional research should be conducted to determine how to successfully incorporate the PS into these matrices.

Chapter 3

3.0.0 Methods

3.1.0 Experimental design

Men and women with moderately elevated plasma LDL cholesterol participated in three 4-week phases, to determine the effects of two different PS preparations delivered in a milk matrix on plasma lipid responses. During phase I all subjects consumed 2% cows' milk with no PS. During phase II all subjects consumed skim cows' milk containing 2.0 grams of unaided PS. During phase III all subjects consumed fat-free cows' milk containing 2.0 grams of triglyceride recrystallized phytosterols (TRP). A first group took the TRP milk immediately after the completion of phase II, and a second group took the TRP milk after a one month washout period (non-testing phase).

To determine total cholesterol, LDL-c, HDL-c, triglycerides, and the total/HDL cholesterol ratio, the average of two fasting blood draws was obtained at baseline and after each

of the 4-week phases. A third fasting blood draw was made if duplicate LDL-c values varied by more than 15%.

Subjects were asked to consume two 8 oz servings of the respective phase's milk, one serving at breakfast and one serving at dinner. Also to maintain their habitual diet and level of physical activity throughout the entire period. A 3-day diet record (2 weekdays and 1 weekend day) was obtained from baseline and at the end of each testing phase. If body weight fluctuated more than 3.0 kg subjects were counseled by research dietitians for caloric adjustments in order to maintain baseline weight.

3.2.0 Subjects

A total of 46 hyperlipidemic, weight stable men and postmenopausal women between the ages of 35-70 years, were recruited from the local area (UConn Storrs, CT). Potential subjects completed screening questionnaires (medical history, diet history and I-Pac). Participants were excluded if they: had a diagnosis of hypertension, type I or II diabetes, liver, kidney, or other metabolic or endocrine dysfunction; currently used cholesterol medications; or were known to abuse alcohol or use tobacco products. Volunteers taking supplements known to affect serum lipid levels such as anabolic steroids, fish oil, niacin, psyllium or NSAIDs were either excluded or asked to maintain their habitual use. A fasting blood draw was performed and subjects with serum LDL-c less than 100 or greater than 209 mg/day were excluded. Subjects were informed of the purpose and possible risks of the investigation prior to signing an informed consent document approved by the University's Institutional Review Board. A total of 20 individuals met the study criteria and finished the protocol. Baseline characteristics of subjects are shown in Table 4.

Table 4. Subject baseline characteristics. (n = 20)

Values are mean ± SD

3.3.0 Milk Supplement

Phase I consisted of commercially available 2% cows' milk obtained from the local Mountain Dairy farm.

Phase II consisted of fat-free cows' milk with 2.0 grams unaided (free) soy sterols.

Phase III consisted of fat-free cows' milk, with 2.0 grams TRP. The TRP was derived from soy and dispersed in the milk prior to ultra-high-temperature treatment. The milk for Phase II and III were supplied by GFA Brands, Inc, and consisted of grade A fat-free (<0.5% fat (<1.24 grams fat / 8.0 oz serving)) with added non-fat milk solids, high oleic sunflower oil and sunflower lecithin.

Characteristics	Baseline
Age (y)	56 ± 9.6
Gender (% male)	65
BMI (kg/m^2)	27 ± 5.2
Weight (kg)	78± 16.9
LDL-c (mg/dL)	134 ± 23.4
HDL-c (mg/dL)	63 ± 21.2
Total Cholesterol (mg/dL)	219 ± 33.0
Triglycerides (mg/dL)	109 ± 60.1
Fat Mass (kg)	24 ± 11
Lean Body Mass (kg)	501 ± 11

3.4.0 Testing Protocol and Weekly Visits

At baseline and after each of the 4-week phases, subjects reported to the Human Performance research laboratory (HPL) at the University of Connecticut for two consecutive,

morning testing procedures. Subjects came 12 hours fasted and 24 hours in abstinences of intense exercise, caffeine and alcohol consumption.

Testing day 1. The morning of testing day 1, subjects rested quietly for ten minutes in the supine position, blood was then obtained from an arm vein and gathered in a serum separator, serum, sodium citrate and Ethylenediaminetetraacetic Acid (EDTA) tube. Once blood was obtained a series of anthropometric measures were taken including height, by averaging three subsequent measures taken with a dual reading height rod (only at baseline); weight, by a digital scale; and body composition, through dual-energy X-ray absorptiometry (Prodigy, Lunar Corporation, Madison, WI). Finally the 3-day diet record was handed in and reviewed, with the subjects, by one of the dietetic staff, for accuracy.

Testing day 2. Subjects returned to the HPL either 24 or 48 hours after the first visit for testing day 2. Subjects rested quietly for ten minutes in the supine position, blood was then obtained from an arm vein and gathered in a serum separator tube. Subjects took home the new weekly milk supply and weekly compliance recording sheet (documenting date and time milk was consumed).

Testing day 3. If testing day 1 and day 2 LDL-c values varied more than 15%, then subjects returned to the HPL for testing day 3. Subjects rested quietly for ten minutes in the supine position, blood was then obtained from an arm vein and gathered in a serum separator tube.

Weekly visits. Subjects also checked into the HPL weekly (not fasted) during each testing phase. At this time subjects were given a week supply of milk supplement and weekly compliance recording sheet, their weight was taken, a questionnaire regarding changes in lifestyle habits (dietary, supplement and physical activity changes) was filled out, finally the previous week's compliance recording sheet was handed in and verbal compliance was obtained.

3.5.0 Analysis

Whole blood was collected into a serum separator, serum, sodium citrate (chilled) and EDTA (chilled) tube. The serum separator and serum tubes clotted at room temperature and then centrifuged with the chilled EDTA tube at 1600 x g for 15 minutes at 4°C, the sodium citrate tube was centrifuged at 2000 x g for 30 minutes at 4°C. The serum separator was sent to a certified medical laboratory (QUEST Diagnostics, Wallingford, CT) for determination of total cholesterol, HDL-C, TG, and calculated LDL-c concentrations; LDL-c was calculated indirectly using the Friedewald equation (77). The serum, sodium citrate and EDTA were aliquoted and stored in an -80°C freezer for later analysis. Diet records were analyzed for energy and nutrient composition and monitored for dietary maintenance using nutrient analysis (NUTRITIONIST PRO™, Version 1.3, First Databank Inc, The Hearst Corporation, San Bruno, CA).

Apolipoprotein A1 was analyzed from sodium citrate plasma (200 fold dilution) by ELISA (Innovative Research, Novi, MI), with sensitivity of 100 ng/mL, intraassay coefficient of variation (CV) of 3.8% and interassay CV of 8.0%. The assay wavelength was read at 450 nm on a Molecular Devices VERSAmax tunable microplate reader.

Apolipoprotein B was analyzed from sodium citrate plasma (20,000 fold dilution) by ELISA (Innovative Research, Novi, MI), with sensitivity of 20 ng/mL, intraassay coefficient of variation (CV) of 4.9% and interassay CV of 10.7%. The assay wavelength was read at 450 nm on a Molecular Devices VERSAmax tunable microplate reader.

3.6.0 Statistics

Only subjects who completed the intervention and were compliant were analyzed. Means and SD were computed and distributions for all dependent variables were examined for approximate normality. Analysis of variance (ANOVA) was used to determine significant differences over time and associations between variables. Bonferroni correction (or LSD-equivalent) was used for all pair-wise comparisons. Covariate analysis was explored to determine

if certain variables produce a significant covariate F score, for these instances ANCOVA with multiple covariates with the model above was used. Significance was set at $p \leq 0.05$.

Chapter 4

4.0.0 Results

Compliance was 98.8% for all subjects, and did not differ between phases. The three milk products were well tolerated, and no adverse side effects were reported. The subjects' physical activity, body mass and composition, and blood glucose and insulin did not significantly change during any of the study phases. Nutrient intake was not significantly different between study phases. Baseline dietary composition is presented in Table 5. Baseline lipids are presented in Table 4.

Table 5. Baseline nutrient analysis.

Dietary Nutrients	Baseline
Kilocalories (Kcal/day)	2006 ± 675
Protein (g)	92 ± 31
Carbohydrate (g)	215 ± 76
Total Fat (g)	81 ± 34
Saturated Fat (g)	28 ± 14
Monounsaturated Fat (g)	21 ± 8
Polyunsaturated Fat (g)	11 ± 6
Trans Fatty Acid (g)	0.6 ± 0.6
Dietary Fiber (g)	18 ± 6
Cholesterol (mg)	329 ± 166

Values are mean ± SD.

Comparison of the lipid panel between phases is presented in Table 6. Plasma lipids did not change significantly between the baseline and control phases (2% milk). There were significant reductions in total and LDL cholesterol between the 2% milk and both the free PS milk (-5.9%, -9.0%) and the TRP milk (-9.0%, -11.%) respectively. Surprisingly, although the effect sizes were small, there were significant reductions in HDL-c between the TRP milk and

both the 2% milk (-8.0%) and the free PS milk (-6.7%), however, there was not a significant difference between the TRP milk and baseline. There were no significant changes in total and LDL cholesterol between the free PS milk and the TRP milk phases. There were also no significant changes in triglycerides, LDL/HDL cholesterol ratios, and total/HDL cholesterol ratios between any of the phases.

Table 6. Average plasma lipid values after each phase.

Parameter	P1 2% milk	P2 Free PS milk	P3 TRP milk	**P1 vs. P2** mean difference p value 95% CI Cohen's d	**P1 vs. P3** mean difference p value 95% CI Cohen's d	**P2 vs. P3** mean difference p value 95% CI Cohen's d
Total cholesterol (mg/dL)	223.6 ± 33	210.4 ± 33	203.4 ± 35	-13.2 .039 (-25.8, -0.50) .4	-20.2 .001 (-33.1, -7.2) .6	-7.0 .136 (-15.3, 1.3) .2
LDL-c (mg/dL)	138.3 ± 23	126.0 ± 23	123.1 ± 24	-12.4 .019 (-23.2, -1.6) .5	-15.2 .002 (-25.6, -4.7) .7	-2.8 1.000 (-11.3, 5.7) .1
HDL-c (mg/dL)	63.8 ± 21	62.9 ± 20	58.9 ± 19	-0.9 1.000 (-4.6, 2.9) .0	-5.1 .029 (-9.8, -0.03) .2	-4.2 .023 (-8.0, -0.4) .2
Triglycerides (mg/dL)	106.9 ± 68	107.3 ± 7	107.4 ± 67	0.4 1.000 (-24.1, 24.9) .0	0.5 1.000 (-18.6, 19.6) .0	.1 1.000 (-18.6, 19.8) .0
LDL/HDL cholesterol ratio	2.41 ± 0.9	2.21 ± 0.9	2.29 ± 0.82	-0.1 1.000 (-0.4, 0.2) .2	-0.1 .144 (-0.4, 0.2) .1	0.1 1.000 (-0.2, 0.3) .0
Total/HDL cholesterol ratio	3.79 ± 1.1	3.82 ± 1.1	3.92 ± 1.1	-0.2 .091 (-0.5, 0.1) .2	-0.1 .126 (-0.5, 0.3) .1	0.1 .082 (-0.2, 0.3) .1

Values are means ± SD; ($p<0.05$) using ANOVA repeated-measures and Bonferroni adjustments

P1, Phase 1; P2, Phase 2; P3, Phase 3; 95% CI, 95% confidence interval.

Apolipoprotein B (apo B) values resulted in a significant decrease between the free PS milk and the TRP milk of -7 mg/dL. The apo A1 values, on the other hand, showed no significant change between any of the milk phases. The apo A1/apo B ratio tended to be higher in the free PS milk and TRP milk when compared to the 2% milk, 5.9% and 6.6% respectively, although not significantly (Table 7).

Table 7. Average Apolipoprotein values after each phase.

Parameter	P1 2% milk	P2 Free PS milk	P3 TRP milk	**P1 vs. P2** mean difference p value 95% CI Cohen's d	**P1 vs. P3** mean difference p value 95% CI Cohen's d	**P2 vs. P3** mean difference p value 95% CI Cohen's d
po B (mg/dL)	99.8 ± 23	100.4 ± 22	93.5 ± 21	0.6 .872 (-6.5, 7.6) .0	-6.33 .104 (-14.1, 1.4) .3	-7.0 .048 (-13.7, -0.1) .3
po A1 (mg/dL)	89.9 ± 19	95.0 ± 21	89.4 ± 13	5.2 .136 (-1.8, 12.1) .3	-0.5 .865 (-6.6, 5.6) .0	-5.7 .130 (-13.1, 1.8) .3
po A1/Apo B ratio	0.94 ± 0.28	0.99 ± 0.35	1.00 ± 0.28	0.1 .282 (-0.1, 0.2) .2	0.1 .207 (0.0, 0.2) .2	0.0 .923 (-0.1, 0.1) .0

Values are means ± SD; ($p<0.05$) using ANOVA repeated-measures
P1, Phase 1; P2, Phase 2; P3, Phase 3; 95% CI, 95% confidence interval.
PS and TRP milks (-6.6%), yet significantly different between the 2% and TRP milks (-6.5%)

These results indicate that 14 subjects had a more favorable LDL-c response to the TRP milk compared to the free PS milk. The average percent change between free sterol and TRP milk for these 14 subjects was -7.3% (Figure 3).

The graph also indicates that there were three subjects who did not respond to either the free PS or the TRP milk, as indicated by LDL-c concentrations above zero for both phases. When these non-responders were removed from the analysis the total and LDL cholesterol trends were the same. The HDL-c trended on the other hand was not significantly different between the free Table 8).

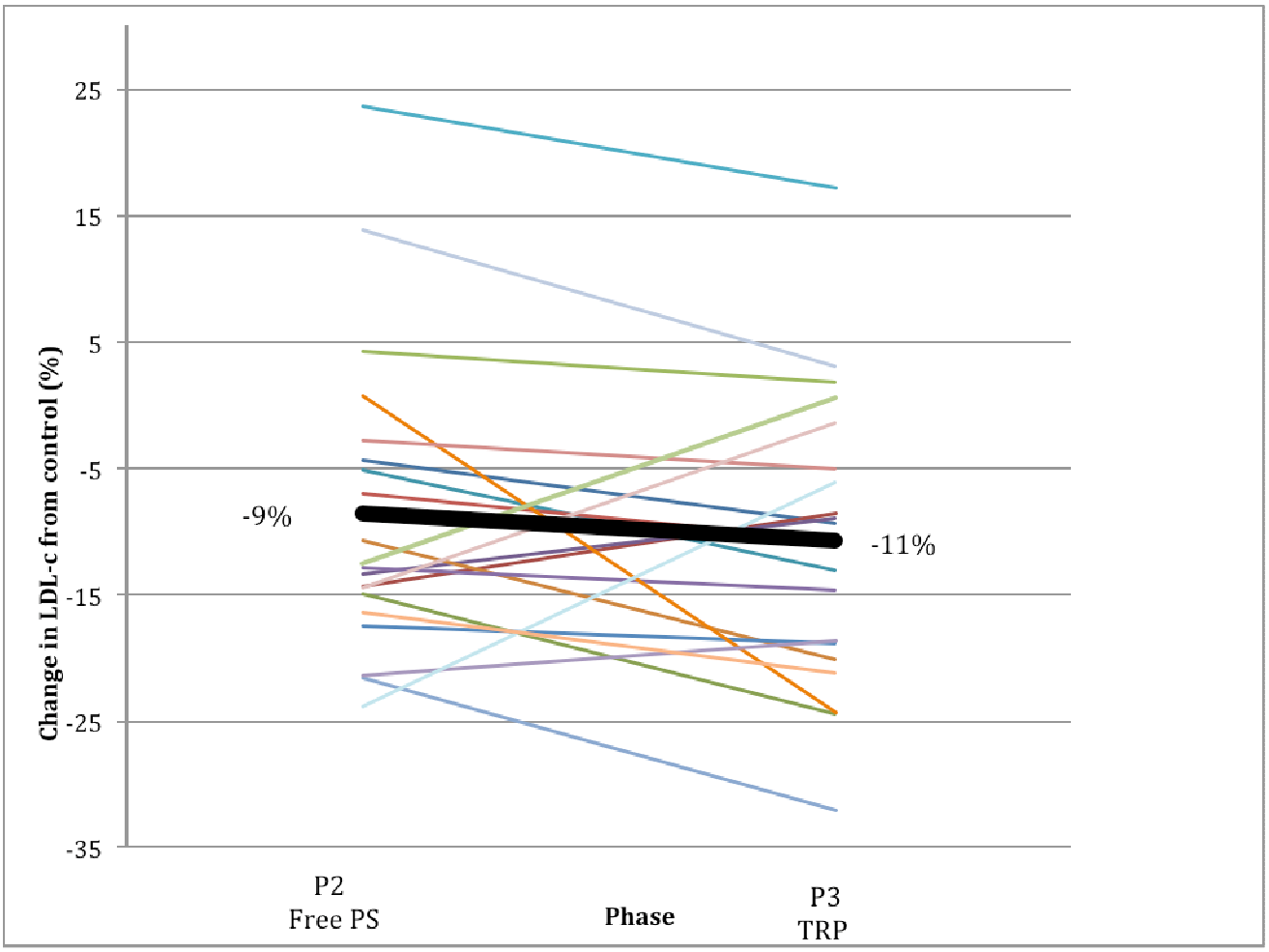

Figure 3. Individual LDL-c response from free PS milk to TRP milk. Percent change from the P1 control (2% milk) (n = 20).

The LDL-c results were also stratified according to the LDL-c classifications specified by the U.S. Department of Health and Human Services (76) (Figure 4). All of the strata resulted in reduced LDL-c in response to the two PS milks. Yet the magnitude in the reduction for the very high group was on average greater than the borderline high and near optimal group 21, 9 and 6 mg/dL respectively. However, when compared to the free PS milk the TRP milk resulted in greater percent changes in the near optimal and borderline high groups -68% (-3 mg/dL) and -42% (-3 mg/dL) respectively, where the very high group only resulted in a 2% (-0.5 mg/dL) reduction.

Table 8. Average plasma lipid values after each phase for responders only (n=17).

Parameter	P1 2% milk	P2 Free PS milk	P3 TRP milk	**P1 vs. P2** mean difference p value 95% CI Cohen's d	**P1 vs. P3** mean difference p value 95% CI Cohen's d	**P2 vs. P3** mean difference p value 95% CI Cohen's d
Total cholesterol (mg/dL)	222.3 ± 27	204.1 ± 28	197.9 ± 30	-18.2 .000 (-27.0, -9.29) .7	-24.4 .000 (-35.4, -13.3) .9	-6.2 .403 (-15.7, 3.3) .2
LDL-c (mg/dL)	138.8 ± 20	121.2 ± 17	119.3 ± 19	-17.6 .000 (-25.4, -9.8,) 1.0	-19.5 .000 (-28.6, -10.4) 1.0	-1.9 1.000 (-12.0, 8.1) .1
HDL-c (mg/dL)	64.2 ± 20	64.3 ± 21	60.0. ± 19	0.1 1.000 (-2.9, 4.6) .0	-4.2 .018 (-7.8, -0.6) .2	-4.3 .066 (-8.7, 0.2) .2
Triglycerides (mg/dL)	96.5 ± 43	93.4 ± 29	93.2 ± 42	-3.2 1.000 (-26.2, 19.8) .1	-3.4 1.000 (-21.7, 14.8) .1	-0.2 1.000 (-23.4, 23.0) .0
LDL/HDL cholesterol ratio	2.34 ± 0.7	2.05 ± 0.6	2.14 ± 0.60	-0.3 .000 (-0.5, -0.1) .4	-0.2 .242 (-0.5, 0.1) .3	0.1 1.000 (-0.2, 0.4) .2
Total/HDL cholesterol ratio	3.70 ± 0.9	3.38 ± 0.7	3.48 ± 0.7	-0.3 .001 (-0.5, -0.1) .4	-0.2 .595 (-0.6, 0.2) .3	0.1 1.000 (-0.2, 0.4) .1

Values are means ± SD; ($p<0.05$) using ANOVA repeated-measures and Bonferroni adjustments.
P1, Phase 1; P2 Phase 2; P3, Phase 3; 95% confidence interval.

The correlation between the baseline LDL-c value and the TRP LDL-c value is shown in Figure 5. The correlation is highly significant (<0.0001) with a Pearson Correlation of 0.844.

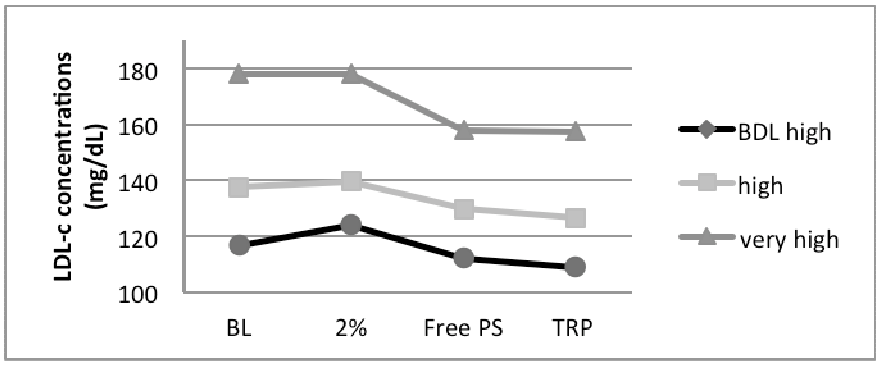

Figure 4. LDL-c responses to the milk products. Stratified according to the U.S. Department of Health and human Services LDL-c classifications; ( ) represents LDL-c concentrations of "near optimal" (100-129 mg/dL) (n of 9), ( ) represents LDL-c concentrations of "borderline high" (130-159 mg/dL) (n=8), ( ) represents LDL-c concentrations of "high to very high" (>160 mg/dL) (n=3).

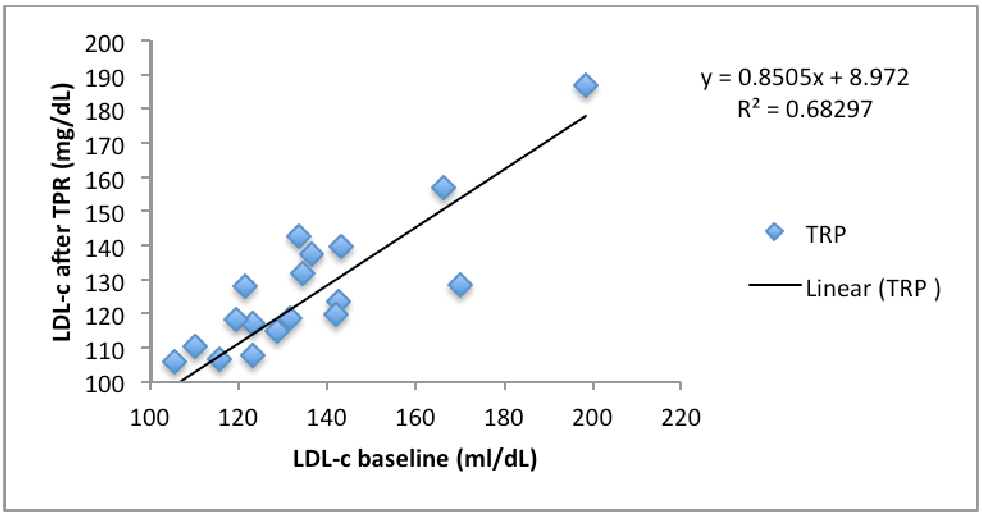

Figure 5. Correlation between baseline LDL-c values and the LDL-c response to TRP, (n=20).

Chapter 5

5.0.0 Discussion

In this study we confirmed that PS added to a non-fat milk matrix significantly lowers both total and LDL cholesterol. The results of the current study's free PS milk are in line with other recent milk with added PS intervention studies, which show significant, yet below the average (10%), reductions in LDL-c (36, 45, 59, 78). In fact, as far as we know, to date, no milk with added PS has lowered LDL-c beyond the average 10%. Other PS added dairy products such

as yogurts and cheese (56, 58), as well as, non dairy products such as margarine(26, 27, 41), mayonnaise (50) and grains (34, 60, 65, 66) have trials which lower LDL-c well above the average. These above average reductions are usually contributed to factors that increase the PS lipid solubility into the food matrix and include using stanol esters (17). Rather than use stanol esters our phase two milk intervention dispersed a fat-soluble novel TRP complex in a non-fat milk matrix. As expected the results showed an above average LDL-c reduction (60).

These results were complemented with the trending reduction in apo. B after the TRP milk intervention. There is one apo, B molecule per all non-HDL molecules (VLDL, IDL and LDL). Reductions are commonly seen in trials that use the lipid soluble stanol ester (26, 28, 29). These results indicate an actual reduction in number of lipoprotein in circulation, and may be attributed to potential liver adaptions. The adaptions respond to the reduced dietary cholesterol absorption and bile acid reabsorption, in an attempt to maintain the hepatic cholesterol pool. First VLDL lipoprotein synthesis is reduced second LDL-c receptor expression is increased (17) facilitating a larger uptake of LDL. The maintenance in apo. B after the free sterol milk intervention despite the reduction in LDL-c indicates that rather than reducing the absolute number of LDL particles, there may have been a change in particle sizes. Even though it is established that significant changes in particle sizes do not occur with a sterol intervention, there does seem to be a consistent insignificant trend showing a reduction in large LDL particles and an increase in small particles (61, 79).

The TRP complex is a favorable addition to a non-fat milk matrix and showed a 7% greater reduction in LDL-c when compared to the free PS non-fat milk in 14 of 20 subjects. The variation in individual responses to PS is still unclear, however, it most likely do to the ability of the PS to reduce enterocyte cholesterol absorption. It is commonly recognized that if the PS are more lipid soluble then their ability to incorporate into the micelle and displace dietary and biliary cholesterol is increased (12), ultimately reducing the absorption of cholesterols and reducing LDL-c concentration. On the contrary the six individuals with an increase in LDL-c, in response

to the TRP, may have an increased ability to absorb PS (11), and because the incorporation of TRP in the micelles is greater these individuals may have absorbed more PS. The removal of the PS from the intestine (3), then allows for an increased in cholesterol incorporation into the micelle, resulting in an increased absorption and reabsorption of the cholesterols (11).

The individual variability in PS and cholesterol absorbability may have also been the case for the three individuals who did not respond to either the free PS or the TRP. However, it is most likely due to an up-regulation of whole body cholesterol synthesis in response to the decrease in cholesterol absorption (80), which is indicative in individuals with an apo E4 allele phenotype (71). When these three subjects were removed from the analysis the responders expressed an above average 11 and 14 percent reduction in total and LDL cholesterol respectively when comparing the TRP to the 2% milk phases.

The whole body cholesterol synthesis is also positively correlated with baseline lathosterol concentrations. Lathosterol concentrations is a cholesterol precursor indicationg that the higher the lathosterol concentration the higher the cholesterol concentration. A positive correlation has also been associated with the baseline lathosterol concentrations and the LDL-c percentage change after a PS intervention (71, 80). These associations therefore, link the baseline LDL-c concentration with the LDL-c change after a PS intervention, which are also supported here. Individuals with a very high baseline LDL-c concentration resulted in reductions twice as high as the individuals with near optimal or borderline high baseline LDL-c concentrations.

An unexpected finding was the group's average reduction in HDL-c after the TRP intervention. The HDL-c was not reduced in our free PS milk phase, and to the best of our knowledge no PS mono-therapy intervention has ever shown a significant reduction in HDL-c. Only a few factors are known to reduce HDL-c concentration and include the dietary fatty acid composition, particularly the consumption of trans fatty acids (81). The dietary characteristics in our study population, as indicated by 3-day diet records during each phase, did not change in trans fatty acid or any nutrient consumption. Therefore, it can be deducted that the triglycerides

used in the TRP matrix may have been manufactured in a way that formed a trans bonds. Fortunately reducing the concentration of trans fatty acid in a food matrix has become more conventional and would be relatively simple to implement (82). It was also noted that when only the responders were analyzed the HDL-c during the TRP intervention was not significantly lower than the free PS milk intervention. This conclusion can be explained by a concept discussed above, individuals who do not respond to PS absorb the PS, which concurrently increases the absorption of the trans fatty acids in the TG recrystallized to the TRP complex, and thereby lowered HDL-c. Furthermore the responders, who do not absorb the PS did not absorb the trans fatty acids as readily and their HDL-c was not significantly lower. The production of apo. A1, which is associated with the HDL molecule was also not reduced in response to the TRP milk intervention, when all subjects were analyzed. These results indicate that the TRP complex did not increase the atherosclerotic risk (83) in responders to the TRP milk intervention.

Chapter 6

6.0.0 Conclusion:

Our results show that replacing 16 oz of 2% milk with 16 oz of non-fat milk with 2.0 g of added novel phytosterols in the form of TRP will significantly lower LDL-c above the average LDL-c reduction of 10%. Further study is needed to elucidate the relationship between the HDL-c response and the fatty acids used in the triglyceride recrystallization.

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Appendix A. Informed Consent document

Consent Form for Participation in a Research Project

Principal Investigator: Jeff S. Volek

Study Title: Cholesterol Lowering Effects of Milk with Added Plant Sterols

Study Sponsor: GFA Brands, Inc.

Invitation to Participate

You are invited to participate in this study that will examine how daily intake of milk with added plant sterols (plant cholesterol) affects the amount of cholesterol in your blood. Plant sterols are known to decreases LDL cholesterol, but how this specific plant sterol contained within milk impacts blood cholesterol levels has not been determined. The plant sterol will be added to milk containing different levels of milk fat and you will be asked to consume the milk twice a day for 12 weeks.

What are the study procedures? What will I be asked to do?

This research study will take place at the University of Connecticut (UConn) in Storrs and will last approximately 12 weeks. For this study, you will be required to follow your normal diet while supplementing with the provided milk. We will require you to visit our lab at least 1 time per week for the 12 week period. This is specifically what will happen during the research study.

Screening Visit: Your first visit will be a 30-minute screening visit, it will involve you filling out a medical, nutrition, dietary supplementation, exercise history and menstrual (for women) questionnaire. We will also determine your height, weight and blood pressure. We will ask you to fast overnight for 12 hours for this screening visit so we can obtain a small blood sample (about 10 mL or 2 teaspoons) to determine your cholesterol level. All blood draws in the study will be obtained by trained personnel. We are looking for men and postmenopausal women between 35 and 70 years of age who have moderately high levels or high levels of LDL cholesterol. You will be excluded if any of the conditions below are true:

Exclusion Criteria:

1) You take cholesterol lowering medications.
2) Your body weight is more than 320 pounds.
3) You have diabetes.
4) You regularly use tobacco products.
5) You plan on changing your physical activity in the next 3 months.
6) Your have lost or gained more than 7 pounds in the last 3 months.
7) You consume alcohol more than 3 drinks/day or 18/week.
8) You have an allergy to milk or are lactose intolerant.
9) Your LDL cholesterol is greater than 210 mg/dL or less than 100 mg/dL at screening.
10) You have been diagnosed with heart disease or have high blood pressure.

If you have a blood cholesterol level over 130mg/dL you will need signed approval from your doctor to participate in this study. If you are taking other medications or supplements known to affect cholesterol levels we may ask you to discontinue use to allow for an adequate washout period before beginning the study, or in some cases if you have been taking a consistent dose for an extended period of time we will have you continue on the same regimen. Your doctor will need to approve your decision to discontinue any medications or supplements and decide a washout period for you.

If you qualify based on the screening visit, we will schedule you to begin phase 1 of the study. This is the sequence of events that will take place.

Baseline Visit 1: For the first baseline visit, we will be asking you to fast for about 12 hours overnight before coming to the laboratory for blood testing and body composition scans. This means no food or drink that contains calories (including coffee) but you should drink plenty of water. We want you to be well hydrated during all tests. You must also avoid alcohol and strenuous exercise for at least 36 hours prior to coming to the laboratory for testing. We will obtain a small amount of blood from your arm using a needle. The amount of blood will be about 80 mL or 1/3 cup. We will also measure your body weight on a scale and your body composition (fat, lean, and bone weight) using a machine that will expose you to a small amount of X-ray radiation. You will lie quietly on a table while a scanning arm passes over your body from head to toe. You must remain still for about 5 min during this test. A certified X-ray technician will perform the scan. These tests will take about 1 hour.

Baseline Visit 2: We will ask you to come back to the laboratory on the following day (or as close as possible to your previous visit) to obtain another blood sample (about 10 mL or 2 teaspoons). For this visit, we will also require that you fast overnight for 12 hours. The reason for this second blood draw is to measure your cholesterol levels again to account for day-to-day variability. If by chance the 2 cholesterol levels are more than 15% apart, we will ask you to come back a third time to repeat the blood draw. This test will take about 30 min.

Milk Supplementation: After baseline testing we will provide you with the milk supplement. You will be drinking 16oz/day (2 cups) of cow's milk every day. You should try to drink one cup with a morning meal and one cup with an evening meal. You will receive your first milk supplement supply at the baseline blood draw, which should last about one week. You will be expected to come in each week for a new milk supply and a weight check. You do not need to be fasted during the milk pick up and weight check. We will also be in contact with you weekly to check that you are consuming your milk and see how you are feeling. The milk supplementation will continue for 12 weeks.

Follow-Up Testing: We will repeat the baseline testing again after 4, 8, and 12 weeks of milk supplementation. At each of these time points, we will measure your body weight and body composition, as well as 2 separate blood draws as described for Baseline Visits 1 and 2.

Diet: You will be asked to follow a diet that is as close to usual as possible over the 12 weeks. We want you to continue your normal diet so that your weight will stay about the same throughout the study. In order to help you with the diet and monitor compliance, we will ask you to complete a 3-day food record before starting, and every 2 weeks for a total of 7 food records. You will meet with a Registered Dietitian and receive instructions on how to complete the food logs (writing down all foods and beverages you consume over the day). You will meet with a Dietitian after each food record to go over the results and make changes accordingly to maintain weight.

Blood Work: The primary markers we will measure in your blood will include cholesterol levels, glucose, insulin, and fat soluble vitamins. The amount of blood taken being taken is relatively small at any one visit. The total amount of blood taken over the entire 12 week project will not exceed 410 mL (less than a pint).

Additional Testing Period. In addition to the 12 week intervention described above, we would like to invite you to participate in an additional 4 week intervention period to study the effects of a slightly different milk supplement containing plant sterols. The same protocols will be followed as described in Baseline Visit 1 and Visit 2. Thus, we will be taking blood and measuring your body weight and body composition before and after the 4 weeks of milk supplementation. The total amount of blood we will obtain during the 4 visits to the lab will be about 180 mL (3/4 cup) and the total amount of testing time will be about 3 hours.

What are the risks or inconveniences of the study?

Managing Hyperlipidemia. The risk of elevated LDL cholesterol can primarily increase chances of developing cardiovascular disease. Dietary modification is a preferred first line of approach, and use of plant sterol has been shown to be effective in many people to lower blood cholesterol levels. The nature of this project is such that a daily dose of plant sterol will be provided to all subjects, thus there is a good chance you may see a decrease in LDL cholesterol and therefore a decrease in risk for heart disease. If your starting LDL cholesterol is greater than 130 mg/dL, you will need to obtain written permission from your personal physician.

Milk with Added Plant Sterols. There have been a large number of research studies examining plant sterols and they have been found to be well tolerated, especially at the dose of 2 g/day used in this study. Their long-term safety has not been established. Plant sterols work by partially blocking the absorption of dietary cholesterol into your blood, and therefore they often lower blood levels of cholesterol in people. Some studies have shown that blood levels of fat soluble vitamins may decrease, but this is unlikely to have any significant affect on your health in a 12 week period.

Blood Draws. Blood draws with a needle may cause discomfort at the puncture site and the development of a slight bruise. You may also experience lightheadedness or fainting during the blood draw. There is a slight risk of infection from these procedures. All possible precautions to avoid infection will be taken including use of sterile disposable needles, drapes and gauze and the practice of aseptic techniques during blood sampling. All blood samples will be obtained by trained people. You should refrain from giving blood during the course of the study.

Body Composition. You will be exposed to a very small amount of radiation by the scanner used to measure your body composition. Exposure to any amount of X-ray radiation, no matter how low, may cause abnormal changes in cells. However, the body continuously repairs these changes and the amount of radiation is very low in this study. The total exposure for a whole body scan is approximately 125 times less than the average radiation from a standard chest x-ray. Thus, the radiation levels are extremely low and the health risk minimal.

What are the benefits of the study?

The results of this study will help to determine the role plant sterols in milk have on responses to cholesterol and general health, and therefore provide a potential therapeutic option for people trying to manage their cholesterol levels within a healthy range. You will be provided with the opportunity to talk with a Registered Dietitian regarding diet during the study. You will also learn your body composition and will possibly improve health status. You will receive your pre and post cholesterol levels and have this information to discuss with your doctor in order to plan for follow-up care if needed. You may not benefit directly from this study.

How will my personal information be protected?

The following procedures will be used to protect the confidentiality of your data. The researchers will keep all study records (including any codes to your data) locked in a secure location. The results of this study will be kept in locked cabinets under the supervision of Dr. Volek. Research records will be labeled with a unique code that will not contain any information that could be linked to your identity. A master key that links names and codes will be maintained in a separate and secure location. The master key will be destroyed after 3 years from study completion. All electronic files (e.g., database, spreadsheet, etc.) containing identifiable information will be password protected. Any computer hosting such files will also have password protection to prevent access by unauthorized users. Only the members of the research staff will have access to the passwords. Data that will be shared with others will be coded as described above to help protect your identity. At the conclusion of this study, the researchers may publish their findings. Information will be presented in summary format and you will not be identified in any publications or presentations.

You should also know that the UConn Institutional Review Board (IRB) and the Office of Research Compliance may inspect study records as part of its auditing program, but these reviews will only

focus on the researchers and not on your responses or involvement. The IRB is a group of people who review research studies to protect the rights and welfare of research participants.

What happens if I am injured or sick because I took part in the study?

In the event you become sick or injured during the course of the research study, immediately notify the principal investigator or a member of the research team. If you require medical care for such sickness or injury, your care will be billed to you or to your insurance company in the same manner as your other medical needs are addressed.

However, if you believe that your illness or injury directly resulted from the research procedures of this study, you may be eligible to file a claim with the State of Connecticut Office of Claims Commissioner. For a description of this process, contact the Office of Research Compliance at the University of Connecticut at 860-486-8802.

Can I stop being in the study and what are my rights?

You do not have to be in this study if you do not want to. If you agree to be in the study, but later change your mind, you may drop out at any time. There are no penalties or consequences of any kind if you decide that you do not want to participate. You will be notified of all significant new findings during the course of the study that may affect your willingness to continue.

You may be withdrawn from the study at any time. Conditions for such a withdrawal may include missed appointments, non-adherence to study procedures, disruptive behavior during study procedures, and/or adverse reactions to the supplement.

Who do I contact if I have questions about the study?

Take as long as you like before you make a decision. We will be happy to answer any question you have about this study. If you have further questions about this project or if you have a research-related problem, you may contact the principal investigator, Jeff S. Volek at 860-486-6712. If you have any questions concerning your rights as a research subject, you may contact the University of Connecticut Institutional Review Board (IRB) at 860-486-8802.

Documentation of Consent:

I have read this form and decided that I will participate in the project described above. Its general purposes, the particulars of involvement and possible risks and inconveniences have been explained to my satisfaction. I understand that I can withdraw at any time. My signature also indicates that I have received a copy of this consent form.

_____ _____ _____

Participant Signature: Print Name: Date:

_____ _____ _____

Signature of Person Obtaining Consent Print Name: Date: