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# Reduction of Obesity-Associated Oxidative Stress by Low-Fat Yogurt Consumption

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# **Reduction of Obesity-Associated Oxidative Stress by Low-Fat Yogurt Consumption**

Diana Michelle DiMarco

B.S., Western Washington University, 2011

A Thesis

Submitted in Partial Fulfillment of the

Requirements for the Degree of

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University of Connecticut

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# APPROVAL PAGE

Master of Science Thesis

## Reduction of Obesity-Associated Oxidative Stress by Low-Fat Yogurt Consumption

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## List of Abbreviations

|  |   |
|--|---|
| ACE: Angiotensin-converting enzyme         | Hcy: Homocysteine   |
| AGE(s): Advanced glycation endproduct(s)   | HPLC: High performance liquid chromatography  |
| ANOVA: Analysis of variance                | IL-1 $\beta$ : Interleukin 1-beta   |
| AU: Arbitrary units                        | IL-6: Interleukin 6   |
| AUC: Area under the curve                  | ICAM: Intercellular adhesion molecule   |
| BCA: Bicinchoninic acid                    | LBP: LPS-binding protein  |
| BMI: Body mass index                       | LDL: Low density lipoprotein  |
| BMR: Basal metabolic rate                  | LPS: Lipopolysaccharide   |
| CAM: Cell adhesion molecule                | LS: Lean soy  |
| CD14: Cluster of differentiation 14        | LY: Lean yogurt   |
| CEL: N-carboxyethyllysine                  | MCP-1: Monocyte chemoattractant protein-1   |
| CML: N-carboxymethyllysine                 | MDA: Malondialdehyde  |
| CRP: C-reactive protein                    | Met: Methionine   |
| CVD: Cardiovascular disease                | MHO: Metabolically healthy obese  |
| Cys: Cysteine                              | NADPH: Nicotinamide adenine dinucleotide phosphate (reduced)                              |
| CysS: Cystine (oxidized cysteine)          | NDSR: Nutrition Data Systems for Research   |
| DTNB: 5,5'-Dithiobis-(2-nitrobenzoic acid) | NF- $\kappa$ B: Nuclear factor kappa-light-chain-enhancer of activated B cells protein 65 |
| DV: Daily value                            | NO: Nitric oxide  |
| EDTA: Ethylenediaminetetraacetic acid      | OS: Obese soy   |
| GSH: Glutathione                           |   |
| GSSG: Glutathione disulfide                |   |

OY: Obese yogurt

PBS: Phosphate-buffered saline

RAGE: Receptor of advanced glycation endproducts

RDA: Recommended dietary allowance

rEI: Reported energy intake

RNS: Reactive nitrogen species

ROS: Reactive oxygen species

sCD14: Soluble CD14

SH: Total thiols

SOD: Superoxide dismutase

TBA: Thiobarbituric acid

TLR4: Toll-like receptor 4

TNF $\alpha$ : Tumor necrosis factor alpha

VCAM: Vascular cell adhesion molecule

## **Abstract**

Excess macronutrient intake leads to an increase in formation of reactive oxygen species (ROS). This causes an imbalance between ROS generation and the ability of the body to neutralize these radicals, a state known as oxidative stress. Previous research suggests the ability of dairy products to attenuate the postprandial response, which may reduce ROS formation. Therefore, this study aimed to elucidate the impact of low-fat yogurt consumption on fasting and postprandial oxidative stress in obese women.

We hypothesized that co-consumption of low-fat yogurt and a high-calorie, high-fat meal would reduce postprandial elevations in oxidative stress in obese women. To test this hypothesis, healthy, lean and obese women co-consumed low-fat yogurt or soy pudding (control) and a high-calorie, high-fat meal on 2 occasions separated by 9-week daily yogurt or pudding consumption. Postprandial blood samples were collected for 4 h during meal challenges and fasting samples were collected at 3 week intervals throughout the duration of the intervention. Fasting and postprandial plasma samples were analyzed for malondialdehyde (MDA), total thiols (SH), and advanced glycation endproducts (AGEs). A secondary aim of this work was to classify changes in dietary intake associated with the intervention; data was based on 3-day self-reported dietary records.

Low-fat yogurt consumption did not lead to changes in BMI, waist circumference, or blood pressure. Consumption of low-fat yogurt attenuated postprandial increases in MDA in obese women at both the initial and final meal challenges. However, 9-week yogurt consumption did not further attenuate the postprandial response and had no effect on fasting MDA concentrations

in obese women. Yogurt and pudding prevented postprandial changes in SH and AGEs, while chronic plasma SH and AGEs were unaffected by 9-week yogurt or soy pudding intake. Daily consumption of low-fat yogurt and soy pudding increased calcium and vitamin D intake and yogurt increased dairy intake in lean and obese women. Therefore, the addition of low-fat yogurt to the diet may be a strategy to increase dairy, calcium, and vitamin D intake without inducing any unfavorable changes in body composition, blood pressure, and postprandial or chronic levels of oxidative stress.



## Introduction

Epidemiological studies support an association between optimal dairy intake and beneficial health outcomes, including favorable changes in body composition and blood pressure, and reduced incidence of certain metabolic diseases. Among obese individuals, regular dairy consumption is also linked to reductions in biomarkers of oxidative stress and inflammation [1-3]. Obesity is associated with increased levels of oxidative stress and the presence of chronic, low-level systemic inflammation, both of which play crucial roles in the development of many chronic diseases, including cardiovascular disease (CVD) [4-6]. Therefore, regular intake of dairy products may attenuate development of these diseases.

Increasingly, atherosclerosis is considered to be a postprandial phenomenon; postprandial elevations in plasma glucose and triglycerides, as well as increases in oxidative stress and inflammation, are considered more accurate markers of future cardiovascular risk than are fasting levels of these biomarkers [7]. Postprandial hyperglycemia, hypertriglyceridemia, oxidative stress, and inflammation can be induced even in healthy individuals by consumption of a high-calorie, high-fat meal, which are common components of the Western diet [6-9]. Excess energy intake overwhelms the metabolic and endogenous antioxidant systems, leading to increased generation of reactive oxygen species (ROS) and reduced ability to neutralize these harmful species [8]. This imbalance between generation and quenching of ROS is known as oxidative stress [10].

The consequences of postprandial oxidative stress include the oxidation of lipids, proteins, and nucleic acids by ROS [10, 11]. Malondialdehyde (MDA) is a product of polyunsaturated fatty

acid oxidation and is a biomarker for lipid peroxidation [10, 12, 13]. Plasma thiols, including glutathione, cysteine, and methionine, are easily oxidized by ROS [11]. Decreases in plasma total thiols (SH) may therefore be a biomarker for oxidative damage [14, 15]. In addition, excess carbohydrate intake results in hyperglycemia, which leads to an increase in the formation of advanced glycation endproducts (AGEs) – aldehyde-adducted lipids, proteins, or nucleic acids [16]. Therefore, plasma AGEs are a biomarker for elevated blood glucose. Postprandial increases in ROS may also trigger postprandial increases in the inflammatory response [17].

Previous research suggests that the postprandial response to a high-calorie, high-fat meal may be modified by co-consumption of certain foods. For example, orange juice favorably impacted the postprandial inflammatory response as well as biomarkers of oxidative stress in healthy individuals [18]. Calcium, protein, and low-fat milk have also been examined and found to attenuate the postprandial glucose and lipid responses [19, 20]. In addition to calcium and protein, low-fat yogurt also contains whey, casein, vitamin D and probiotics, all of which are known to have beneficial impacts on health. In particular, average intake of calcium and vitamin D is below recommendations for many women [21]. Therefore, regular consumption of low-fat yogurt may decrease baseline levels of oxidative stress while increasing calcium and vitamin D intake in obese women. Co-consumption of low-fat yogurt with a high-calorie, high-fat meal may also attenuate postprandial increases in oxidative stress in obese women. This research aims to examine these outcomes.

The study population consists of healthy, lean (BMI 18.5-27 kg/m<sup>2</sup>; n = 66) and obese (BMI 30-40 kg/m<sup>2</sup>; n = 66), premenopausal women between the ages of 21 and 55. The primary outcome of this research is to examine the impacts of low-fat yogurt intake on intestinal barrier function and inflammation. The secondary outcomes of this research are to examine the impacts of low-

fat yogurt consumption on postprandial and chronic oxidative stress. These secondary outcomes are the focus of the upcoming chapters; my colleagues will elaborate upon the acute and chronic impacts of yogurt consumption on intestinal barrier function and inflammation at a later date.

The primary hypothesis for this study is that co-consumption of low-fat yogurt with a high-calorie, high-fat meal will reduce postprandial inflammation in obese women and, to a lesser extent, in lean women by improving intestinal barrier function. Additionally, sustained consumption of low-fat yogurt will reduce chronic inflammation in obese women and will further attenuate the postprandial inflammatory response. These modulations will also be observed to a lesser degree in lean women. Reductions in intestinal barrier dysfunction and systemic inflammation will be evident by suppression of fasting levels of and postprandial increases in lipopolysaccharide (LPS), LPS-binding protein (LBP), and soluble CD14 (sCD14), as well as the expression of certain pro-inflammatory cytokines.

The secondary hypothesis for this study is that co-consumption of low-fat yogurt with a high-calorie, high-fat meal will reduce postprandial oxidative stress in obese women. In addition, sustained intake of low-fat yogurt will reduce fasting levels of oxidative stress and will further attenuate the postprandial oxidative response in obese women. This will be evident by reductions in MDA and AGEs and increases in plasma SH. Lastly, intake of dairy products, calcium, and vitamin D will be significantly increased by daily yogurt consumption. However, because this intervention aims to maintain an isocaloric diet, changes in calorie, carbohydrate, fat, and protein intake are expected to be negligible.

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# **Chapter 1:**

## **Literature Review**

## 1.1 Background

Cardiovascular disease (CVD) is the leading cause of death in the United States, responsible for an estimated 1 in 3 fatalities [1]. Overweight and obesity are associated with marked increases in the prevalence of CVD and its associated complications [1]. The current obesity rate among adults in the United States is close to 35%, with the combined rate of overweight and obesity nearing 70% [1]. Obesity is also closely associated with a myriad of metabolic disruptions, including insulin resistance and/or diabetes, hyperglycemia, hyperlipidemia, hypercholesterolemia, and hypertension [1-3]. Together, elevated fasting triglycerides, fasting glucose, blood pressure, waist circumference, and low HDL cholesterol form the criteria for metabolic syndrome, a chronic state characterized by the presence of any 3 of these 5 conditions [2]. Most often, obesity is the main factor in the development of metabolic syndrome [4].

Though there are many drugs and medical interventions designed to modulate these conditions, treatments are not without side effects and often present serious complications [5]. Therefore, dietary means of reducing the negative health outcomes associated with obesity are of utmost importance. One approach is weight loss; however, this is often an unsuccessful endeavor. A complementary approach is to examine the ability of certain foods to reduce the occurrence of the metabolic changes associated with obesity and the development of atherosclerosis, thus reducing future CVD risk.

The hypothesis that the development of atherosclerosis is a postprandial phenomenon has gained greater acceptance in recent years [6]. The postprandial period is the period following nutrient intake during which changes occur in the body that enable it to metabolize, transport, and store these nutrients. The postprandial period may last up to 8 h following consumption of a meal and

encompasses changes such as increases in metabolic processes, blood glucose and triglycerides, and oxidative and inflammatory stress [7]. Postprandial dysmetabolism is characterized by above-normal increments of these changes and is an independent risk factor for future cardiovascular events [8]. Postprandial dysmetabolism is often seen in obese individuals; however, it can be induced, even in healthy individuals, by consumption of a high calorie and/or high-fat meal [6, 9]. This is crucial because postprandial glucose and triglyceride concentrations are more closely associated with risk of CVD and coronary events than are fasting values [6, 10].

Also of concern for individuals who consume a Western diet is the fact that the majority of the day is spent in the postprandial state [11]. In many cases, the effects of the previous meal have not subsided by the time the next is consumed, resulting in a sustained postprandial period. For individuals with postprandial dysmetabolism, this translates to extended periods of time in which the body is exposed to these unfavorable metabolic changes. Therefore, strategies to attenuate postprandial dysmetabolism are potentially beneficial for reduction of CVD risk.

## **1.2 Inflammation**

Acute inflammation is a component of the body's natural response to injury, pathogens, or other undue stress [12]. Initiation of inflammation involves the activation of toll-like receptor (TLR) signaling, activation of the pro-inflammatory gene transcription factor nuclear factor  $\kappa$ B (NF- $\kappa$ B), and endoplasmic reticulum stress. The result is an increase in the synthesis and release of pro-inflammatory cytokines, including tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), interleukin 6 (IL-6), interleukin 1 $\beta$  (IL-1 $\beta$ ), the chemokine monocyte chemoattractant protein-1 (MCP-1), and C-reactive protein (CRP), an acute-phase reactant synthesized by the liver in response to increased



IL-6 production [12]. Once the injury is healed or the pathogen eliminated, the pro-inflammatory pathways are inhibited and the inflammation is reduced. This is known as acute inflammation.

Chronic inflammation is defined as sustained, low-level inflammation and is now known to be a component of many metabolic diseases [12, 13]. Certain autoimmune diseases, as well as conditions involving sustained tissue injury, are contributing factors. In addition, chronic inflammation is associated with obesity, as adipocytes are main contributors to cytokine production [13]. Indeed, macrophage infiltration into adipose leads to release of pro-inflammatory cytokines, which contribute not only to inflammation but also to obesity-associated insulin resistance via modification of the insulin receptor signaling pathway [12]. While some cytokines act locally to recruit leukocytes and other inflammatory mediators to the site of the injury or to the adipose, others may enter into systemic circulation, causing inflammatory changes elsewhere in the body [12].

Inflammation can also be induced in the postprandial period as a result of energy intake [8, 14]. Excess macronutrient intake leads to an increase in free-radical generation; these species are natural byproducts of carbohydrate and lipid metabolism which, in high amounts, can damage cells and tissues, eliciting an immune response to repair the damage [15]. This postprandial inflammation may contribute to chronic inflammation [12]. In addition, high-calorie, high-fat meals challenge the intestine and may compromise intestinal barrier function [16]. Studies support an association between intestinal barrier dysfunction and inflammation; therefore, high-calorie, high-fat meals may induce intestinal barrier-mediated inflammation [16].

Obesity is also associated with reduced integrity of the intestinal barrier, though the mechanisms involved remain unclear. Perhaps obesity-associated intestinal barrier dysfunction occurs due to

the constant stress of consuming excess calories and/or fat. Alternatively, obesity is known to be associated with an altered intestinal bacterial profile [17]. As these microbes contribute to the intestinal immune response, obesity-associated changes in the microbiota may lead to reductions in these normal protective mechanisms [18]. It is also possible that alterations in intestinal microbiota are a causal factor in the development of obesity [19]. Clearly much remains unknown in this area of research.

Regardless of the cause, disruptions in intestinal barrier function lead to increased translocation of lipopolysaccharide (LPS) into the bloodstream. LPS is a component of the membranes of Gram-negative bacteria and elicits a strong immune response [18, 20]. It is always present in the gut microbiota but is not typically absorbed into the bloodstream [21]. However, in cases of compromised intestinal barrier function, the integrity of tight junctions may be compromised, allowing LPS to be absorbed paracellularly [20, 21]. LPS is also incorporated into chylomicrons; thus, a meal high in fat may facilitate LPS uptake [20, 21]. In addition, a chronic high-fat diet has been shown to alter the intestinal microbiota, in particular by reducing the presence of *Bifidobacterium* spp [21]. Bifidobacteria may act to improve intestinal barrier function and are inversely associated with plasma LPS in mice [16]. Therefore, a chronic high-fat diet may increase intestinal permeability by both acute and chronic mechanisms [16, 21].

Once in the bloodstream, LPS elicits a strong immune response. This begins with the association of LPS with LPS-binding protein (LBP), which transports LPS to the CD14/MD-2/TLR4 receptor complex on the surface of macrophages and myeloid cells [18, 22]. LPS binding triggers a pro-inflammatory cascade that ultimately results in increased NF- $\kappa$ B expression, promoting inflammation [20].

Reductions of postprandial and chronic intestinal barrier dysfunction can be assessed by measuring expression of LPS, LBP, NF- $\kappa$ B, and certain pro-inflammatory cytokines, including IL-6 and TNF $\alpha$ . In addition, CD14 expression may be indicative of changes in intestinal barrier function. CD14 is a membrane-bound protein; however, it also exists in a soluble form (sCD14), which is expressed in many cells, including hepatocytes and adipocytes [18, 22]. sCD14 is present in plasma due to secretion from cells or by cleavage of membrane-bound CD14 derived from macrophages [18]. sCD14 is thought to be a protective mechanism, competing with CD14 for LPS binding and acting to prevent LPS-CD14 triggered inflammation [22]. Therefore, increased sCD14 expression is indicative of increased LPS exposure.

### **1.3 Oxidative stress**

Oxidative stress is defined as an imbalance between the generation and neutralization of oxidative species in the body [7]. Oxidative species are natural byproducts of human metabolism. Due to their high reactivity and potential to damage cells and tissues, the body has endogenous antioxidant defense systems designed to neutralize these species. When oxidant production exceeds the capacity of these systems, the excess oxidants cannot be neutralized and are free to interact with cells and tissues, resulting in oxidative damage. Oxidative stress is linked to the pathogenesis of atherosclerosis as well as chronic inflammation [15, 23].

However, some changes in the oxidative balance of the body are normal and, in fact, integral to proper control of many enzymes, cytokines, and body systems [24-26]. For example, transcription of NF- $\kappa$ B can be activated by reactive oxygen species (ROS) [27, 28]. Many other signaling pathways, under the control of protein kinases and/or phosphatases, are also regulated

by the redox state of plasma [24, 26, 28]. These pathways are involved in normal physiological processes, such as cellular signaling, repair, or apoptosis [26]. Recent studies also show that transient increases in ROS in adipocytes may actually perpetuate insulin signaling while decreasing further lipid accumulation [4, 29]. Therefore, such regulated short-term alterations in oxidant concentration are a normal and necessary aspect of proper physiological function. However, a prolonged state of imbalance does not translate to sustention of these effects [4].

The consequences of dysregulated oxidative stress are widespread and numerous. ROS and reactive nitrogen species (RNS) can interact with and oxidize LDL, nitric oxide (NO), proteins, lipids, and nucleic acids, causing irreversible damage [30]. ROS and RNS may also regulate the activities of certain transcription factors, expression of pro-inflammatory genes, and subsequent synthesis of pro-inflammatory cytokines [15, 31].

Oxidative stress is induced through nutrient intake, which leads to an increase in metabolic processes such as glycolysis and fatty acid oxidation [8]. Under normal circumstances, the oxidative species generated through these processes are neutralized by the body. However, when excessive nutrient intake occurs, it overwhelms the capacity of these metabolic systems and, subsequently, mitochondrial oxidative phosphorylation, leading to an increase in free radical generation [8]. These excess free radicals cannot be neutralized properly. This is known as postprandial oxidative stress.

A consequence of postprandial oxidative stress is endothelial dysfunction – an impairment in endothelium-dependent vasodilation and vascular integrity [11]. This is also an early indicator of CVD. Vasodilation is controlled by numerous endothelial-derived factors, including NO. NO is also responsible for inhibiting platelet aggregation and monocyte adhesion to the vascular walls,

as well as decreasing capillary permeability, all of which are critical to the maintenance of vascular integrity [11]. NO is particularly sensitive to the presence of ROS and can be easily oxidized, reducing its bioavailability and leading to loss of proper endothelial function [15, 23]. Endothelial dysfunction is apparent even in healthy individuals in as little as a few hours following fat consumption [32]. Because of the frequency of meal intake, the effects of the previous meal will not have subsided by the time the next is consumed, leading to a prolongation of the postprandial state. This in turn translates to extended periods of endothelial dysfunction.

A second byproduct of postprandial oxidative stress is lipid peroxidation. Oxidative modification of LDL particles is an early step in the formation of atherosclerosis [7]. In addition, malondialdehyde (MDA) – a product of polyunsaturated fatty acid oxidation – can react with the amino groups of lipoproteins, forming MDA-adducted LDL particles [7]. Oxidized phospholipids can also accumulate in LDL particles [7]. Increases in free and protein-bound MDA are measurable in plasma during the postprandial period [27, 33].

Excess nutrient intake may also lead to oxidative damage via the formation of advanced-glycation endproduct(s) (AGE(s)), non-enzymatic modifications of proteins, lipids, and nucleic acids by aldehydes [30]. Endogenously, glucose is the most common aldehyde involved in AGE formation. Nutrient-induced ROS production also leads to oxidation of plasma thiols, including glutathione (GSH), methionine (Met), and cysteine (Cys). When ROS react with thiols, oxidized glutathione (GSSG), homocysteine (Hcy), and cystine (CysS), are generated [34]. These oxidized species often dimerize, forming disulfide bridges. To counteract this, additional thiols may be released into the bloodstream to restore the plasma antioxidant capacity [6, 25]. This is measurable in the postprandial period.

Due to the nature of these reactions, chronic elevations in glucose or triglycerides in the plasma may lead to elevated levels of MDA, AGEs, and/or oxidized thiols. This is indicative of elevated levels of oxidative stress and thus, increased risk of oxidative damage. Therefore, dietary interventions aimed at reducing baseline plasma glucose and lipids may reduce oxidative stress. Likewise, strategies to blunt the postprandial increases in plasma glucose and triglycerides triggered by excess nutrient intake may reduce postprandial oxidative changes that are associated with atherosclerotic changes in the body.

#### **1.4 Connections between inflammation and oxidative stress**

That co-consumption of antioxidants (such as fruit juice) can attenuate postprandial inflammation induced by a high-fat meal supports a connection between oxidative state and inflammation [35]. There are multiple methods by which this modulation may occur.

Inflammation is the body's natural response to injury; therefore, damage caused by oxidative species activates the immune response [24]. This includes the release of pro-inflammatory cytokines and macrophage recruitment to the affected area [24]. Reduction of oxidative species will reduce these damages, thus attenuating the inflammatory response.

A second pathway connecting inflammation and oxidative stress is via lipid peroxidation, which occurs naturally in the body in apoptotic cells as part of the programmed cell death process [36].

Lipid peroxidation elevates the immune response to properly carry out the apoptotic process.

However, lipid peroxidation in non-apoptotic cells can also occur in cases of elevated oxidative stress, such as that associated with obesity. Indeed, lipid peroxidation is positively correlated with BMI and waist circumference [4]. Though transient lipid peroxidation is necessary in cases

of apoptosis, excessive or sustained exposure to lipid peroxides upregulates the immune response [37, 38]. As such, long-term pro-oxidative conditions may lead to a steady state of chronic, low-level inflammation [14]. This type of inflammation is associated with the development and progression of many chronic diseases, including atherosclerosis, vascular diseases, and metabolic syndrome [39, 40]. It is also associated with obesity [13].

ROS and RNS also modulate cytokine release. In the postprandial state, NO reacts with the superoxide radical to generate peroxynitrite, a highly reactive species that oxidizes proteins and lipids [7]. Peroxynitrite, along with oxidative byproducts of meal intake (oxidized glucose and fatty acids), stimulates the release of inflammatory cytokines as well as cell adhesion molecules (CAMs), which promote monocyte adhesion to the endothelium [7, 26]. These oxidative species may also interact with and damage the endothelium, similarly triggering an immune response. Adherent monocytes then migrate into the subendothelial space, where they differentiate into macrophages and begin to take up circulating lipids [41]. In particular, uptake of oxidized LDL is an unregulated process; excess uptake leads to foam cell formation. Concurrently, recruitment of more macrophages, as well as proliferation of smooth muscle cells, are promoted by macrophage proinflammatory cytokine release, which further damages the endothelium [42]. Thus begins the cyclical process of lesion and, ultimately, plaque formation [41, 42]. In this way, oxidative stress is directly related to inflammation.

Additionally, in adipose tissue, ROS production leads to a dysregulation in adipokine production triggered by macrophage infiltration [4, 43]. In adipocytes ROS increase NADPH oxidase 4 activity, which leads to continued ROS production, perpetuating this cycle [4]. ROS damage-induced macrophage infiltration into adipocytes also contributes to increased proinflammatory cytokine production, including induction of IL-6 and MCP-1 synthesis. Moreover, adipose tissue

seems to be the main mediator of circulating inflammatory cytokine concentration; increasing adiposity is associated with increased plasma proinflammatory cytokines [4, 6]. Adipose may also be the main contributor to circulating oxidative species and byproducts, including lipid peroxides [4]. The result of these processes is a state of chronically-elevated oxidative stress and inflammation in overweight and obese individuals.

Because oxidative stress and inflammation are so closely connected, attenuation of one may subsequently reduce the other. Decreases in chronic oxidative stress may reduce cellular injury, thereby reducing activation of the inflammatory response. Likewise, reductions in oxidative stress, particularly in adipose, may attenuate ROS-induced dysregulation of cytokine production. In the postprandial period, reduction of nutrient-induced ROS generation will limit oxidative damage and, subsequently, inflammation. Therefore, dietary interventions aimed at reducing both baseline and postprandial oxidative stress and inflammation, particularly among obese individuals, are of great interest.

### **1.5 Induction of postprandial oxidative stress and inflammation**

It has long been established that certain dietary alterations can reduce plasma glucose, lipids, and/or cholesterol, as well as blood pressure, over the long term. However, due to the emerging understanding of the connection between postprandial dysmetabolism and atherosclerosis, research is shifting to focus on the extent to which dietary interventions can attenuate these changes. It is well documented that just one instance of excess nutrient intake can trigger postprandial changes in the body [9, 24]. This includes meals containing high amounts of



carbohydrates, fat, and/or calories. High-carbohydrate meals lead to postprandial hyperglycemia. High-fat meals lead to postprandial hypertriglyceridemia and may increase the absorption of LPS, which was discussed previously [20].

To further examine these postprandial phenomena, Miglio, et al. designed an intervention to examine the 8 h postprandial response to a high-fat meal among overweight and obese individuals [6]. The meal was comprised of fried eggs, fried potatoes, cheese, a roll, and a high-sugar beverage, totaling approximately 1400 calories, including 82 g of fat, 123 g of carbohydrates, and 51 g of protein. Following the meal, they observed increases in plasma glucose, triglycerides, total cholesterol, TNF $\alpha$ , and IL-6. Glucose levels peaked within the first hour and returned to their baseline values rather rapidly. However, triglyceride, cholesterol, TNF $\alpha$ , and IL-6 concentrations remained elevated throughout the entire study period, indicating that postprandial changes associated with a high-fat meal can last most of the day [6]. In a review by Sanders, the author suggests that as little as 40-50 g of fat in a single meal is sufficient to induce these changes. Subsequent meals may further prolong these postprandial alterations [9]. A pair of studies suggests that an increase in plasma triglycerides of as little as 20-50 mg/dL may be sufficient to reduce endothelial function and increase expression of CAMs in healthy individuals. Ceriello, et al. report that a high-fat meal or a high glucose load each increase CAM levels postprandially and that, when combined, this effect is additive and may endure for greater than 4 h [44].

Additionally, dietary interventions have been designed to examine the impact of long-term dietary patterns on the postprandial response. A study by Sanders, et al. revealed that high oleic acid and high trans-fat diets, consumed for two weeks, increased postprandial lipemia to a greater extent than a high carbohydrate diet [45]. Therefore, long-term dietary habits may alter

the postprandial response. One food group that has generated much interest for its potential health effects is dairy. In recent years, dairy consumption has been examined in relation to overall health status, weight, body composition, and inflammation. More recently, dairy has been examined for its potential impacts on metabolism, particularly during the postprandial state.

### **1.6 Positive health outcomes associated with chronic dairy consumption**

Epidemiological and intervention studies show strong inverse associations between low-fat dairy consumption and weight, body composition, blood pressure, and inflammatory biomarkers, as well as incidence of inflammatory diseases and all-cause mortality [46-48]. Analysis of data from NHANES III found that the odds of being in the highest quartile for body fat were reduced to 0.75, 0.40, and 0.16, respectively, in the three highest quartiles of calcium intake ( $p < 0.0009$ ) [49]. An epidemiological study conducted in Iran divided individuals into quartiles by dairy consumption and found that individuals in the upper quartile had a significantly lower risk for overweight or obesity and those in the third quartile had a lower risk for being obese [50]. The top two quartiles contained proportionally fewer individuals with  $BMI > 30 \text{ kg/m}^2$  versus individuals in the normal ( $< 25$ ) range. Likewise, individuals with a  $BMI < 25$  consumed significantly more dairy products and calcium than their overweight or obese counterparts [50]. A later study by the same group divided Iranian women into quintiles based on low-fat dairy consumption and found that those in the highest quintile had a lower BMI and waist circumference as well as circulating concentrations of IL-6 and soluble vascular cell adhesion molecule (VCAM). [51]. However, in this study, there were no associations with total dairy

consumption, suggesting that perhaps low-fat dairy products confer beneficial effects not demonstrated by their full fat counterparts.

An observational study involving individuals with metabolic syndrome found that consumption of dairy products (excluding cheese), cheese, and an increased calcium density of the diet (defined as amount of calcium per 1000 calories) were all associated with lower diastolic blood pressure at the 9-year follow-up period [52]. Dairy consumption and calcium density were also inversely associated with the incidence of type-2 diabetes at follow-up. Tavani, et al. examined the relationship between dairy intake and risk of acute myocardial infarction among Italian adults [53]. Weekly consumption of  $\geq 7$  cups of milk,  $\geq 7$  cups of yogurt,  $\geq 350$  g of cheese, and daily consumption of  $\geq 1000$  mg calcium were each associated with reduced odds of acute myocardial infarction when compared with individuals consuming  $< 1$  cup of milk per week or  $< 800$  mg calcium per day [53].

A cross-sectional study of Greek adults found a relationship between moderate (11-14 servings per week) and high ( $>14$  servings per week) dairy consumption and lower total cholesterol and triglycerides versus individuals with low ( $< 8$  servings per week) dairy consumption [40].

Greater dairy consumption was also inversely correlated to concentrations of certain inflammatory markers; CRP and Hcy were correlated with low-fat milk, TNF $\alpha$  and IL-6 with low-fat and full-fat milk, and TNF $\alpha$  with low-fat yogurt. CRP, IL-6, and TNF $\alpha$  were also inversely associated with general dairy consumption; individuals with moderate dairy consumption had 16%, 5%, and 12% lower levels of these biomarkers, respectively, compared with individuals in the low dairy group, and those with high dairy consumption had 29%, 9%, and 20% lower concentrations, respectively, versus low dairy consumers [40]. Because this was

observational data, other lifestyle factors may also contribute to these observations. For example, the CARDIA study, a cohort study of American adults, found dairy consumption to be positively associated with consumption of fruits, vegetables, and whole grains, and inversely associated with intake of soft drinks, all of which are considered to be healthier dietary habits [54]. The CARDIA study also found dairy consumption to be associated with a > 67% reduction in incidence of metabolic syndrome at 10-year follow-up among overweight individuals consuming > 5 servings of dairy per day versus those consuming < 1.5 servings per day [54]. The odds of having elevated triglycerides, blood pressure, BMI, and abnormal glucose metabolism were also reduced among individuals with greater daily dairy consumption [54]. As this study was also observational, dairy consumption alone cannot be attributed as the sole effector of these outcomes. Nevertheless, consuming greater amounts of dairy appears to be associated with positive health outcomes.

As a result of these observations, numerous intervention studies have been conducted on the health benefits of dairy consumption. Indeed, dairy consumption is associated with beneficial changes in biomarkers linked to many diseases in animal and human studies [6, 55-57]. These changes include reductions in fat absorption and deposition, increases in fatty acid  $\beta$ -oxidation, increases in fat excretion, reductions in blood pressure, reductions in plasma glucose, and reduced CRP [55-57].

Reductions in fat absorption and deposition and increases in fat oxidation and fecal fat excretion in both animals and humans have been attributed to the calcium content of dairy products [3, 55]. Calcium may interfere with fat absorption, disrupting micelle formation in the small intestine by reducing bile acid solubility [58]. Dietary calcium also moderates circulating levels of 1,25-dihydroxyvitamin D (calcitriol), the hormone responsible for controlling intracellular calcium

concentration [3, 49]. When calcitriol levels are high, it stimulates cellular uptake of calcium. Increased intracellular calcium in adipocytes increases fatty acid synthase and decreases lipolysis, thereby favoring storage of lipids in adipose tissue. Increased dietary calcium intake reduces circulating calcitriol, reducing calcium uptake by adipocytes and reversing the inhibition of lipolysis. Intracellular calcium also increases expression of uncoupling protein [3]. Together, these modulations lead to increased lipolysis and fatty acid  $\beta$ -oxidation and reduced adipocyte lipid accumulation [3, 49].

Reductions in blood pressure associated with dairy consumption are attributed to the presence of bioactive angiotensin-converting enzyme (ACE)-inhibitory peptides found in milk, particularly in the casein fraction, which comprises approximately 80% of milk protein [59]. These peptides are known as casokinins [60]. Individual casokinins, and the evidence supporting their bioactivity, are reviewed elsewhere [59]. Nevertheless, most ACE-inhibitory peptides exert their activity in a similar manner – by binding one of the two active sites of ACE. ACE catalyzes the conversion of angiotensin I to angiotensin II, leading to vasoconstriction and triggering the release of aldosterone, which acts to increase sodium (and therefore water) retention by the kidneys [59]. This leads to an increase in blood pressure. Inhibition of this hormone arrests this process; therefore, consumption of dairy peptides may have a hypotensive effect.

It is noteworthy that intake of supplemental calcium does not confer similar benefits to those associated with dairy consumption [61]. This may account for the seemingly conflicting results of numerous intervention trials examining the influence of calcium on postprandial metabolic parameters. Perhaps other components of dairy interact favorably with calcium to increase its absorption or bioactivity, thus requiring calcium to be fully intact to achieve maximum effect. In addition to calcium, dairy contains many bioactive components, including whey and casein,

short-chain fatty acids, vitamins and minerals, and, in fermented products, probiotics [40, 43, 56, 57]. An intervention study examined the interplay between calcium and protein [62]. Overweight participants consumed a low-calcium normal-protein (LC/NP), high-calcium normal-protein (HC/NP), or high-calcium high-protein (HC/HP) diet for one week each, with low-fat dairy products as the main source of each nutrient. The HC/NP diet led to a significant increase in fecal fat and energy excretion. This translated to an increase of 5.4 g of fat excretion per 1000 mg of calcium [62]. Both systolic and diastolic blood pressure also decreased on both HC diets, though the reductions did not reach significance. Triglycerides, fatty acids, cholesterol, and insulin were unchanged; this may be attributed to the short duration of each dietary period. The authors attributed the lack of increased fat excretion on the HC/HP diet to the potential for calcium to bind casein, thereby inhibiting its ability to modulate fat absorption in the intestines [62]. However, formation of these casein phosphopeptide-calcium complexes may act to increase calcium absorption [60].

A dietary intervention conducted by Zemel, et al. compared the impact of dairy versus soy on oxidative stress and chronic inflammation in overweight and obese individuals [43]. Twenty healthy, overweight or obese men and women participated in the study. Subjects were placed on a dairy-free (soy protein isolate) or adequate dairy (nonfat dry milk powder) diet for 28 days, separated by an approximately 28-day washout period. Soy or dairy products consisted of smoothies that were consumed 3 times daily. Aside from the smoothies, diets were designed to be similar in macronutrient and caloric content. The adequate dairy diet led to significant reductions in plasma MDA and urinary 8-isoprostanes, markers of oxidative stress. Changes were measurable within the first week of the intervention. No changes in either biomarker were observed during the soy diet. The adequate dairy diet also lead to reductions in inflammation, as

indicated by reduced expression of TNF $\alpha$  and IL-6, while the soy diet led to increases in these biomarkers. MCP-1 and CRP were significantly decreased (-20% and -57%, respectively) upon consuming the adequate dairy diet. LDL cholesterol was also significantly decreased by the adequate dairy diet [43]. Due to the nature of the intervention, it is not possible to determine what components of dairy are responsible for these changes [43].

Stancliffe, et al. examined the influence of adequate dairy consumption on oxidative stress in overweight and obese individuals with metabolic syndrome [63]. Individuals were placed on a low dairy (< 0.5 servings per day) or adequate dairy (> 3.5 servings per day) diet for 12 weeks. Two of the daily servings on the adequate dairy diet were milk or yogurt. Plasma samples were collected following weeks 1, 4, and 12. Systolic blood pressure was significantly lowered following just 1 week on the adequate dairy diet, with further reductions seen after 12 weeks. Diastolic blood pressure was also significantly reduced by 12 weeks. Plasma MDA and oxidized LDL were also decreased following week 1, and continued to decrease throughout the intervention. By week 12, individuals in the adequate dairy diet exhibited a 35% reduction in MDA and a 25% reduction in oxidized LDL. The adequate dairy diet also led to reductions in inflammatory biomarkers as early as week 1, with additional reductions by the end of the intervention. This was indicated by reductions in TNF $\alpha$ , IL-6, CRP, and MCP-1 [63].

However, Wennersberg, et al. did not observe any differences in inflammatory markers, plasma glucose, or insulin following 6 months of 3 to 5 servings of dairy per day versus habitual lower intake ( $\leq 2$  servings per day) [64]. Possible explanations for the lack of reduction in inflammatory biomarkers include the high baseline dairy intake among individuals in the intervention group (about half already consumed > 700 mg calcium per day) and the fact that individuals in the control group continued their normal dairy intake patterns (< 2 servings per

day) [64]. However, the authors did observe an increase in serum cholesterol in the dairy group, an outcome not reported in other studies. This warrants further investigation.

The above data support a long-term association between dairy intake and positive health outcomes, including changes in body composition, body weight, and reductions in baseline levels of inflammation and oxidative stress. However, it is important to note that these positive health outcomes associated with increased dairy and/or calcium consumption are often most beneficial for individuals with suboptimal calcium intake. This ‘suboptimal’ threshold of calcium consumption has been set at 700-800 mg per day, an intake level that a large portion of the population fails to meet [55, 64]. Therefore, a dietary intervention that incorporates dairy products would be a means of increasing daily calcium intake, particularly in individuals who fall below the 700-800 mg daily threshold. Indeed, studies have shown that increasing calcium consumption to optimal levels can lead to increases in weight and fat loss during energy restriction and may also reduce blood pressure in hypertensive individuals [55, 64].

Increased dairy intake has also been associated with increased intakes of protein, fat, saturated fat, and cholesterol [64]. It is important to take into consideration that the saturated fats present in dairy products are primarily stearic acid and short- and medium-chain fatty acids, which are not associated with increases in plasma cholesterol [65]. Vitamin D is also an important component of dairy products. Due to its fat solubility, vitamin D can be sequestered in adipose tissue [66]. Therefore, its bioavailability may be reduced in obese individuals, necessitating increased dietary intake. Regular dairy consumption would be a means of reaching this goal.

Though numerous studies support the benefits of dairy on body composition, body weight, and oxidative and inflammatory parameters, which components of dairy products are responsible for



these changes and by what mechanisms these effects are conferred remains unknown.

Additionally, data is lacking on the postprandial impacts of dairy consumption on intestinal barrier function and biomarkers of inflammation and oxidative stress. Furthermore, the anti-inflammatory and antioxidative effects of individual dairy products, including low-fat yogurt, have not been well characterized.

### **1.7 Attenuation of postprandial oxidative stress and inflammation by dietary intervention**

Many foods have been examined for their ability to modulate postprandial metabolism, including orange juice, protein, individual components of dairy, and a variety of dairy products [23, 48, 67-69]. A 2010 study found orange juice to have a favorable effect on postprandial inflammation and oxidative stress among healthy, normal weight (BMI 20-25) individuals [23]. In this intervention, a high-carbohydrate, high-fat meal was consumed along with either a glucose drink, orange juice, or water [23]. The meal consisted of egg-muffin and sausage-muffin sandwiches and two hash brown patties, totaling 900 calories, 81 g carbohydrates, 51 g fat, and 32 g protein. A statistically significant postprandial rise in blood glucose was observed following the meals consumed with water or glucose but not following those consumed with orange juice. Orange juice also blunted postprandial increases in ROS generation, TLR4 and p38 MAP kinase expression, and circulating LPS [23]. While the nutrient profile of orange juice is markedly different from that of low-fat dairy products, the results of this intervention are indicative of the ways in which certain foods can positively influence the postprandial response.

Whey protein comprises approximately 20% of the protein content of milk and milk products [67]. Bioactive peptides in whey include lactalbumin,  $\beta$ -lactoglobulin, immunoglobulin,

lactoperoxidase, and a large proportion of branched chain amino acids [68]. An intervention trial on healthy individuals found that consumption of whey protein or whey protein isolate prior to a meal led to a reduction in postprandial glucose and insulin [67]. The first intervention consisted of men consuming 0, 10, 20, 30, or 40 g of whey protein (WP) concentrate 30 minutes prior to consumption of pizza. Pizza was consumed until subjects were full but within a 20-minute time limit. The second portion of the trial consisted of men and women consuming 0, 5, 10, 20, or 40 g of WP concentrate or 10 g whey protein hydrolysate (WPH) followed 30 minutes later by 12 kcal/kg body weight of pizza, to be consumed in 20 minutes. In trial 1, ingestion of 20, 30, and 40 g doses of WP significantly reduced postprandial plasma glucose concentrations versus the control at 30 minutes, and all doses led to significantly lower plasma glucose at 50 and 65 minutes postprandially versus the control. Glucose AUC was significantly reduced in all WP supplemented groups as well [67]. In trial 2, post-meal and cumulative postprandial plasma glucose was significantly reduced by all WP doses when compared to the control. WP also decreased total food intake in trial 1, which would similarly serve to reduce postprandial glucose. Cumulative glucose AUC was not reduced by WPH consumption [67]. This suggests that the protein needs to be fully intact for these postprandial effects to be observed.

Ingestion of 54 g of supplemental whey, casein, or a glucose control (two 27 g capsules dissolved in water, one prior to breakfast and one prior to dinner) daily for 12 weeks was linked to a significant decrease in both systolic and diastolic blood pressure in overweight and obese individuals [68]. However, inflammatory parameters – as indicated by IL-6, CRP, and TNF $\alpha$  – were unchanged [68]. It is unknown what components of whey and casein mediate these hypotensive actions, but this study does suggest that the proteins need not necessarily be consumed as complete dairy products to exert a beneficial effect on blood pressure. Following

these outcomes, the authors designed a second study to examine whether whey and casein would exert similar effects on an acute basis [48]. Overweight and obese women consumed a high-fat meal containing approximately 600 calories, with 45 g of either whey protein isolate, sodium caseinate, or glucose (control) dissolved in water. However, no changes in inflammatory biomarkers were observed over the 6-h postprandial period in either treatment group when compared with the control [48].

Another dietary intervention tested the intake of casein, a commercial mixture of mono- and oligosaccharides, or both, in concert with a high-fat meal [69]. The study was a crossover design involving healthy males and females. Both interventions delayed the peak and lowered total triglyceride concentration in the bloodstream. Though the oligosaccharides exhibited a much stronger effect than the casein, when combined the effect was even greater [69]. Casein and casein + oligosaccharides also lowered plasma free fatty acid concentration following the high-fat meal. As yogurt contains both casein and oligosaccharides, it may possess the ability to attenuate the postprandial lipemic response.

An intervention study comparing the influence of dairy and supplemental calcium on postprandial metabolism found that only dairy calcium successfully attenuated the postprandial lipid response [61]. In the trial, healthy men consumed a low dairy calcium (LC), medium dairy calcium (MC), high dairy calcium (HC), or calcium carbonate (CC) supplemented meal, and the postprandial response was monitored for 7 h. Chylomicron triglyceride AUC was lower following the MC and HC meals than the CC meal, suggesting a reduction in fat absorption in these meals [61]. This is in agreement with previous research establishing the impact of calcium on fat absorption and fecal fat excretion. These results also support the notion that dairy calcium is more efficacious than supplemental calcium in modulating the postprandial response.

To examine the benefits of intact dairy products, Ballard, et al. designed an intervention to test the impact of low-fat dairy milk versus rice milk on the postprandial endothelial response in individuals with metabolic syndrome [27]. Low-fat milk has a low glycemic index that would limit postprandial hyperglycemia and any associated increases in oxidative stress. Indeed, glucose increased to a lesser extent and returned to baseline more rapidly following dairy milk than rice milk consumption [27]. Examination of oxidative biomarkers revealed that low-fat milk decreased oxidative stress and preserved NO bioavailability, thereby helping to maintain normal vascular function. MDA increased postprandially during the rice milk trial but not the dairy milk trial, suggesting that low-fat milk may also mitigate postprandial lipid peroxidation [27].

van Meijl, et al. examined the impact of milk on postprandial lipemia and glycemia in overweight and obese men in response to a high-fat meal [56]. The ingredients of interest were calcium and protein. The intervention consisted of 4 meals consumed on 4 days, each separated by at least 3 days. The meal consisted of a butter cake and a 500 mL beverage: either skim milk, water with lactose, water with lactose and calcium, and water with lactose, calcium, and milk protein. Fat content was 44 g, carbohydrate was 109 g, and calorie content was between 870 and 950 in each meal. Calcium and protein content varied by design between each intervention meal. Plasma glucose and triglycerides were measured for 6 h postprandially. The calcium meal decreased the triglyceride AUC by 48% versus the milk meal. The protein meal reduced maximal plasma glucose concentration versus the control meal, and the milk meal also reduced peak plasma glucose, though the reduction did not reach significance [56]. This suggests that calcium and protein need not be fully intact in milk in order to lower postprandial triglycerides or glucose; in fact, reductions did not reach significance when the calcium and protein were

consumed as milk. Therefore, it is possible that the health benefits attributed to dairy products are due to components other than calcium or protein.

Clearly, the ability of dairy products to attenuate the postprandial response requires further examination. In addition, the connections between postprandial reductions in glucose and triglycerides and corresponding changes in oxidative stress have not been well characterized.

### **1.8 Potential health benefits of yogurt consumption**

In addition to whey, casein, proteins, and other bioactive components found in dairy products, yogurt also contains probiotics – live microorganisms that, when consumed in sufficient quantities, confer a benefit to the host. Probiotic consumption has been linked to improved intestinal health, alterations in the indigenous intestinal microflora and changes in the metabolic profile of the host [70, 71]. More recently, animal studies have shown connections between probiotic consumption, the intestinal microflora profile of the host, and neurological changes. This is reviewed in detail elsewhere [72, 73] Alterations in microbiota composition are also associated with obesity and other metabolic disorders; this was reviewed by Chen, et al. [74] and Walsh, et al. [75].

Probiotics themselves possess the ability to act as indirect antioxidants both by trapping ROS in the intestine and by stimulating the synthesis of cellular antioxidants, including glutathione peroxidase (GPx) and superoxide dismutase (SOD) [57]. Indeed, a dietary intervention study examined the 3-week intake of a low-fat dairy diet, a non-fermented dairy diet, and a fermented dairy diet on various cardiovascular outcomes [46]. TNF $\alpha$ , MCP-1, IL-1 $\beta$ , and VCAM were all

non-significantly decreased following the fermented dairy versus low-fat dairy diet. However, both the low-fat and fermented dairy diets resulted in a significant reduction in IL-6 concentrations. Sphingomyelin, a biomarker indicative of early atherosclerosis, was also decreased by the low-fat dairy diet, though not by the fermented dairy diet [46].

## **1.9 Examination of postprandial and chronic biomarkers of oxidative stress**

### **Malondialdehyde:**

MDA is a product of polyunsaturated fatty acid oxidation and an established biomarker for the measurement of lipid peroxidation [76]. The structure of MDA is shown in **Figure 1.1**. MDA increases during the postprandial period [27, 33, 77]. MDA can react with amino groups of lipoproteins, generating MDA-adducted LDL particles [7]. These adducts often exhibit epitopic similarities to antigens; they are recognized as such by the immune system and are, therefore, pro-inflammatory [37]. Thus, dietary means of reducing MDA formation may reduce inflammation.

Long-term calcium supplementation and/or dairy intake has been shown to decrease MDA in both mice and humans [13]. Interestingly, supplemental calcium reduced MDA to a greater extent than dietary calcium in obese mice. The high-calcium diet also reduced NADPH oxidase and ROS production [13]. Since MDA is a product of lipid oxidation, decreases in ROS generation should reduce its concentration. A second study by Zemel, et al. found that 28-day consumption of a dairy-rich diet by overweight and obese individuals led to a reduction in

oxidative stress, as indicated by a 22% reduction in plasma MDA [43]. This reduction was seen after just 1 week on the high-dairy diet.

An intervention by Ballard, et al. comparing the influence of low-fat milk versus rice milk on the postprandial response found that 435 mL of rice milk was associated with a 17-33% increase in MDA from 30-120 minutes while MDA concentration following intake of 475 mL of low-fat milk did not change [27]. Low-fat milk also resulted in a smaller MDA AUC in the 3 h postprandial period compared with rice milk ( $p < 0.01$ ). Finally, MDA AUC was correlated with glucose AUC ( $p < 0.01$ ;  $r = 0.75$ ), suggesting that postprandial hyperglycemia is associated with MDA formation [27]. As excess plasma glucose is associated with increased ROS generation, this would seem a logical correlation. Therefore, consumption of low-fat yogurt may reduce postprandial plasma glucose, thereby attenuating formation of MDA.

### **Advanced glycation endproducts:**

AGEs are natural byproducts of human metabolism, formed by the non-enzymatic reaction of aldehydes with exposed amino groups of lipids, proteins, and nucleic acids, to form covalently-modified proteins [30, 78, 79]. Such proteins include collagen and plasma lipoproteins, of which lysine and arginine side chains are the most reactive for this process [30, 80]. There is a well-established correlation between diabetes and/or renal dysfunction and elevated plasma AGEs. Increased production of AGEs has also been observed in cases of hypertriglyceridemia and hyperglycemia; as such, high plasma AGEs are associated with obesity [81]. As obesity is often comorbid with some or all of the above conditions, the reduction of plasma AGEs in obese individuals may have simultaneous beneficial impacts on insulin sensitivity, renal function, and hypertriglyceridemia. Increased plasma AGEs is also observed in concert with higher levels of

oxidative stress and inflammation [30, 81]. Indeed, oxidative stress exacerbates the formation of AGEs and vice versa [82]. Thus, any potential means of reducing postprandial oxidative stress would tend to decrease formation of postprandial AGEs.

Elevated levels of AGEs also lead to activation of RAGE, a pattern recognition receptor that recognizes AGE-adducted proteins [83]. Activated receptors trigger a variety of pro-oxidative and pro-inflammatory pathways, including the synthesis of CAMs and activation of NF- $\kappa$ B [81, 83, 84]. However, it is important to note that most studies of RAGE activation have been conducted in animals and much remains unknown with regard to its mechanisms of activation and action [85].

AGEs are always present in some quantities the body but in addition, about 10% of those ingested are absorbed in the intestine [83]. AGEs are present in higher amounts in animal-based foods (meat, eggs) than in fruits and vegetables. Dairy products may have AGEs as well, though the water content of the products tends to impede their formation [79]. The preparation methods of foods can also greatly increase the exogenous supply of AGEs. In particular, hot, dry cooking methods such as frying, grilling, and broiling increase formation of AGEs more so than wet cooking methods such as boiling and steaming [79].

Once absorbed, exogenous AGEs enter the bloodstream, where they form crosslinks to proteins, lipids, and nucleic acids [30]. Crosslinks formed between AGEs and endogenous proteins are irreversible and damaging, with greater amounts of damage accumulating on proteins that have a lower turnover rate [30]. Examples include collagen and LDL, leading to an increase in arterial stiffness, decreased LDL uptake, and an overall increase in endothelial dysfunction. It is necessary to note that the eventual degradation of these bound AGEs leads to formation of other



highly reactive intermediates, which, upon entering circulation, are once again available to crosslink with plasma proteins [80]. Two commonly measured AGEs in plasma are N-carboxymethyllysine (CML) and N-carboxyethyllysine (CEL) [30]. They can be measured individually or in combination with each other and the numerous other AGE species present in plasma to give a more thorough indication of plasma AGE status. The structures of CML and CEL are shown in **Figure 1.2**.

Postprandial effects of AGE consumption include increased endothelial dysfunction and decreased adipokine secretion [30]. A study examining the impacts of a high-AGE meal in both diabetic and non-diabetic patients found significant increases in plasma AGE concentrations beginning 1 to 2 h postprandially and peaking 4 to 6 h after meal consumption [80]. Elevated levels in healthy subjects were observed as much as 18 h following consumption of the high-AGE meal; AGE levels in diabetic subjects remained elevated for much longer.

Long-term impacts of high-AGE diets are still somewhat unknown. However, existing studies show that extended consumption of a high-AGE diet leads to elevated plasma AGE levels [83]. In a study reviewed by Sebekova and Somoza, 21 healthy volunteers consumed either a high- or low-AGE diet for 1 week each; the only difference in the diets was the content of AGEs [86]. After one week, fasting serum concentration of CML was elevated in those individuals on the high-AGE diet.

Due to reduced clearance of glucose, AGE production is higher in individuals with diabetes; methods to decrease AGE absorption and formation may thus be particularly beneficial to these individuals [30, 79]. While the exact quantitative definition of a low-AGE diet has not been established, dietary reduction of AGE intake has been shown to positively impact levels of

oxidative stress and insulin resistance [85]. In a study in which obese participants were placed on either a high-AGE (14,090 kU/day) or low-AGE (3,302 kU/day) diet, consumption of the low-AGE diet led to improvements in renal function and the inflammatory profile after a 2-week intervention [87].

Endogenously, glucose is the most available and commonly-used sugar in AGE formation [30, 81]. By this logic, it stands to reason that a reduction in fasting blood glucose and/or postprandial glycemia may reduce formation of AGEs. Previous studies show a reduction in postprandial hyperglycemia following a meal supplemented with dairy products [10, 27]. A randomized, double-blind study comparing the postprandial effects of consumption of rice milk versus a low-fat dairy milk revealed a blunted postprandial increase in plasma glucose and glucose AUC, with values returning to baseline much more rapidly following dairy milk than rice milk [27]. An intervention study by van Meijl, et al. found that the addition of milk or milk protein to a high-fat meal attenuated the postprandial rise in plasma glucose levels [10]. Finally, a third study showed that consumption of whey protein prior to a high-carbohydrate meal led to a dose-dependent reduction in postprandial blood glucose levels [67].

A study examining long-term calcium and vitamin D supplementation found a significant decrease in fasting plasma glucose levels in non-diabetic adults [88]. Although these results cannot be attributed to dairy consumption, calcium and vitamin D are present in dairy products and the effects of calcium are also known to be much more profound when the mineral is from dairy versus a supplemental form [61]. Because dairy consumption can reduce postprandial glycemia, it may also possess the ability to limit postprandial formation of AGEs.

**Total thiols:**

Protein thiols are indicators of oxidative and nitrosative stress, as well as plasma antioxidant status [89, 90]. They are preferentially oxidized in cases of increased oxidative stress; a higher concentration of oxidized thiols is often interpreted as an increase in oxidative damage [90, 91]. This occurs via the oxidation of sulfhydryl groups on cysteine, methionine, and glutathione residues, forming disulfide bridges [90]. Plasma thiols include GSH, glutathione disulfide (oxidized glutathione - GSSG), Cys and cystine (oxidized cysteine - CysS), Met and its oxidized form, Hcy [27]. Structures of these molecules are depicted in **Figure 1.3**. GSH is synthesized from other sulfur-containing amino acids, of which Cys concentration is the rate-limiting factor [34]. GSSG and CysS are formed by the reaction of hydrogen peroxide or peroxynitrite with GSH and Cys, respectively. It has been shown that CysS concentrations exhibit no variations in association with meal consumption [25]. However, Cys and GSH do vary in relation to meal consumption and meal composition, as well as endogenous oxidative conditions [34, 92].

It is noteworthy that thiol concentration and oxidation status is known to vary throughout the day, as well as in response to meals [25]. For example, plasma Cys concentration varies between its minimum in the early morning and its maximum in the evening. This cycle is in addition to peaks occurring 2 to 3 h following each meal [25]. CysS concentration also shows diurnal fluctuations; this was seemingly not in correlation to meal consumption but rather on a 12 h cycle with maximum levels at midday and minimum levels in the middle of the night [25]. Plasma GSH also increased following meal consumption, though this occurred much later than with Cys [25]. Lowest GSH oxidation levels were present in the early morning and evening.

It is possible to measure total thiols (SH), which includes GSH, Cys, Met, and protein and non-protein thiols [6]. Protein and non-protein thiols often dimerize in the presence of increased

oxidative stress as a protective mechanism. These disulfides are indicative of an elevated oxidative burden, a state which is also measurable by a decrease in the ratio of SH:disulfides [6].

There are many biological pathways whose regulation relies on redox status, including thiol redox state [26]. Postprandial increases in ROS trigger increased generation of CysS; the oxidation status of Cys/CysS is known to modulate adhesion of monocytes to endothelial cells via activation of CAMs [25, 26]. Thiol oxidation is also involved in the modulation of NO activity. Increased oxidative stress leads to the oxidation of thiols by ROS and RNS, triggering subsequent NO dysregulation [89, 93]. As such, preservation of normal levels is necessary for maintenance of the endogenous antioxidant defense system and proper endothelial function.

It is well established that consumption of a high-fat meal decreases endogenous antioxidant status in healthy individuals, leading to an increase in ROS [94]. As these ROS promote thiol oxidation, it is reasonable to expect that meal consumption may trigger this phenomenon. Indeed, Miglio, et al. found that consumption of a high-fat meal significantly increased plasma total thiols, with a peak 2 to 4 h post-ingestion [6]. The caloric content of the high-fat meal used in this intervention is slightly greater than that used in the present study, but the macronutrient compositions are similar [6]. As this is the case, we can expect to see an increase in total thiols 2-4 h postprandially. However, as observed by Miglio et al., SH levels remained elevated for 8 h postprandially [6].

It is important not to discount the potential impact of protein intake on thiol concentration. There is evidence that ingestion of proteins rich in sulfur-containing residues can raise plasma thiol levels [25]. It is also important to note that Cys groups are prone to oxidation and are

preferentially oxidized, altering the plasma thiol redox status [6]. To counteract this, GSH from the tissues is released into the plasma; this may result in an increase in plasma thiols.

Finally, postprandial hyperglycemia increases ROS generation and, hence, thiol oxidation [90].

One clinical study found an inverse relationship between postprandial glucose levels and reduced protein thiols [90]. Therefore, reductions in postprandial plasma glucose levels may also lead to a reduction in oxidative damage to proteins, resulting in a restoration of normal protein thiol levels. As this is known to be a potential impact of dairy consumption, low-fat yogurt may blunt the postprandial increase in thiol oxidation following consumption of a high-fat meal.

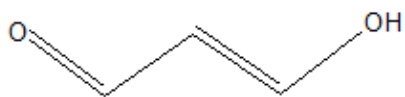
## **1.10 Conclusions**

Atherosclerosis is primarily a postprandial phenomenon, the development of which is associated with postprandial dysmetabolism as a result of excess nutrient intake. Among these changes are increases in oxidative stress and inflammation, conditions that are closely linked. Excess nutrient intake may also compromise intestinal barrier function, leading to increased LPS entry into the bloodstream, which elicits a strong inflammatory response.

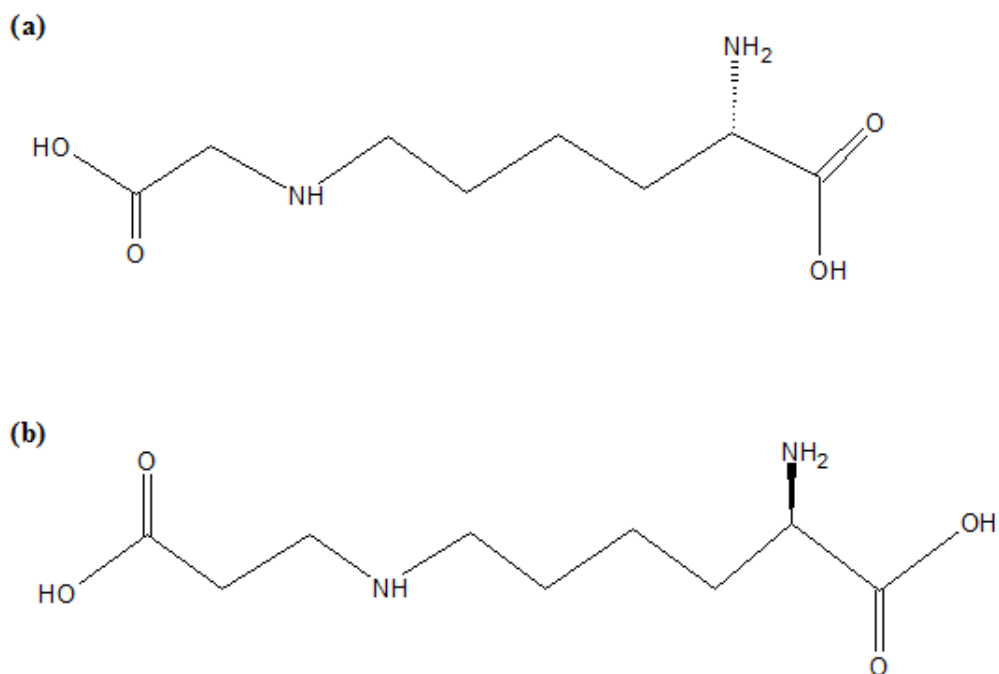
Among the suspected causes of postprandial oxidative stress are increases in plasma glucose and triglycerides, as well as excess ROS generated by nutrient metabolism. These oxidative species can damage cells and tissues; this injury leads to activation of the inflammatory response. ROS also promote CAM synthesis and subsequent promotion of monocyte adhesion to the vascular walls, an early step in the pathogenesis of atherosclerosis. Both circulating glucose and ROS can also interact with nucleic acids, proteins and lipids; resultant byproducts include AGEs, MDA,

and oxidized thiols. However, it is important to note that these three biomarkers do not provide a full picture of postprandial oxidative changes in the body. The body's endogenous antioxidant enzymes – including GPx and SOD – are also key regulators of oxidative stress that may be altered by intake of a high-fat meal [94]. In addition, urinary isoprostanes and 8-deoxyguanosine, a marker of DNA oxidation, are well-established markers of oxidative stress. Uric acid has also recently been shown to decrease in healthy subjects following a high-fat meal, and may also be indicative of oxidation status [94]. Finally, conflicting research on postprandial plasma thiols suggests that regulation of oxidant status is far more complicated, as dietary thiols may contribute to plasma thiols [6]. Furthermore, additional thiols may be released from cells in response to oxidation of existing plasma thiols to maintain redox balance [6]. Therefore, numerous factors may need to be examined to elucidate the full impact of low-fat yogurt consumption on oxidative stress.

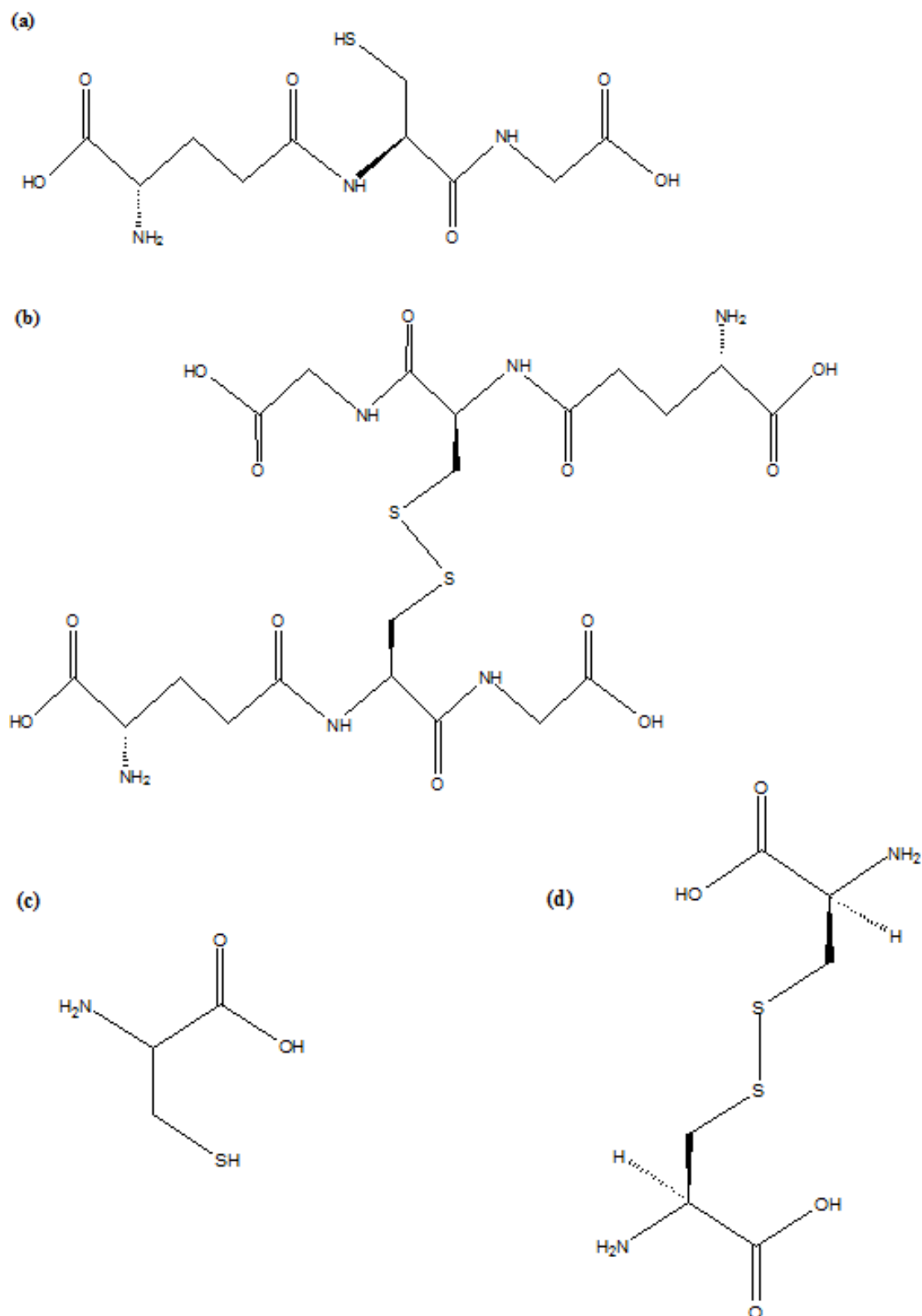
Nevertheless, as evidence of the consequences of postprandial stress become more apparent, dietary strategies to reduce these changes are of necessity. Past studies link chronic dairy consumption to many beneficial health outcomes, including decreased odds of obesity, reductions in blood pressure, postprandial glucose, and triglycerides, and reductions in inflammation. Postprandially, dairy and its individual components have been shown to reduce plasma glucose and triglycerides, as well as certain inflammatory biomarkers. However, the impacts of individual dairy products remain largely unexplored.



**Figure 1.1.** Structure of malondialdehyde (MDA).



**Figure 1.2.** Structure of two common plasma advanced glycation endproducts (AGEs): (a) N-carboxymethyllysine, and (b) N-carboxyethyllysine.



**Figure 1.3.** Structure of four common plasma thiols: (a) glutathione, (b) glutathione disulfide, (c) cysteine, and (d) cystine.



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## **Chapter 2:**

### **Experimental Design**



## 2.1 Introduction

The overall goal of this intervention study is to determine the impact of yogurt consumption on intestinal barrier function in obese individuals. Obesity is associated with compromised intestinal barrier function; animal and human studies support this connection [1, 2]. A compromised intestinal barrier may promote chronic, low-level systemic inflammation. Obesity is also associated with a chronically elevated inflammatory status, which is a contributing factor in the development and pathogenesis of many diseases [2, 3]. In addition, postprandial inflammation contributes to chronic inflammation [2]. Indeed, human trials show that ingestion of a high-calorie and/or high-fat meal acutely compromises intestinal barrier function and increases translocation of lipopolysaccharide (LPS) from the intestine into the bloodstream [4-6]. Once in the bloodstream, LPS elicits a strong inflammatory response, triggering the expression of many pro-inflammatory cytokines [7, 8]. Chronic intake of high-fat foods also contributes to intestinal barrier dysfunction and chronic inflammation by unfavorably altering the makeup of the intestinal microbiota [9]. In their unaltered state, these microbes contribute to the normal protective mechanisms of the intestinal barrier and reduce LPS translocation [9]. Due to the frequency of meal consumption and the prevalence of high-fat foods in the Western diet, many individuals spend large portions of the day in a state of elevated intestinal barrier dysfunction and/or inflammation [10]. Therefore, dietary approaches to reduce postprandial and chronic inflammation are of importance.

Human trials support the role of low-fat dairy in decreasing chronic inflammation in obese individuals [13, 14]. In addition, numerous observational and intervention studies suggest the potential benefits of dairy consumption over both the short and long term in lean, overweight, and obese individuals [13-17]. However, the impact of low-fat yogurt consumption on intestinal

barrier function and inflammation in the obese population has not been elucidated. This research was designed to examine these associations. Secondary outcomes of this study were to examine the impact of low-fat yogurt consumption on oxidative stress in obese women and to characterize changes in dietary intake associated with this intervention.

## **2.2 Materials and Methods**

### ***2.2.1 Subject recruitment and screening***

This study was approved by the Institutional Review Board at the University of Connecticut, approval number H12-168. The study was registered at [www.clinicaltrials.gov](http://www.clinicaltrials.gov), identifier number NCT01686204. Recruitment began in September 2012; as of August 2014, the study was still in progress. A complete timeline of the study is depicted in **Figure 2.1**.

Healthy, lean (BMI 18.5-27 kg/m<sup>2</sup>; n = 66) and obese (BMI 30-40 kg/m<sup>2</sup>; n = 66), premenopausal women between the ages of 21 and 55 were targeted for recruitment from the University of Connecticut in Storrs, CT and surrounding areas. Only women were selected for the study in order to reduce the potential for confounding variables. For example, it has been shown that men and women have different metabolic responses to a high-fat meal [19-22]. Addition of carbohydrates to a high-fat meal reduced postprandial plasma triglycerides in women but not men [22]. Plasma triglyceride responses following meals high in unsaturated and saturated fats also differed between genders, and the plasma triglyceride AUC was significantly higher in men versus women [20]. Finally, plasma interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF $\alpha$ ) increased to a greater degree in women than men following intake of a high-

calorie, high-fat meal [21]. Hormonal changes associated with menopause may increase oxidative stress and facilitate the establishment of a pro-atherogenic lipid and cholesterol profile [23]. Therefore, only premenopausal women were recruited for participation in this study. In addition, only healthy women were recruited; this was to eliminate the potential for confounding inflammatory status, as many diseases have some component of inflammation. Also, anti-hypertensives and other medications may interfere with or obscure the mechanisms by which yogurt modulates postprandial changes.

Recruitment methods included posting flyers in academic buildings, dining halls, and residence halls, submitting written ads to university email groups, and placing ads on TV monitors around campus. Additional methods included advertising in local and regional publications, public buses, attending various health fairs and promotional events, and posting flyers in area businesses and other public locations.

Following initial contact with potential participants, a phone screening interview was arranged for interested individuals. The phone screening consisted of a 15 to 20 minute call, during which potential participants received a detailed explanation of our study aim and the procedures.

Participants were also asked a series of health questions to ensure eligibility. Eligibility criteria included a self-reported lack of cancer, diabetes, high blood pressure ( $\geq 140/90$  mm Hg), high cholesterol ( $\geq 240$  mg/dL), cardiovascular disease, arthritis, anemia, gastric conditions, vegetarianism, allergies to dairy, soy, or egg products, and participation in a weight-loss regimen. The use of medications for high blood pressure, blood sugar, gastric conditions, estrogen replacement therapy, and regular use of anti-inflammatory drugs were also causes for disqualification. Medications for cholesterol, hypothyroidism, mental health conditions, and the

use of contraceptives were allowed. Individuals who met all inclusion criteria and remained interested in participating were scheduled for a screening visit (Visit 1).

At Visit 1, written informed consent was obtained from all participants, following which a health questionnaire was completed to ensure that the individual met the inclusion criteria for our study. Then, anthropometric measurements were taken. Measurements included height, weight, waist circumference, and blood pressure. Height and weight were measured to the nearest half centimeter and tenth of a kilogram, respectively, with a Detecto 758C scale, from which BMI was calculated. Waist circumference was measured by hand with a measuring tape, and blood pressure was measured using an Omron HEM-780 monitor for lean individuals and an Omron BP710 monitor for obese individuals. Two measurements were taken; the first after 5 minutes of sitting upright in a phlebotomy chair, and the second at least 3 minutes following the first.

All individuals who attended Visit 1 cleared and were enrolled in the study. Following anthropometric measurements, subjects were provided with a binder of information, including reminder lists for each visit, a guide to dairy serving sizes, and instructions and supplemental materials for completing dietary records. Subjects were also trained in completing dietary records. Following Visit 1, subjects were randomized to either the yogurt or soy pudding (control) group.

Two weeks prior to the second study visit, all participants were asked to begin the washout period, during which no foods containing probiotics could be consumed. Participants were provided with an extensive list of probiotic-inclusive foods. This restriction endured for the entire length of the study. Participants were also asked to limit their dairy consumption to  $\leq 4$  servings per day for the duration of the study. Finally, participants were asked to complete 3 days

of dietary records. This was to ensure compliance to the dietary restrictions as well as to provide a broad overview of the individual's typical dietary patterns. Diets were recorded during the washout period, on nonconsecutive days, and included 1 weekend day. All food and beverages (including water) consumed during a 24 h period were recorded. Participants were instructed to provide as much information as possible about each food item and to save the packaging of any items about which they were unsure. If individuals consumed food from a dining hall or restaurant, they were asked to make their best estimation of serving size. Home-prepared recipes were to be broken down into each individual component to increase the accuracy of the record. Individuals were provided with serving sizes and typical amounts of common foods to assist with their estimations. Finally, participants were asked to refrain from taking any dietary supplements during their enrollment in the study.

### ***2.2.2 Dietary intervention and sample collection***

Visit 2 took place after a 2-week washout period. This visit constituted the initial acute portion of the dietary intervention. Participants were instructed to avoid caffeine consumption and vigorous exercise for 24 h prior to Visit 2, to fast for 12 h, and to consume sufficient amounts of water to avoid light-headedness during the blood draw.

Visit 2 was conducted in the morning, typically beginning between 7:00 a.m. and 9:30 a.m., and lasted approximately 5 h per person. Upon arrival at the study center, subjects gave verbal confirmation that they had met the pre-visit requirements, and that they were well hydrated. Dietary records were collected, and anthropometric measurements were taken and recorded. Protocol for anthropometrics was identical to that described for Visit 1. Next, 50 mL of venous blood (30 mL in sodium heparin vacuum tubes and 20 mL in EDTA vacuum tubes) was

collected from the antecubital vein either through a catheter or via a single-stick insertion. After blood collection, tubes were gently inverted 8-10 times; 2 heparin tubes were placed at room temperature for white blood cell separation while the other 3 tubes were placed on ice.

Processing occurred within 30 min of obtaining the samples. Briefly, samples were centrifuged at 1500 x g for 15 min at 4°C. Plasma was aliquotted into labeled 2 mL cryogenic vials and flash-frozen in liquid nitrogen. All samples were stored at -80°C until further analysis.

Following the fasting blood draw, participants were informed of their randomization to either the low-fat yogurt the soy pudding group, and were asked to consume 8 oz. of the snack. The yogurt was Yoplait Original, offered in strawberry, strawberry banana, raspberry, and peach flavors. This particular type of yogurt was chosen because it is widely available and representative of most commercial low-fat yogurts. The soy pudding was Zensoy Organic, offered in chocolate, vanilla, and chocolate/vanilla swirl flavors. This pudding was selected as a control because it is dairy-free, isocaloric, contains no probiotics, and is similar in nutrient content to the yogurt. In addition, previous studies have found soy to have no impact on modulating chronic oxidative stress and inflammation or postprandial metabolism in obese individuals [14, 24]. The soy pudding was also selected because it is similar in texture to the yogurt and both products are packaged in the same size of container and can be consumed in a similar manner. This is important because these qualities may influence how and when participants consume the snacks [25]. Texture is also associated with feelings of satiety [25]. The nutrient content of both snacks is listed in **Table 2.1**.

After 10 min and ingestion of 8 oz. of yogurt or pudding, participants were given a high-calorie, high-fat meal. The meal consisted of 2 Jimmy Dean sausage egg muffin sandwiches and 2 hash brown patties (Stop & Shop brand). Ketchup was also provided; approximately 20% of subjects

opted to add it to the breakfast in moderate quantities. The breakfast (excluding yogurt or pudding and ketchup) contained approximately 960 calories. With the yogurt or pudding, total calorie content of the breakfast was 1180 calories. Complete nutrient composition of the breakfast is listed in **Table 2.2**. Participants were supervised throughout consumption of the meal and when they finished, the time was recorded. Subjects were allowed only water during and after the meal.

Following consumption of the high-fat meal, blood samples were collected 1, 2, 3, and 4 h postprandially. At each time point, 10 mL of heparinized blood and 10 mL of EDTA blood was collected. As before, tubes were gently inverted 8-10 times and placed on ice for up to 30 min prior to centrifugation. After collection of the last blood sample, Visit 2 was complete. At this time, subjects were instructed to resume their normal dietary and exercise habits, but to continue to avoid probiotic foods, refrain from taking dietary supplements, and limit dairy consumption. For individuals in the yogurt group, the yogurt constituted 2 servings of dairy; an additional 2 were allowed each day. The pudding was dairy-free; individuals in this group were allowed a full 4 servings of dairy each day. Once Visit 2 was completed, caffeine was allowed at all times except 24 h prior to each visit. Alcohol was acceptable when consumed in moderation.

Prior to their departure from Visit 2, participants were provided with yogurt or pudding supplies to last approximately 1 week. Subjects visited the study center every 7 to 10 days for the duration of the study to restock yogurt or pudding supplies. The intervention period was 9 weeks in length. This 9-week period constituted the chronic portion of the intervention.

During this period, participants were required to consume 12 oz. of yogurt or soy pudding each day. Product lids were saved and returned to us weekly to ensure compliance, and it was

necessary for subjects to maintain at least 80% compliance in order to continue their participation. Additionally, at each visit to the study center subjects were monitored for illness, health status, were asked for verbal confirmation of consumption of the products, and were encouraged to voice any other questions or concerns they might have. It was also noted how and when subjects preferred to consume their yogurt/pudding each day.

At the end of week 5 and again at the end of week 8, subjects visited the study center in the morning for anthropometric measurements and to provide a fasting blood sample. For these visits (Visit 3 and Visit 4), participants arrived fasted and with no caffeine or vigorous exercise within the previous 24 h. Anthropometric measurements were taken as before, following which 40 mL (20 mL in heparinized vacuum tubes and 20 mL in EDTA vacuum tubes) of venous blood was collected via the single stick method. This was the duration of each visit.

Between Visit 4 and Visit 5, subjects were again asked to complete 3 days of dietary records. Guidelines were identical to those described previously, and completed records were used to assure compliance to all dietary restrictions as well as adherence to yogurt or pudding consumption. Visit 5 took place at the end of week 11. This visit comprised the final day of the intervention and the second acute portion of the trial. The procedure for this visit was identical to that for Visit 2, as described above.

Following Visit 5, subjects' participation in the study was complete. All dietary records were entered into the Nutrition Data Systems for Research (NDSR) dietary analysis software following Visits 2 and 5. The program analyzes daily dietary intakes and provides output files of nutrient intake by meal, food, food group, and total daily intake. This data was used to examine changes in dietary intake associated with chronic yogurt and soy pudding consumption in lean



and obese individuals. Fasting blood samples from each visit were used to examine the impact of chronic yogurt consumption on biomarkers of oxidative stress in obese individuals. Visit 2 and 5 fasting and hourly blood samples were used to examine the impacts of co-consumption of yogurt with the high-calorie, high-fat meal on postprandial oxidative stress in obese individuals. Individual biomarkers, expected outcomes, and results will be discussed in later chapters.

### ***2.2.3 Statistical Analysis***

The study was powered to detect an 8.2% difference in sCD14 (the primary outcome), a 29% difference in LPS, and a 21% difference in toll-like receptor 4 (TLR4) [4, 31]. For fasting plasma samples, the study was powered to detect an 11% difference in IL-6 and a 5% difference in TNF $\alpha$  [14]. Level of significance was set at  $\alpha = 0.05$  with a two-sided 95% confidence interval. Attrition rate was expected to be 10%.

Analysis of meal consumption time was done by paired and unpaired t-test using GraphPad Prism 5.0. Level of significance was set at  $P = 0.05$ . Statistical analysis of dietary intake and oxidative stress variables will be discussed in upcoming chapters.

## **2.3 Results and Discussion**

### ***2.3.1 Enrollment***

A full chart detailing study enrollment is provided in **Figure 2.2**. Initial rates of reply to our emails and/or voicemails were less than 50%, and among those who scheduled a phone screening interview, around 50% were not available at their specified time and never returned our calls. Of those who began the phone screening, approximately 15% of lean and 23% of obese individuals did not clear. The most common reasons for disqualification included being postmenopausal,

having a hysterectomy, high blood pressure or diabetes, a BMI that fell outside of our range, or vegetarianism. Of those who cleared the phone screening, 11% failed to attend Visit 1; some individuals gave health or scheduling-related reasons while others failed to contact us in advance. When attempts to make contact failed, these individuals were dropped from the study. As of June 2014, 121 individuals had cleared the phone screening and 106 attended Visit 1. All individuals who attended Visit 1 cleared the screening process and were enrolled in the study.

Between Visit 1 and Visit 2, 11 lean individuals and 8 obese individuals (15% of enrolled individuals in each group) withdrew from the study. Reasons for withdrawal included scheduling conflicts, loss of interest, and changes in health status. Additionally, 2 lean and 2 obese participants voluntarily withdrew from the study between Visit 2 and Visit 3; of these, 3 were unable to comply with the yogurt/pudding consumption and the fourth was unable to attend her scheduled visits. A participant in the obese yogurt group was removed from the study prior to Visit 4 due to a lack of compliance. As of June 2014, 47 lean individuals (24 assigned to the yogurt group and 23 to the pudding group) and 35 obese individuals (17 assigned to the yogurt group and 18 assigned to the pudding group) had completed the study.

### ***2.3.2. Participant characteristics***

Both lean and obese participants were primarily Caucasian (77% and 80%, respectively), unmarried (83% and 54%, respectively), and in college or graduate school (87% and 54%, respectively). There were no statistically significant differences in height between lean and obese participants. However, obese participants were significantly older ( $P < 0.0001$ ), and had a larger BMI ( $P < 0.0001$ ) and waist circumference ( $P < 0.0001$ ) than the lean participants. Additionally, systolic blood pressure ( $P = 0.0002$ ) and diastolic blood pressure ( $P = 0.0025$ ) were significantly

higher in the obese study population. However, no changes in any anthropometric parameters changed as a result of the intervention in either lean or obese individuals. Initial participant characteristics are listed in **Table 2.3**.

### ***2.3.3 Nutritional changes of study products***

In September 2013, Zensoy discontinued the vanilla (and therefore chocolate/vanilla swirl) formulation of the soy pudding and from then on, only chocolate pudding was available. There were also slight changes to the product in terms of ingredients and nutrient content. Notably, calcium decreased (15%-6% DV), saturated fat increased (0-0.5 g), potassium increased (30-150 mg), and vitamin B12 decreased (25%-6% DV) per 4 oz. container. Also, sea salt was replaced by evaporated salt. Subjects enrolled at the time noted an improvement in taste and texture.

The nutrient profile of the breakfast sandwiches also changed midway through the study. Initially, nutrient content of each sandwich totaled 330 calories, 20 g fat, 14 g protein, and 800 mg sodium. In October 2013, each sandwich increased by 20 calories and 1 g of fat, while protein and sodium content decreased by 1 g and 100 mg, respectively. This brought the total for each sandwich to 350 calories, 21 g fat, 13 g protein, and 700 mg of sodium. Carbohydrate content did not change. Overall, the high-calorie, high-fat breakfast increased by 40 calories and 2 g fat while protein decreased by 2 g and sodium by 200 mg. In the context of the entire breakfast, the impact of these changes is expected to be negligible.

### ***2.3.4 Observational outcomes***

Initially, many individuals expressed a preference towards being randomized to the yogurt group, possibly due to their unfamiliarity and/or reluctance towards consuming the soy pudding. Most

participants did not consume much soy as a part of their normal diet and of those that did, none mentioned having previously consumed this particular type of pudding. However, reactions to the pudding were largely favorable, though preference for the vanilla pudding was highly polarized whereas most participants did not mind the other two flavors. Overall compliance to yogurt/pudding consumption was nearly 90%, and only 1 person was removed for failure to comply. Three others voluntarily withdrew due to compliance issues; of these, 2 were due to a dislike of the pudding, and the third individual could not consume 12 oz. of yogurt per day.

Despite the strong desire to be placed in the yogurt group, individuals in both groups reported fatigue of consuming each product by about week 6 of the intervention. At this time, dietary consumption patterns often changed as well. Initially, individuals expressed common tendencies: (1) eating one 4 oz. container of yogurt or pudding with each meal, (2) eating all 12 oz. of yogurt at breakfast; or (3) eating all 12 oz. of yogurt or pudding for dessert following dinner. As the study progressed, more subjects seemed inclined to consume all 12 oz. at the same time and mixed with other foods to simply “get it over with.” Making the product into smoothies was common in the yogurt group but much less so in the pudding group. In fact, only 1 subject reported making smoothies out of the pudding, while many individuals reported consuming the yogurt in this manner.

Notably, consumption of the high-calorie, high-fat meal was not asked to be completed within a certain timeframe, and varied from 20 min to nearly 2 h, with an average of  $38.2 \pm 13.74$  min at Visit 2 and  $34.44 \pm 12.94$  min at Visit 5, though this difference was not significant ( $p = 0.086$ ).

There was also no difference in meal consumption time between lean and obese individuals.

Though these differences are not significant, with such a range of times it would be interesting to examine the association between the speed of meal consumption and postprandial parameters.

### ***2.3.5 Strengths and limitations***

Strengths of this study include the large sample size and the enrollment of only healthy, premenopausal women. This eliminated numerous confounding variables associated with gender differences in postprandial metabolism and fasting levels of oxidative stress and inflammation. This also eliminated documented differences in oxidative stress and metabolic parameters between pre- and postmenopausal women, thus reducing individual variation in biomarkers. The use of healthy women eliminated potential effects of conditions that may have complicated the blood draw and/or medications that may have blunted or interfered with the effects of the yogurt.

All participants were healthy, without signs of cardiovascular disease, diabetes, or any other metabolic conditions. Studies show that 10-25% of obese individuals are classified as metabolically healthy obese (MHO) [34]. These individuals are obese in terms of their BMI but do not experience the metabolic abnormalities that are typically associated with obesity, though the causal factors behind this phenomenon are not well understood [34]. This subpopulation was the one from which our study participants were recruited. Due to this difference, and the inclusion of only MHO individuals in our study, outcomes may not be readily extrapolated to non-MHO individuals, who constitute the majority of the obese population. In addition, these MHO individuals may not exhibit the same degree of postprandial dysmetabolism as non-MHO individuals. Therefore, the impact of yogurt consumption on postprandial metabolism in non-MHO individuals remains to be seen.

The use of Yoplait Original yogurt is a strength of this study, as this brand is formulated similarly to most commercial low-fat yogurts. Therefore, any beneficial health outcomes associated with this study could be feasibly translated into the average person's diet. However,

the use of Yoplait Original is a limiting factor in that it contains more added sugars and less protein and probiotics than other commercial regular or Greek-style yogurts. For individuals with impaired glucose tolerance, the increased sugar intake associated with incorporation of low-fat yogurt into the diet may be detrimental. In addition, any benefits of probiotic consumption may be somewhat limited due to the presence of fewer numbers and strains of the bacteria than in yogurts formulated with probiotics.

Perhaps the greatest limitation of this study is that it is not a crossover design. This is due to the lack of evidence in regards to washing out the intervention effects. Even with this data available, the length of the study would limit feasibility. Due to the nature of the intervention, the study is also not blinded. Additionally, it remains to be seen whether the soy pudding was an adequate control snack. Because the pudding was only offered in the chocolate flavor for the majority of the intervention, it is possible that individuals were not consuming the 2 snacks in a similar manner. For instance, many participants seemed to prefer to consume the yogurt with breakfast while pudding consumption, likely attributable to its chocolate flavor, seemed to take place primarily later in the day and often as a snack or dessert rather than as part (or all) of a meal.

A final limitation is that where the women were in their menstrual cycle was not taken into consideration. Previous studies show differences in certain metabolic parameters in association with phases of the cycle [35, 36]. For example, insulin sensitivity exhibits variation throughout the cycle. Insulin sensitivity was reduced following an oral glucose tolerance test in women in the luteal phase of their cycle [36]. Oxidative stress levels also vary, with highest levels occurring in the luteal phase [35]. This can be attributed to the antioxidant capacity of estrogen and the natural fluctuations of the hormone throughout the cycle. Indeed, one study found both urinary hydrogen peroxide and thiobarbituric acid-reactive substances to be increased during the

luteal phase of the cycle [35]. Because of this, previous studies examining inflammation and oxidative stress required the women to begin participation in the intervention during the follicular phase of the cycle to eliminate this potential source of variation in oxidative stress biomarkers [22, 29, 37]. As this is not something we controlled for in this study, it may confound observed changes in biomarkers of oxidative stress, particularly in fasting plasma samples.

**Table 2.1.** Nutritional profile of the low-fat yogurt and soy pudding snacks.

|                          | <b>Low-fat<br/>yogurt</b> | <b>Soy pudding<br/>(initial)</b> | <b>Soy pudding<br/>(final)</b> |
|--------------------------|---------------------------|----------------------------------|--------------------------------|
| <b>Serving size</b>      | 4 oz                      | 4 oz                             | 4 oz                           |
| <b>Calories</b>          | 110                       | 110                              | 110                            |
| <b>Calories from fat</b> | 10                        | 10                               | 10                             |
| <b>Fat (g)</b>           | 1                         | 1                                | 1                              |
| <b>Saturated fat (g)</b> | 0.5                       | 0                                | 0.5                            |
| <b>Cholesterol (mg)</b>  | 5                         | 0                                | 0                              |
| <b>Carbohydrates (g)</b> | 22                        | 22                               | 22                             |
| <b>Sugars (g)</b>        | 17                        | 18                               | 18                             |
| <b>Protein (g)</b>       | 3                         | 3                                | 3                              |
| <b>Potassium (mg)</b>    | 0                         | 30                               | 150                            |
| <b>Calcium (mg)</b>      | 150                       | 150                              | 60                             |
| <b>Vitamin D (mcg)</b>   | 2.25                      | 2.25                             | 2.25                           |
| <b>Vitamin B12 (mcg)</b> | -                         | 0.6                              | 0.14                           |



**Table 2.2.** Nutritional profile of the high-calorie, high-fat meal.

|                          | <b>Sandwich</b> | <b>Hash brown patty</b> | <b>Meal total</b>                   |
|--------------------------|-----------------|-------------------------|-------------------------------------|
| <b>Serving size</b>      | 1 sandwich      | 1 patty                 | 2 sandwiches + 2 hash brown patties |
| <b>Calories</b>          | 330-350         | 130                     | 920-960                             |
| <b>Calories from fat</b> | 180-190         | 60                      | 480-500                             |
| <b>Fat (g)</b>           | 20-21           | 7                       | 54-56                               |
| <b>Saturated fat (g)</b> | 8               | 1                       | 18                                  |
| <b>Cholesterol (mg)</b>  | 125             | 0                       | 250                                 |
| <b>Carbohydrates (g)</b> | 26              | 15                      | 82                                  |
| <b>Fiber (g)</b>         | 1               | 2                       | 6                                   |
| <b>Protein (g)</b>       | 13-14           | 1                       | 28-30                               |
| <b>Sodium (mg)</b>       | 700-800         | 300                     | 2000-2200                           |
| <b>Potassium (mg)</b>    | 290             | 170                     | 920                                 |

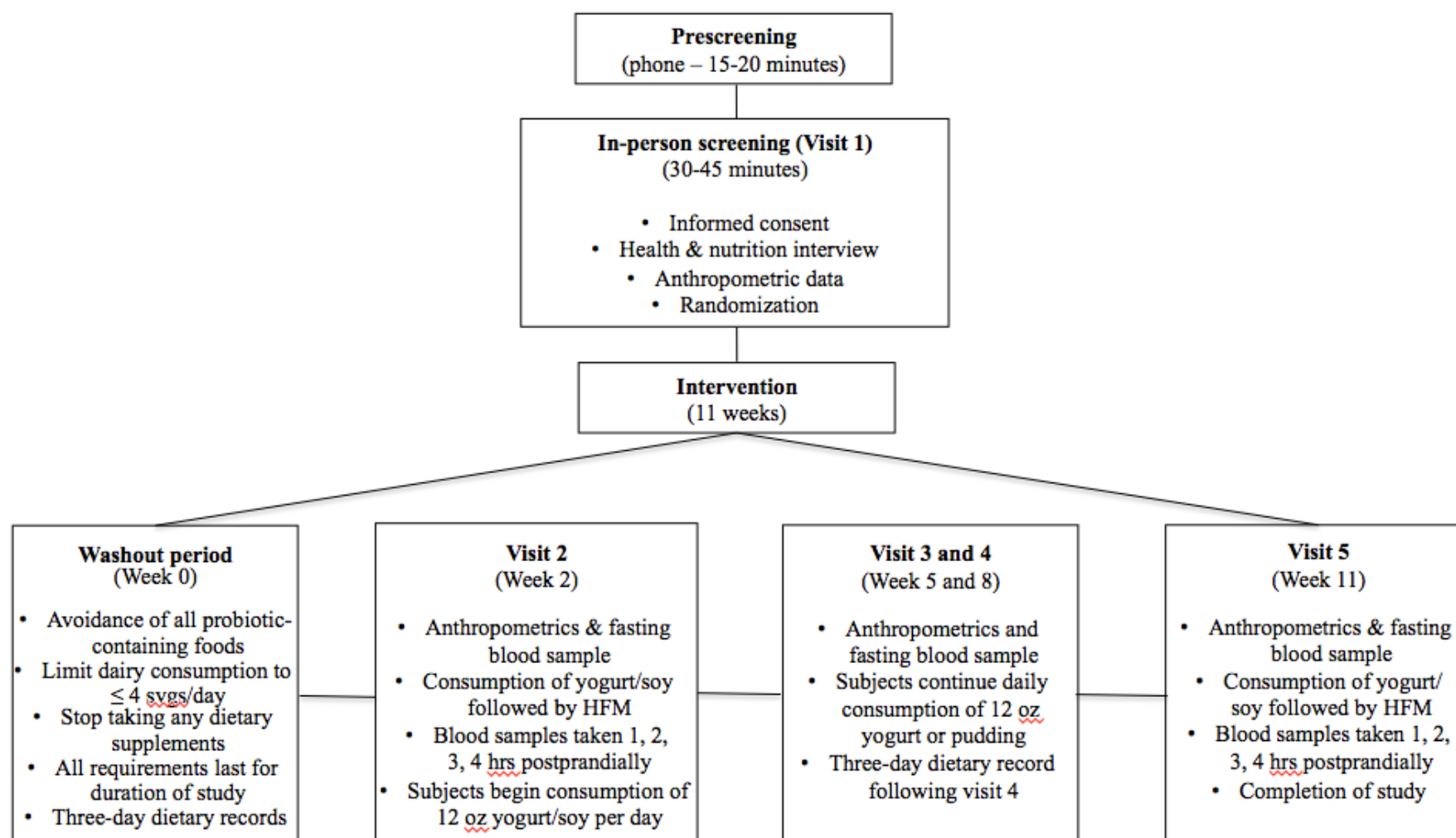
**Table 2.3.** Initial anthropometric characteristics of lean and obese study participants.

|   | <b>LY</b><br><b>(n = 24)</b> | <b>LS</b><br><b>(n = 23)</b> | <b>OY</b><br><b>(n = 17)</b> | <b>OS</b><br><b>(n = 18)</b> |
|---|------------------------------|------------------------------|------------------------------|------------------------------|
| <b>Age (y)</b>                          | 24.8 ± 5.0                   | 23.9 ± 3.7                   | 33.6 ± 11.8 **               | 31.3 ± 8.5 ‡                 |
| <b>Weight (kg)</b>                      | 63.2 ± 8.5                   | 60.1 ± 7.7                   | 92.8 ± 9.7 **                | 92.5 ± 12.3 ‡                |
| <b>BMI (kg/m<sup>2</sup>)</b>           | 23.0 ± 2.5                   | 22.0 ± 2.0                   | 33.9 ± 3.2 **                | 34.1 ± 2.8 ‡                 |
| <b>Waist circumference (cm)</b>         | 73.3 ± 7.7                   | 69.8 ± 6.8                   | 93.5 ± 8.1 **                | 94.7 ± 7.2 ‡                 |
| <b>Systolic blood pressure (mm Hg)</b>  | 103 ± 11                     | 107 ± 12                     | 113 ± 9 *                    | 117 ± 12 †                   |
| <b>Diastolic blood pressure (mm Hg)</b> | 71 ± 8                       | 75 ± 10                      | 78 ± 7 *                     | 79 ± 6 †                     |

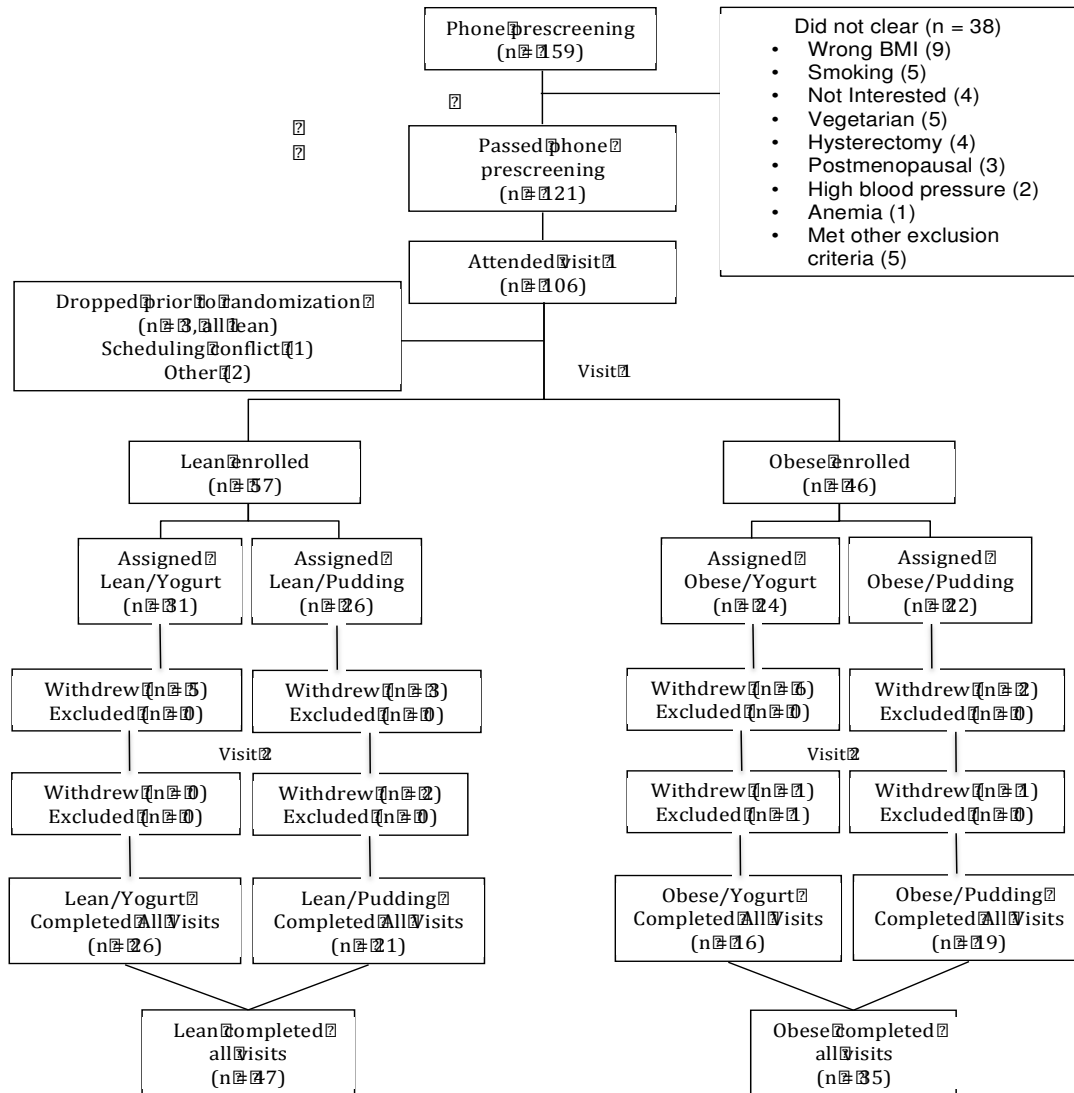
LY = lean yogurt, LS = lean soy, OY = obese yogurt, OS = obese soy. Between-group analysis

by unpaired t-test. \*  $P < 0.05$  from LY, \*\*  $P < 0.0001$  from LY, †  $P < 0.05$  from LS, ‡  $P <$

0.0001 from LS.



**Figure 2.1.** Study design.



**Figure 2.2.** Diagram of study progress, including recruitment, enrollment, and completion of participants as of August 2014.

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**Chapter 3:**  
**Changes in Nutrient Intake Associated with Daily**  
**Consumption of Low-Fat Yogurt**

### 3.1 Introduction

The main goal of this intervention is to determine the impact of low-fat yogurt consumption on intestinal barrier function in obese women. However, when conducting a dietary intervention, it is necessary to record any alterations from normal dietary habits that may arise as a result of the intervention. Therefore, a secondary aim of this study is to examine the impact of daily consumption of low-fat yogurt and soy pudding on dietary intake patterns in both lean and obese women. This chapter will focus on self-reported changes in intake of calories, macronutrients, and micronutrients relevant to dairy products that may change as a result of the intervention.

Epidemiological studies support an inverse association between calcium intake and body fat, and between low-fat dairy consumption and BMI, blood pressure, and all-cause mortality [1-4]. In the postprandial period, co-consumption of dairy products is associated with reductions in fat absorption and deposition, increases in fatty acid  $\beta$ -oxidation, and greater fecal fat excretion [6, 7]. While many of these modulations cannot be directly associated with individual components of dairy products, calcium is known to interfere with fat absorption and increase fecal fat excretion by disrupting micelle formation [8]. Therefore, co-consumption of calcium-containing foods with a high-fat meal may limit fat absorption, thereby modulating the postprandial effects of the meal. Chronically, increased calcium consumption may also foster favorable changes in body composition due to promotion of fatty acid  $\beta$ -oxidation.

It is important to note that 700-800 mg/day of non-supplemental calcium sources are needed to achieve its benefits [7, 9]. For individuals who regularly consume < 700 mg/day, increasing calcium intake to > 700 mg/day is associated with reductions in blood pressure and increases in weight and fat loss when combined with an energy-restricted diet [7, 9]. Individuals that

consume > 700 mg/day may not receive the same benefits from increased calcium intake as those who consume less. However, the recommended dietary allowance (RDA) for adult women is 1000 mg/day of calcium and many women, particularly those over the age of 50, do not reach this amount [10]. Therefore, incorporation of low-fat yogurt into the diet may be a means to reach this goal, as adequate calcium intake is necessary for maintenance of many facets of physiological function [10].

Studies suggest that absorption of calcium is vitamin D dependent [12, 13]. Indeed, the biosynthesis of intestinal calbindin, a protein required for active calcium uptake, is modulated by vitamin D [14]. Though calcium is also absorbed by passive diffusion, this occurs only when intake is high. Therefore, under circumstances of normal calcium intake, adequate vitamin D is critical to maintain calcium absorption [10]. Low serum 25-hydroxyvitamin D and calcium intake are associated with an increased risk for developing type-2 diabetes [15]. Though vitamin D is also synthesized by the body in response to ultraviolet light, latitude and season play large roles in daily sun exposure [10]. Particularly during winter months or in climates that lack exposure to intense sunlight, vitamin D synthesis by the body is likely to be low. Therefore, dietary intake will need to increase to supplement this deficit. The low-fat yogurt and soy pudding used in the present study are fortified with vitamin D; incorporation into the diet would contribute to adequate vitamin D intake.

In addition to calcium and vitamin D, dairy products contain many bioactive components that may contribute to the observed health benefits, including casein, whey, vitamins, minerals, short-chain fatty acids, probiotics (in fermented products), and certain bioactive peptides [5, 16-18]. Despite the established health benefits of dairy consumption and the identification of these bioactive components, much remains unknown. For example, the impact of consumption of

individual dairy products on various health outcomes has not been established. No studies specifically examine the impacts of low-fat yogurt consumption on postprandial and chronic inflammatory parameters in obese women. This study aims to examine these associations. In establishing these outcomes, it is also necessary to monitor changes in dietary intake associated with the intervention, as dietary changes may be important contributors to the observed results.

## **3.2 Materials and methods**

### ***3.2.1 Dietary restrictions***

Two weeks prior to commencement of the dietary intervention, participants began a washout period. During this period, participants were asked to stop taking any dietary supplements, limit their dairy intake to no more than 4 servings per day, and refrain from consuming any foods containing probiotics. This included yogurt and any yogurt-based product, coleslaw, sauerkraut, miso, tempeh, kimchi, probiotic milk products, and other specialty items fortified with probiotic bacteria, including teas and chocolates. At their initial screening visit, participants were provided a list of items, as well as instructions to examine food labels for indications of probiotic cultures. Adherence was ensured by verbal affirmation and completion of dietary records. The protocol for recording dietary intake will be discussed in more detail below.

### ***3.2.2 Dietary intervention***

A complete explanation of this intervention is provided in Chapter 2. Briefly, the intervention period began at week 2, following the 2-week washout. During the intervention, participants were asked to maintain the same restrictions as during the washout period while continuing with

their normal dietary and exercise patterns. The intervention was 9 weeks in length, during which participants were asked to consume either 12 oz. of low-fat yogurt (Yoplait Original in strawberry, strawberry-banana, raspberry, or peach flavors) or dairy-free soy pudding (Zensoy Organic in chocolate, vanilla, or chocolate-vanilla swirl flavors) each day. Participants were required to maintain 80% compliance as assessed by product lids returned on a weekly basis. Compliance to dietary restrictions was assessed by verbal affirmation and analysis of a second set of dietary records, completed prior to the final study visit (week 11).

In September 2013, Zensoy reformulated the soy pudding and eliminated the vanilla and chocolate-vanilla swirl flavors, leaving only chocolate pudding. The ingredient profile was also slightly modified, thereby altering the nutrient profile. Saturated fat content increased (0-0.5 g), potassium increased (30-150 mg), calcium decreased (15%-6% DV), and vitamin B12 decreased (25%-6% DV) per 4 oz. container. Of greatest importance is the change in calcium content. Prior to the reformulation, both the yogurt and soy snacks contained the same amount of calcium (45% DV per 12 oz.), such that all subjects received the same amount of calcium as a result of the intervention. Following the reformulation, participants in the soy group received only 18% DV calcium per 12 oz. of pudding. However, this reformulation occurred early in study. Therefore, the majority of participants consumed the reformulated, lower calcium version of the pudding.

### ***3.2.3 Dietary records***

As discussed in Chapter 2, materials to record dietary records were provided to subjects during their screening visit, along with instructions and a detailed example chronicling how to complete the dietary records. The records included time of intake, food item, and amount. Supplemental materials were provided, listing common serving amounts and examples of 0.5 cup and 1 cup of

food to assist with estimation of intake. The instructions highlighted the importance of specificity when recording the diet, i.e. reporting brand names and qualifiers listed on the labels, cooking methods, and any additives, particularly fats and salt used in cooking and additions to coffee. Recipes needed to be broken down into individual ingredients while components of purchased meals needed to be estimated to the best of the subject's ability. Participants were also instructed to save the packaging of any products that might cause confusion; these were handed in along with the completed records.

Dietary records were collected from each subject at weeks 2 and 11. Each set consisted of 3 days of records, 2 during the week and 1 on a weekend, from non-consecutive days, so as to receive a broad overview of the subject's typical diet. Records turned in at week 2 took place during the washout period and should have included no probiotic-containing products and  $\leq 4$  daily servings of dairy. Those from week 11 were from the final 3 weeks of the intervention period, and should have included the yogurt or pudding products but no other probiotic foods and  $\leq 4$  daily servings of dairy. All dietary information was self-reported. On site, records were checked to ensure comprehensibility and specificity; where this was lacking, subjects were asked to provide necessary details to the best of their ability.

#### ***3.2.4 Entry of dietary records***

Dietary intake data was recorded using Nutrition Data System for Research (NDSR) software versions 2011, 2012, and 2013, developed by the Nutrition Coordinating Center (NCC), University of Minnesota, Minneapolis, MN. Final calculations were completed using NDSR version 2013. The NDSR time-related database updates analytic data while maintaining nutrient profiles true to the version used for data collection. Subjects' name, identification number,

gender, and age range were recorded, along with the date of each record. NDSR allows for the selection of meal time and meal type (breakfast, lunch, dinner, snack, beverage only). All records were assumed to be accurate and representative of the participant's normal diet.

Entering the records was more challenging than anticipated. While fruits, vegetables, and other common items were fairly simple to locate and record, packaged foods, restaurant or commercial meals, and university meals proved much more complicated. Because many of our participants were university students, meals purchased from dining halls, campus markets, and local restaurants were common. However, nutritional information for these meals was often difficult to locate. Additionally, new, local, and uncommon products were often not listed in the database.

Frequently, upon entering individual records into the database, it became evident that the provided data were not in agreement with the level of specificity required by NDSR. When possible, subjects were contacted and asked to clarify the composition of foods. If the subject was unable to be reached or was unable to provide more specific information, alternate steps were taken. Where possible, the ingredient and/or nutrition fact labels of these foods were searched online. A picture of the ingredient labels could sometimes be located on the product website, and restaurants often provide basic nutrition facts for their menu items. When this information was accessible, it was compared to that of the products listed in NDSR, and the most similar alternative was selected. For restaurant menu items, the ingredients, measurements, and preparation methods were established as accurately as possible via the information provided. When dietary information could not be located, a visual comparison of foods was performed, whereby the recorded food and the options in the database were searched online, and the most similar alternative was selected based on pictures of the food itself, the packaging, any labels or claims, and any other available information. In these cases, the original food and the database

substitute were recorded. For cases in which preparation methods or the list of ingredients were not detailed enough to allow for accurate estimation, the 'unknown' option was selected in NDSR.

To ensure consistency, a standardized list was created for common items. These were items that were assumed by most subjects to be of a normal size. Items included many fruits and vegetables, slices of pizza, eggs, and bread slices. All items were assumed to be of medium size, unless expressly specified by the participant. Items such as butter and peanut butter, which are most commonly salted, were assumed to be so unless otherwise specified. Food preparation methods were also standardized.

### ***3.2.5 Analysis of dietary records***

Analysis of dietary intake was also conducted using the NDSR software. The database provided a comprehensive list of daily nutrient intake that included calories, all macro- and micronutrients, as well as individual amino acids, fatty acids, and artificial sweeteners. For our analysis, we elected to focus on caloric intake, macronutrients, and a select few micronutrients that were likely to be impacted by yogurt consumption. These nutrients included fat, saturated fat, carbohydrates, protein, cholesterol, calcium, vitamin D, and vitamin C. NDSR also provided output reports of dietary intake by food group. NDSR defined a serving of dairy as 8 fl. oz. of milk, 1 cup of yogurt, 1.5 oz. of cheese, 2 oz. of cream cheese, and 0.5 cups of ice cream. A serving of fruit was defined as 1 medium piece, or approximately 0.26 cups dried fruit. Vegetable servings was highly varied; 0.39 cups carrots, 0.5 cups broccoli, 1 cup lettuce or corn, and 1 medium potato were each equivalent to a serving of vegetables. These reports were



examined for associations between changes in macro- and micronutrient intake and changes in consumption of foods from certain food groups.

### ***3.2.6 Statistical analysis***

A participant in the lean soy group was excluded from the analysis due to missing records. Final sample size was  $n = 24$  in the lean yogurt (LY) group,  $n = 22$  in the lean soy (LS) group,  $n = 17$  in the obese yogurt (OY) group, and  $n = 18$  in the obese soy (OS) group.

All statistical analysis was conducted using GraphPad Prism 5.0 software. This included analysis of macro- and micronutrient intake and intake by food group. Between-group analysis was conducted by repeated-measures two-way ANOVA, using time and treatment as factors; Bonferroni post-tests were carried out where appropriate. Within-group analysis was conducted by paired t-test as indicated in tables and figures. Significance level for all statistical tests was set at  $P < 0.05$ .

## **3.3 Results**

### ***3.3.1 Changes in macronutrient intake***

For this analysis, calorie, carbohydrate, fat, saturated fat, cholesterol, and protein intake were of interest. Among lean individuals, there were no significant changes in calorie, fat, saturated fat, protein, or cholesterol intake following consumption of the yogurt or soy pudding (**Table 3.1**). However, there was a significant time effect for carbohydrate intake in the lean group ( $P = 0.0009$ ) (**Figure 3.1 (a)**). Carbohydrate intake increased in the LY group, from  $197 \pm 39$  g/day

(mean  $\pm$  SD) at week 2 to  $224 \pm 54$  g/day at week 11 ( $P = 0.0260$ ), and in the LS group, from  $201 \pm 54$  g/day at week 2 to  $232 \pm 46$  g/day at week 11 ( $P = 0.0146$ ).

Among obese individuals, there were no differences in calorie, fat, or saturated fat intake (**Table 3.2**). As with lean individuals, there was a significant time effect for carbohydrate intake in obese individuals from the intervention ( $P = 0.0089$ ) (**Figure 3.2 (a)**). Carbohydrate intake increased in the OY group ( $210 \pm 72$  g/day at week 2 to  $246 \pm 54$  g/day at week 11;  $P = 0.0162$ ). However, this increase was not observed among the OS group. There was a weak trend towards a significant time effect for protein intake among obese individuals ( $P = 0.0824$ ) (**Figure 3.2 (b)**). In fact, despite the protein content of the yogurt (9 g per 12 oz.), protein intake decreased significantly in the OY group, from  $81 \pm 17$  g/day at week 2 to  $72 \pm 13$  g/day at week 11 ( $P = 0.0349$ ). However, this change was not observed in the OS group. Interestingly, there was also a significant time effect observed for cholesterol intake ( $P = 0.0106$ ) (**Figure 3.2 (c)**); intake decreased significantly in the OY group, from  $337 \pm 159$  mg/day at week 2 to  $216 \pm 87$  mg/day at week 11 ( $P = 0.0066$ ). The decrease in cholesterol intake in the OS group did not reach significance.

### ***3.3.2 Changes in micronutrient intake***

For micronutrient analysis, calcium, vitamin D, and vitamin C were nutrients of interest. Though vitamin C is not found in either snack in significant quantities, it may favorably impact calcium absorption and therefore is of interest [19]. Changes in intake of all 3 nutrients were observed as a result of the intervention (**Table 3.3** and **Table 3.4**).

There was a significant time effect ( $P < 0.0001$ ) and a treatment-time interaction ( $P = 0.0147$ ) for calcium intake among lean individuals (**Figure 3.3 (a)**). Calcium intake increased

significantly in the LY group, from  $696 \pm 214$  mg/day at week 2 to  $1026 \pm 230$  mg/day at week 11 ( $P < 0.0001$ ). Though calcium intake increased modestly in the LS group during the intervention period, it did not reach statistical significance ( $P > 0.05$ ). This was expected, as the yogurt contained more calcium than the soy pudding (45% DV versus 18% DV per 12 oz.) following the reformulation of the pudding. Because this change was made early in the study, more participants consumed the reformulated version than the initial version. Therefore, this supports our hypothesis that calcium intake would increase to a larger degree in the LY group than in the LS group.

Among lean individuals, there was a significant time effect for vitamin D intake ( $P < 0.0001$ ) (**Figure 3.3 (b)**). Vitamin D intake significantly increased in both the LY and LS groups. This was expected, as both the yogurt and soy pudding were fortified with vitamin D (45% DV per 12 oz.). In the LY group, intake of vitamin D increased from  $4.07 \pm 3.13$  mcg/day at week 2 to  $9.91 \pm 2.40$  mcg/day at week 11 ( $P < 0.0001$ ). In the LS group, vitamin D intake increased from  $5.71 \pm 3.72$  mcg/day at week 2 to  $11.18 \pm 3.31$  mcg/day at week 11 ( $P < 0.0001$ ). Interestingly, individuals in the LY group showed a decrease in vitamin C intake from week 2 to week 11 ( $102 \pm 46$  mg/day to  $70 \pm 49$  mg/day;  $P = 0.0056$ ) (**Figure 3.3 (c)**). There was also a weak trend towards decreasing vitamin C intake in the LS group ( $P = 0.0756$ ).

As with lean individuals, there was a significant time effect for calcium intake among obese individuals ( $P = 0.0002$ ), though there was no significant treatment effect (**Figure 3.4 (a)**). Calcium intake increased from  $860 \pm 462$  mg/day at week 2 to  $1178 \pm 253$  mg/day at week 11 ( $P = 0.0089$ ) in the OY group and from  $801 \pm 225$  mg/day at week 2 to  $1013 \pm 369$  mg/day at week 11 ( $P = 0.0102$ ) in the OS group. There was also a significant time effect for vitamin D intake among obese individuals ( $P < 0.0001$ ) (**Figure 3.4 (b)**). In the OY group, vitamin D intake

increased from  $4.43 \pm 3.97$  mcg/day at week 2 to  $10.22 \pm 1.94$  mcg/day at week 11 ( $P < 0.0001$ ) and in the OS group from  $5.01 \pm 4.27$  mcg/day at week 2 to  $9.79 \pm 2.06$  mcg/day at week 11 ( $P = 0.0011$ ). Unlike that observed in lean individuals, there was no change in vitamin C intake in either the OY or OS groups (**Figure 3.4. (c)**). Individuals in all groups were exceeding the RDA for vitamin C intake prior to the intervention, and there was no difference in initial vitamin C intake between lean and obese individuals that may account for these differences. This will be discussed in more detail below.

### ***3.3.3 Changes in intake by food group***

To better describe the basis for observed changes in macro- and micronutrient intake, dietary intake by food group was examined, using the Food Group Totals output reports in NDSR.

Though participants were asked to limit their dairy intake to  $\leq 4$  servings per day throughout the washout and intervention periods, dairy intake in lean individuals increased over time ( $P = 0.0055$ ), as shown in **Table 3.5**. There was also a significant treatment effect among both lean ( $P = 0.0038$ ) and obese ( $P = 0.0226$ ) individuals and a significant time-treatment interaction for dairy intake among lean ( $P < 0.0001$ ) and obese ( $P = 0.0023$ ) individuals (**Figure 3.5**).

Dairy intake (including the daily yogurt consumption) increased among individuals in the LY group, from  $1.13 \pm 0.66$  servings/day at week 2 to  $2.15 \pm 0.54$  servings/day at week 11 ( $P < 0.0001$ ) (**Figure 3.5 (a)**). This change was also observed among obese individuals; intake in the OY group increased from  $1.67 \pm 1.40$  servings/day at week 2 to  $2.57 \pm 0.90$  servings/day at week 11 ( $P = 0.0130$ ) (**Figure 3.5 (b)**). The soy pudding was dairy-free; no increases in dairy consumption were observed in either the LS or OS groups. Interestingly, despite the 4 daily servings of dairy limit imposed upon both groups, there was a slight but insignificant decrease in

dairy consumption in both the LS and OS groups. Thus, participants were found to adhere to the requirement to consume  $\leq 4$  dairy servings/day.

To examine the unexpected decreases in vitamin C intake, fruit and vegetable intake was assessed (**Table 3.6** and **Table 3.7**). Interestingly, there was a significant time effect for both fruit ( $P = 0.0217$ ) and vegetable ( $P = 0.0001$ ) intake among lean individuals (**Figure 3.6**). Fruit intake in the LY group decreased from  $1.96 \pm 1.20$  servings/day at week 2 to  $1.19 \pm 1.11$  servings/day at week 11 ( $P = 0.0031$ ) (**Figure 3.6 (a)**) and there was a weak trend towards decreased vegetable intake ( $P = 0.0909$ ) in this group (**Figure 3.6 (b)**). There were no changes in fruit intake in the LS group. However, vegetable intake in the LS group decreased significantly, from  $3.94 \pm 1.64$  servings/day at week 2 to  $2.59 \pm 1.27$  servings/day at week 11 ( $P = 0.0001$ ).

Among obese individuals, there was a significant time effect only for fruit consumption ( $P = 0.0067$ ) (**Figure 3.7 (a)**). Intake in the OY group decreased from  $1.81 \pm 1.56$  servings/day at week 2 to  $1.00 \pm 0.95$  servings/day at week 11 ( $P = 0.0318$ ). No change was observed in the OS group. Likewise, no significant changes in vegetable intake were observed in either group (**Figure 3.7 (b)**).

### **3.4 Discussion**

#### ***3.4.1 Macronutrient intake***

Because the intervention was designed to be isocaloric, the participants were encouraged to substitute the intervention snacks into the diet rather than adding them onto normal intake was stressed. Therefore, it was anticipated that calorie and macronutrient intake would not change as

a result of the intervention. Indeed, intake of calories, fat, and saturated fat did not change significantly in any group. Though calorie intake increased by 60-130 calories per day in all groups, this did not reach significance. Moreover, the intervention snacks contributed 330 calories per day. Therefore, it appears that most participants were fairly successful in substituting the snacks into their diets. In addition, as discussed in Chapter 2, no significant changes in weight or BMI were recorded.

However, carbohydrate intake increased significantly in 3 of the 4 intervention groups (LY, LS, OY) and protein and cholesterol intakes decreased in the OY group. A likely explanation for the increase in carbohydrate intake is the carbohydrate content of the intervention snacks.

Consumption of 12 oz. of yogurt or soy pudding per day resulted in the addition of 66 g of carbohydrates to the diet. Although the RDA for carbohydrate intake for women is 130 g/day, average intake is 180-230 g/day. This intake, though in excess of the RDA, is necessary in order to meet caloric needs while consuming appropriate proportions of each macronutrient [20]. 66 g is a substantial portion of this amount; therefore it is likely that the foods for which the yogurt or pudding was substituted did not contribute an equivalent amount of carbohydrates to the diet, leading to the observed increase. In addition, because fat and protein intake remained largely unchanged, the minor increases in calorie intake were probably a result of this increased carbohydrate intake.

Though protein intake remained fairly stable in 3 of the groups, intake decreased significantly in the OY group. This was unexpected, as the yogurt contributed 9 g protein per 12 oz. In addition, the fact that this occurred only in 1 group is noteworthy, as both the yogurt and pudding contained the same amount of protein. There was also a significant decrease in cholesterol intake in the OY group only. As the yogurt and pudding contained negligible amounts of cholesterol,

this decrease was not directly related to the intervention snacks. However, many individuals reported consuming the yogurt as breakfast, often in tandem with granola or as a smoothie, while the pudding was not commonly consumed in this manner. Therefore, it is possible that consumption of other protein and/or cholesterol-containing breakfast foods, such as eggs, decreased in the OY group. However, this change was not seen in the LY group. Clearly there were differences in dietary substitution patterns between lean and obese individuals; this is something that should be examined more closely at a later date.

### ***3.4.2 Micronutrient intake***

As expected, changes in micronutrient intake were observed in all intervention groups. Calcium intake increased significantly in 3 of the 4 intervention groups (LY, OY, OS) and vitamin D intake increased significantly in all groups.

During the washout period, women in all 4 study groups consumed approximately 700-800 mg/day of calcium. The yogurt provided 45% DV (450 mg) calcium per day. Inclusion of 12 oz. of yogurt into the diet helped individuals in the LY and OY groups to increase their daily calcium intake by > 300 mg/day. Interestingly, this is less than the amount provided by the yogurt, suggesting that daily consumption of 12 oz. of yogurt resulted in a reduction in consumption of other calcium sources. Indeed, dairy intake increased by approximately 1 serving/day in each group; 12 oz. of yogurt is approximately 1.5 servings of dairy. Therefore, consumption of non-yogurt dairy products decreased by approximately 0.5 servings/day during the intervention in both the LY and OY groups.

Though the soy pudding contained much less calcium than the yogurt, incorporation of the pudding into the diet helped individuals in the OS group to significantly increase their intake.

The soy pudding was fortified with calcium (18% DV per 12 oz.), which added 180 mg/day from the intervention. The mean increase in calcium intake in the OS group was  $213 \pm 311$  mg/day. This also suggests that the decrease in non-intervention-snack dairy intake observed in the LY and OY groups did not take place in the OS group. Indeed, the OS group reported only a small reduction in dairy intake; though it did not reach significance, intake decreased by  $0.37 \pm 0.94$  servings per day. Therefore, incorporation of 12 oz. of soy pudding into the diet helped obese women increase calcium intake.

Interestingly, the increase in calcium intake in the LY group fell short of significance. Despite the 180 mg calcium contributed by the soy pudding, intake increased by just under 100 mg/day in this group, suggesting that calcium from other sources decreased during the intervention. However, dairy intake decreased insignificantly, by a mere  $0.26 \pm 0.87$  servings/day. This reduction is less than that observed in the OS group and yet the increase in calcium intake in the OS group did reach significance. Therefore, the types of dairy products being eliminated in each group may have been different. It is also possible that the yogurt or pudding were consumed in place of non-dairy sources of calcium, such as vegetables, fortified juices, or fortified non-dairy milks. This will be discussed in more detail below.

Regardless, incorporation of low-fat yogurt into the diet significantly increased dairy and calcium intake in lean and obese women. This is important, because consumption of dairy products is associated with numerous positive health outcomes [1, 2, 4, 5, 11, 17, 21]. In addition, increasing calcium consumption from suboptimal ( $< 700$  mg/day) to optimal ( $> 700$  mg/day) leads to beneficial changes in body mass, composition, and various health outcomes [7, 22]. Though individuals in this study were already reaching this threshold without addition of the yogurt or pudding, low-fat yogurt may be a means of increasing calcium intake among



individuals who regularly consume suboptimal amounts of calcium. In addition, though the optimal amount of calcium in terms of various body composition and inflammatory outcomes is 700 mg/day, the RDA for women age 19-50 y is 1000 mg/day [10]. This is because calcium is also necessary for bone health and other physiological functions [10]. Prior to the intervention, average calcium intake in all groups was below the RDA. Addition of low-fat yogurt to the diet helped lean and obese women meet the RDA for calcium intake.

Among obese women, the soy pudding was also successful in raising calcium intake to reach the RDA. However, some studies support the benefit of dairy calcium over other forms of calcium in various health outcomes [23]. Though the reasons for this are not known, it has been hypothesized that the other bioactive components of dairy may work in tandem to carry out the health benefits associated with calcium [24, 25]. For example, casein and calcium can interact, forming a casein-phosphopeptide-calcium complex that may increase calcium absorption [25]. In addition, a study by Zemmel, et al. compared the impact of dairy versus soy on biomarkers of chronic oxidative stress and inflammation in overweight and obese individuals and found no significant changes in the soy intervention [18]. However, the dairy-consuming group had reductions in biomarkers of oxidative stress and inflammation [18]. Finally, many Americans do not consume the suggested 3 servings of dairy per day [26]. This suggests that yogurt, but not the soy pudding, would be a more effective method of increasing calcium intake as it may also help individuals reach current recommendations for dairy intake.

The low-fat yogurt and soy pudding were both fortified with vitamin D (45% DV per 12 oz.); intake increased in all intervention groups. The 45% DV is equivalent to 6.75 mcg vitamin D provided by the intervention snacks. The RDA for vitamin D for women age 19-50 y is 15 mcg/day [10]. Initial intake in each group averaged 4-6 mcg/day, while final intake averaged 9.8-

11.2 mcg/day, an average increase of approximately 5.5 mcg/day. This is slightly less than the 6.75 mcg/day provided by the yogurt and pudding. Because fortified dairy products are common sources of vitamin D, the aforementioned reduction in intake of non-yogurt dairy products may explain this discrepancy among the LY and OY groups. However, as with calcium intake, individuals in the LS and OS groups reported only minor decreases in dairy intake. Therefore, as before, non-dairy sources of vitamin D (including fish, and fortified juices, cereals, and non-dairy milk) must have contributed to intake prior to the intervention but were substituted out of the diet during the intervention period.

While daily yogurt and soy pudding consumption significantly increased vitamin D intake, intake still did not reach the RDA; therefore, other dietary strategies are needed to assure adequate vitamin D intake in both lean and obese women. Adequate vitamin D is necessary for modulation of calcium uptake and intracellular calcium concentration (which regulates the balance of fatty acid storage and breakdown). This is especially important among obese individuals. Vitamin D is a fat-soluble vitamin and is sequestered by adipose tissue, reducing its bioavailability [27]. Therefore, obese individuals may need to consume more vitamin D than lean individuals. Though data from randomized controlled trials is scarce and results are mixed, some studies suggest an inverse relationship between serum 1,25-dihydroxyvitamin D concentrations and risk of cardiovascular-related deaths in older individuals [28]. Clearly adequate vitamin D intake is important; however, among women in the present study intake was lacking. Incorporation of low-fat yogurt or fortified soy pudding into the diet successfully increased vitamin D intake in lean and obese women. Nevertheless, intake of these products needs to be combined with other vitamin D-rich foods, such as fish, fortified cereals or juices, or other dairy products, to reach the RDA.

Though many studies support the health benefits of overall dairy consumption, low-fat dairy products in particular are associated with lower BMI and waist circumference, as well as lower concentrations of certain inflammatory biomarkers in epidemiological studies [4, 21]. A Greek study found inverse associations between low-fat milk and C-reactive protein (CRP), low-fat milk and homocysteine, and low-fat yogurt and TNF $\alpha$  [5]. A fourth study reported increased reductions in inflammatory biomarkers in individuals on a diet containing fermented dairy products compared with those on a low-fat dairy diet [1]. Therefore, because it is low-fat and contains calcium, vitamin D, and probiotics, low-fat yogurt may be of particular benefit.

The low-fat yogurt and soy pudding used in the present study were not sources of vitamin C. However, vitamin C may facilitate calcium absorption [19]. Therefore, intake was examined closely. Interestingly, addition of yogurt or soy pudding to the diet decreased vitamin C intake in both the LY and LS groups. The decrease observed in the LY group was unexpected, as many individuals reported consuming the yogurt as smoothies, and fruit is a typical component of smoothies, as well as a main contributor to vitamin C intake. However, some individuals added milk and/or protein powder to their smoothies instead. It is possible that the addition of yogurt and pudding to the diet resulted in elimination of other foods. Fruit juice and vegetables also contain vitamin C; if yogurt and pudding consumption replaced such foods, vitamin C intake would then decrease. This will be further discussed below.

### ***3.4.3 Intake by food group***

During the washout and intervention periods, dairy consumption was limited to  $\leq 4$  servings per day in both the yogurt and soy groups. Initial intake in all groups was low – between 1.1-1.7 servings/day. Interestingly, initial intake in the obese groups was higher than in the lean groups.

Though these differences did not reach significance, it is worth noting that epidemiological studies report lower dairy intake among obese individuals compared to lean individuals [11, 29]. However, this was not the case for individuals enrolled in the present study.

As a result of the intervention, dairy intake increased in the yogurt groups by approximately 1 serving/day in both the LY and OY groups, to  $2.15 \pm 0.54$  and  $2.57 \pm 0.90$  servings/day, respectively. This suggests that, rather than replacing consumption of other dairy products with the yogurt, individuals tended to consume the yogurt in addition to their normal dairy intake. However, because calorie intake did not significantly decrease, the 330 calories contributed by the yogurt and soy pudding must have been removed from the diet by way of eliminating foods from other food groups.

Indeed, fruit intake decreased in all groups, though only the decreases in the LY and OY groups reached significance. Vegetable intake also significantly decreased in the LS group and there was a trend towards decreased intake in the LY group. This may be explained by variability in consumption of the intervention snacks. Yogurt, due to its fruit flavor, tended to be consumed early in the day, often as breakfast. If the yogurt was being consumed as a meal replacement, it may have led to a reduction in consumption of other breakfast foods, of which fruit is often a component. On the other hand, consumption of the soy pudding, likely due to its chocolate flavor, most often took place later in the day. Many participants reported consumption of the pudding as a snack, or as dessert after lunch or dinner. Vegetables are a common snack or component of lunch and dinner; perhaps the pudding was consumed in their place. On the other hand, these changes in vegetable intake were only observed among lean individuals.

Therefore, as discussed briefly above, there were significant differences in the ways in which individuals chose to substitute the yogurt and pudding into their diets. Though no changes in calorie consumption reached the level of significance, there were slight increases in all groups. Moreover, these increases were larger among lean individuals. It is possible that the obese women were more concerned about gaining weight as a result of the intervention and were therefore more careful to maintain their normal fruit and vegetable intake while reducing consumption of other foods. In addition, the nutritional counseling provided to participants during the intervention was minimal. Perhaps a more intense counseling session would have led to less divergence in the ways in which the intervention snacks were substituted into the diet.

Regardless, these outcomes support the notion that it is necessary to monitor dietary changes associated with any dietary intervention. Though there are many positive health outcomes associated with consuming adequate dairy, vitamin D, and calcium, there is also ample evidence supporting the importance of adequate fruit and vegetable intake [30-32]. Therefore, incorporation of low-fat yogurt or soy pudding into the diet should be done in tandem with the maintenance of adequate fruit and vegetable intake.

#### ***3.4.4 Strengths and limitations***

The main strength of this intervention is that the low-fat yogurt provided is representative of most commercial yogurts. It is widely available and more affordable than Greek-style or other specialty yogurts. Therefore, increasing calcium, vitamin D, and dairy intake by addition of low-fat yogurt to the diet is a strategy that would be easily translatable to the general population. A second strength is the use of a comprehensive, in-depth dietary assessment program. NDSR is a widely-used and well-established diet analysis program [33]. It has numerous options, including

foods from many specific restaurants and brands. It also requires a high degree of specificity for each food item entered, thereby increasing the accuracy of the nutritional analysis. However, a downside to this high specificity is that many individuals are not able to sufficiently report these parameters; therefore, the “unknown” option was selected when entering some items, reducing accuracy of the analysis.

The well-documented National Health and Nutrition Examination Survey (NHANES) is a nationwide, multi-year study that examines, among other parameters, health status and dietary intake in the United States. As of 2013, data was available for nine NHANES study periods [34]. To gather information on nutrient intake data, researchers primarily utilized the 24 h dietary recall interview method [34]. However, a recent review examining the accuracy of such reporting methods found that, particularly among women, misreporting of intake was highly prevalent. This misreporting was found in three forms; underreporting intake, over-reporting intake, and underreporting intake as a result of simultaneously increased calorie intake and physical activity levels. Of these, underreporting was the most prevalent, while over-reporting was the least common [34]. Degree of underreporting also tended to be directly associated with body weight [35]. In addition, underreporting was more highly associated with nutrients deemed “unhealthy,” i.e. fat and sugar, while over-reporting was associated with “healthy” nutrients, such as protein [34, 36]. Focus groups discussing the recording of dietary intake and found that many overweight and obese women were embarrassed of their diets and therefore consciously ate less on days during which they recorded intake [37]. This would also tend to bias the reliability of dietary records.

Archer, et al. defined underreporting as the ratio between reported energy intake (rEI) and basal metabolic rate (BMR); a value  $< 1.35$  was indicative of an rEI not physiologically compatible

with survival [34]. This value is based on previous assessments examining the reporting of energy intake compared with BMR, energy expenditure, body weight, and other parameters [38]. An examination of all NHANES dietary intake data revealed that the average rEI:BMR ratio was 1.19, supporting the notion that underreporting is widespread [34]. Furthermore, underreporting tended to increase with BMI. Over the course of all NHANES surveys, no more than 43% of overweight and obese women reported intake values that fell within the plausible range. When this category was modified to include only obese women, this number declined to just 31%. Even among normal weight women, rEI:BMR scores often fell below 1.35, with just 48 to 65% of women reporting intakes compatible with physiological survival [34].

It is important to note that determination of BMR is not an exact science, and individual variation is common due to a variety of factors. Nevertheless, it is important to examine dietary intake data for plausibility. Indeed, many of the problems faced by NHANES researchers are apparent in our research. Many daily intakes totaled less than 1500 calories, while the recommended for healthy females age 20-50 y ranges from 1800-2400 calories/day, depending on age and physical activity level [39]. Anything less than this would be expected to lead to weight loss, which was not observed among any of the intervention groups in the present study. Average calorie intake among lean participants ranged from approximately 1000 to 2400 (average  $1598 \pm 353$ ) calories per day while among obese participants, intake ranged from approximately 800 to 2600 (average  $1865 \pm 442$ ) calories per day. While we did not determine BMR or individual energy expenditures, it is clear based on normal caloric recommendations and weight status that many individuals underreported their intake.

Possible reasons for this inconsistency include improper estimations, measuring, or reporting, discrepancy between consumed foods and those available in the database, and the potential

impact of recording one's diet on daily food intake. As discussed above, fear of being judged may lead individuals to underestimate foods widely considered to be unhealthy, or to simply avoid recording their diet on days in which such foods were consumed. Due to the tedious nature of recording dietary intake, individuals may also be inclined to selectively report days in which their food choices are less complex and/or easier to properly measure or estimate. All of these factors may contribute to inaccuracies and may also reduce the representativeness of the records. Training in proper estimation, measuring, and reporting methods has been shown to decrease underreporting. Therefore, a short course in dietary intake reporting could be incorporated to future studies to reduce the prevalence of underreporting.

Another limitation we faced was with NDSR. As with any diet analysis software, it is not possible to include all existing food items. Sometimes the actual food consumed could not be found, thereby reducing the accuracy of the analysis. Additionally, a large portion of our study population consisted of university students, faculty, and staff, many of whom frequently dined on campus. While the university is fairly comprehensive in its listing of nutritional information for food items purchased at markets, food consumed in the dining halls is much more difficult to analyze. These items are not often served in measured quantities, dish size varies, and there can be numerous options for additional toppings, garnishes, condiments, etc. Unfortunately, this greatly complicated estimations of intake. Additionally, previous research shows that reported nutrient information from restaurants is often highly inaccurate [40]. As most of our participants consumed at least 1 meal from a restaurant during their 6 days of dietary records, this is a potential source of inaccuracy. Because many restaurant foods are not listed in NDSR, we were often required to look up nutrition data on the restaurant's website and find the closest match in



NDSR. However, if the data listed on the website was inaccurate, so too was the substituted food.

To overcome these limitations, a more in-depth dietary record should be developed that includes more specific questions. There are also programs designed to assist individuals in determining the most accurate means of recording each food. Ideally, participants would be provided with adequate training in proper measuring and estimation techniques and would enter their dietary intake directly into the computer program with the assistance of researchers. This would allow for participants to become familiar with the qualifiers associated with certain foods (i.e. what part of the chicken was eaten, how was it prepared, was the amount consumed based on the before or after cooking amount), thereby increasing their awareness of the specificity necessary for accurate reporting. Participants could also be provided with a scale or other measuring implements to assist with their estimations.

Lastly, it remains to be seen whether the soy pudding was an adequate control snack. Benefits of the pudding are that it was dairy-free, lower in calcium, isocaloric, and similar in texture to the low-fat yogurt. This is important, as texture is known to impact feelings of satiety [41]. Because the pudding was dairy-free, dairy consumption in the yogurt group increased significantly, while dairy consumption in the soy group remained largely unchanged. However, the fortification of the pudding with vitamin D led to similar increases in intake in both intervention groups; therefore any oxidative and inflammatory outcomes cannot be linked to the vitamin D content of the yogurt. In addition, it appears that the yogurt and soy pudding were not consumed in similar manners, likely due to the more dessert-like flavor of the pudding. Therefore, the patterns by which each product was substituted into the diet varied. This also led to reductions in fruit and vegetable intakes in some of the intervention groups, while intake in the others remained stable.

Perhaps other non-dairy control snacks or alternate flavors of soy pudding should be examined in an effort to reduce these differences.

**Table 3.1.** Self-reported changes in macronutrient intake of lean women during a two-week washout period (initial) during which probiotic-containing foods were eliminated from the diet and dairy consumption was limited to  $\leq 4$  servings/day and during a dietary intervention (final) consisting of daily consumption of 12 oz. low-fat yogurt or soy pudding.

| <b>Nutrient</b>          | <b>Group</b> | <b>Initial</b> | <b>Final</b> | <b>Change</b> |
|--------------------------|--------------|----------------|--------------|---------------|
| <b>Calories</b>          | LY           | 1556 ± 352     | 1692 ± 532   | 136 ± 442     |
|                          | LS           | 1644 ± 257     | 1764 ± 289   | 120 ± 352     |
| <b>Fat (g)</b>           | LY           | 56.4 ± 20.3    | 56.5 ± 28.5  | 0.13 ± 24.1   |
|                          | LS           | 64.0 ± 16.7    | 59.0 ± 15.7  | - 5.0 ± 20.7  |
| <b>Saturated Fat (g)</b> | LY           | 18.5 ± 8.6     | 18.2 ± 9.0   | - 0.3 ± 10.2  |
|                          | LS           | 20.9 ± 7.15    | 19.2 ± 6.17  | - 1.7 ± 7.7   |
| <b>Cholesterol (mg)</b>  | LY           | 256 ± 116      | 253 ± 176    | - 4 ± 134     |
|                          | LS           | 254 ± 145      | 225 ± 162    | - 29 ± 126    |
| <b>Carbohydrates (g)</b> | LY           | 197 ± 39       | 224 ± 54*    | 27 ± 57       |
|                          | LS           | 201 ± 54       | 232 ± 46*    | 30 ± 53       |
| <b>Protein (g)</b>       | LY           | 70.8 ± 20.5    | 71.3 ± 29.5  | 0.6 ± 25.1    |
|                          | LS           | 71.9 ± 19.8    | 73.8 ± 18.9  | 1.9 ± 18.5    |

LY = lean yogurt (n = 24); LS = lean soy (n = 22). Data are mean ± SD. Within-group analysis by paired t-test. \*  $P < 0.05$  from Visit 2.

**Table 3.2.** Self-reported changes in macronutrient intake of obese women during a two-week washout period (initial) during which probiotic-containing foods were eliminated from the diet and dairy consumption was limited to  $\leq 4$  servings/day and during a dietary intervention (final) consisting of daily consumption of 12 oz. low-fat yogurt or soy pudding.

| <b>Nutrient</b>          | <b>Group</b> | <b>Initial</b> | <b>Final</b> | <b>Change</b>           |
|--------------------------|--------------|----------------|--------------|-------------------------|
| <b>Calories</b>          | OY           | 1802 ± 499     | 1895 ± 386   | 92.9 ± 380              |
|                          | OS           | 1924 ± 385     | 1987 ± 524   | 63.4 ± 571              |
| <b>Fat (g)</b>           | OY           | 72.5 ± 26.3    | 74.1 ± 38.1  | 1.5 ± 41.7              |
|                          | OS           | 76.8 ± 19.0    | 68.8 ± 25.7  | - 8.0 ± 26.9            |
| <b>Saturated Fat (g)</b> | OY           | 25.6 ± 9.3     | 23.0 ± 10.5  | - 2.6 ± 10.7            |
|                          | OS           | 25.9 ± 8.3     | 23.7 ± 12.7  | - 2.2 ± 8.3             |
| <b>Cholesterol (mg)</b>  | OY           | 337 ± 159      | 216 ± 87*    | - 130 ± 171             |
|                          | OS           | 273 ± 119      | 244 ± 148    | - 29 ± 166 <sup>§</sup> |
| <b>Carbohydrates (g)</b> | OY           | 210 ± 72       | 246 ± 54*    | 36 ± 56                 |
|                          | OS           | 235 ± 58       | 264 ± 76     | 29 ± 82                 |
| <b>Protein (g)</b>       | OY           | 81.0 ± 16.7    | 71.9 ± 13.4* | - 9.1 ± 16.3            |
|                          | OS           | 81.9 ± 26.9    | 75.2 ± 21.0  | - 6.7 ± 32.6            |

OY = obese yogurt (n = 17); OS = obese soy (n = 18). Data are mean ± SD. Within-group

analysis by paired t-test. \*  $P < 0.05$  from Visit 2, §  $P < 0.05$  from OY.

**Table 3.3.** Self-reported micronutrient intake of lean women before and during a 9-week dietary intervention providing 12 oz. of low-fat yogurt or soy pudding daily.

| <b>Nutrient</b>     | <b>Group</b> | <b>Initial</b> | <b>Final</b> | <b>Change</b> |
|---------------------|--------------|----------------|--------------|---------------|
| <b>Calcium (mg)</b> | LY           | 696 ± 214      | 1030 ± 230** | 330 ± 341     |

|                        |    |             |               |                       |
|------------------------|----|-------------|---------------|-----------------------|
|                        | LS | 838 ± 265   | 930 ± 207     | 92 ± 288 <sup>§</sup> |
| <b>Vitamin D (mcg)</b> | LY | 4.07 ± 3.13 | 9.91 ± 2.40** | 5.85 ± 2.72           |
|                        | LS | 5.71 ± 3.72 | 11.2 ± 3.31** | 5.47 ± 3.35           |
| <b>Vitamin C (mg)</b>  | LY | 102 ± 46    | 69.8 ± 48.6*  | - 32.0 ± 51.3         |
|                        | LS | 102 ± 57    | 75.0 ± 38.9†  | - 27.3 ± 68.5         |

LY = lean yogurt; LS = lean soy. Data are mean ± SD. Within group analysis by paired t-test. \*

$P < 0.05$  from initial, \*\*  $P < 0.001$  from initial, §  $P < 0.05$  from LY, †  $P < 0.10$  from initial.

**Table 3.4.** Self-reported micronutrient intake of obese women before and during a 9-week dietary intervention providing 12 oz. of low-fat yogurt or soy pudding daily.

| <b>Nutrient</b>     | <b>Group</b> | <b>Initial</b> | <b>Final</b> | <b>Change</b> |
|---------------------|--------------|----------------|--------------|---------------|
| <b>Calcium (mg)</b> | OY           | 860 ± 462      | 1180 ± 253*  | 318 ± 440     |
|                     | OS           | 801 ± 225      | 1010 ± 369*  | 213 ± 311     |

|                        |    |             |               |               |
|------------------------|----|-------------|---------------|---------------|
| <b>Vitamin D (mcg)</b> | OY | 4.43 ± 3.97 | 10.2 ± 1.94** | 5.80 ± 3.66   |
|                        | OS | 5.01 ± 4.27 | 9.79 ± 2.06*  | 4.78 ± 5.15   |
| <b>Vitamin C (mg)</b>  | OY | 85.1 ± 46.5 | 72.95 ± 36.5  | - 12.2 ± 53.4 |
|                        | OS | 93.3 ± 53.0 | 92.4 ± 53.0   | - 0.88 ± 60.3 |

OY = obese yogurt; OS = obese soy. Data are mean ± SD. Within group analysis by paired t-test.

\*  $P < 0.05$  from initial, \*\*  $P < 0.001$  from initial.

**Table 3.5.** Self-reported dairy intake of lean and obese women during a 2-week washout period (initial) during which probiotic-containing foods were eliminated from the diet and dairy consumption was limited to  $\leq 4$  servings/day and during a dietary intervention (final) consisting of daily consumption of 12 oz. low-fat yogurt or soy pudding.

| Group | Initial | Final | Difference |
|-------|---------|-------|------------|
|-------|---------|-------|------------|

|              |                            |             |               |                            |
|--------------|----------------------------|-------------|---------------|----------------------------|
| <b>Lean</b>  | <b>Yogurt<br/>(n = 24)</b> | 1.13 ± 0.66 | 2.15 ± 0.54** | 1.03 ± 0.91                |
|              | <b>Soy<br/>(n = 22)</b>    | 1.35 ± 0.61 | 1.09 ± 0.76   | - 0.26 ± 0.87 <sup>§</sup> |
| <b>Obese</b> | <b>Yogurt<br/>(n = 17)</b> | 1.67 ± 1.40 | 2.57 ± 0.90*  | 0.91 ± 1.33                |
|              | <b>Soy<br/>(n = 18)</b>    | 1.60 ± 0.80 | 1.22 ± 1.01   | - 0.37 ± 0.94 <sup>§</sup> |

Data are mean ± SD. Between group analysis by two-way ANOVA; within group analysis by paired t-test. \*  $P < 0.05$  from Visit 2, \*\*  $P < 0.0001$  from Visit 2, §  $P < 0.05$  from yogurt group.

**Table 3.6.** Self-reported fruit and vegetable intake of lean women before and during a 9-week dietary intervention providing 12 oz. of low-fat yogurt or soy pudding daily.

| <b>Food Group</b>               | <b>Yogurt</b>  |              | <b>Soy</b>     |              |
|---------------------------------|----------------|--------------|----------------|--------------|
|                                 | <b>Initial</b> | <b>Final</b> | <b>Initial</b> | <b>Final</b> |
| <b>Fruit<br/>(servings/day)</b> | 1.96 ± 1.20    | 1.19 ± 1.11* | 1.83 ± 1.15    | 1.65 ± 1.20  |

|                                      |             |              |             |              |
|--------------------------------------|-------------|--------------|-------------|--------------|
| <b>Vegetables<br/>(servings/day)</b> | 3.12 ± 1.38 | 2.47 ± 1.36† | 3.94 ± 1.64 | 2.59 ± 1.27* |
|--------------------------------------|-------------|--------------|-------------|--------------|

Data are mean ± SD. Within group analysis by paired t-test. \*  $P < 0.05$  from initial, †  $P < 0.10$  from initial.

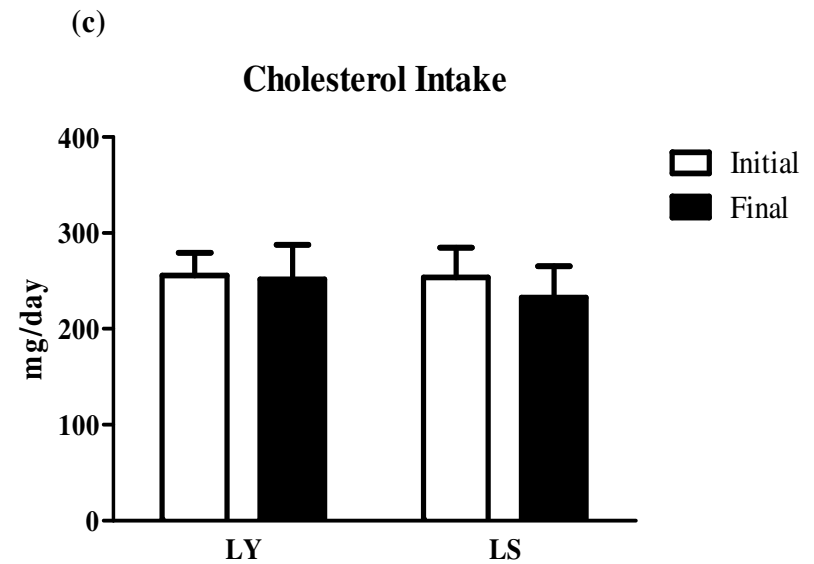
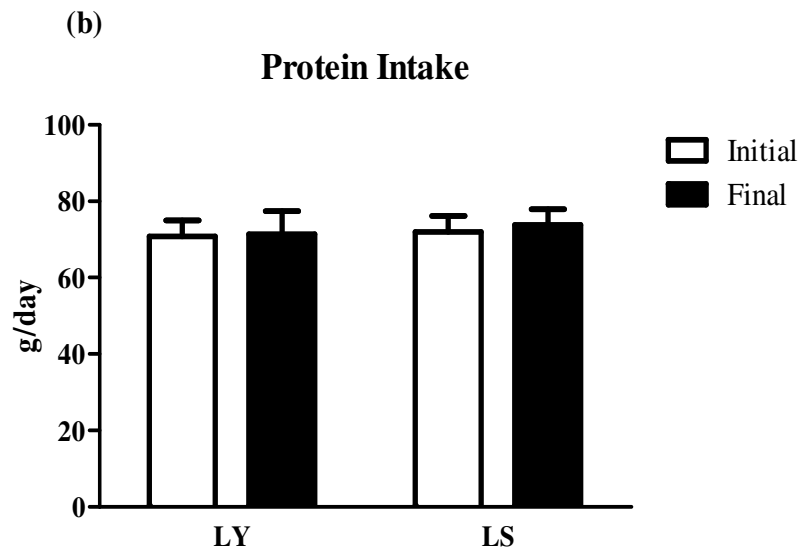
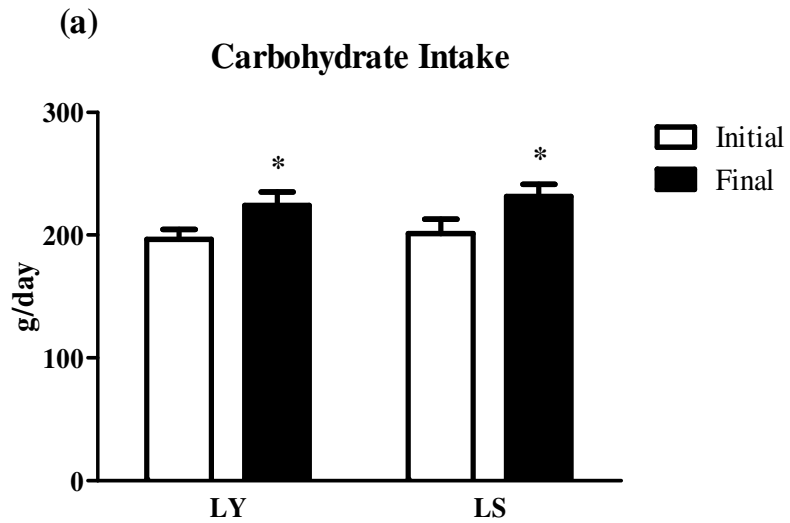
**Table 3.7.** Fruit and vegetable intake of obese women before and during a 9-week dietary intervention providing 12 oz. of low-fat yogurt or soy pudding daily.

| <b>Food Group</b>               | <b>Yogurt</b>  |              | <b>Soy</b>     |              |
|---------------------------------|----------------|--------------|----------------|--------------|
|                                 | <b>Initial</b> | <b>Final</b> | <b>Initial</b> | <b>Final</b> |
| <b>Fruit<br/>(servings/day)</b> | 1.81 ± 1.56    | 1.00 ± 0.95* | 1.51 ± 0.73    | 1.09 ± 0.73† |

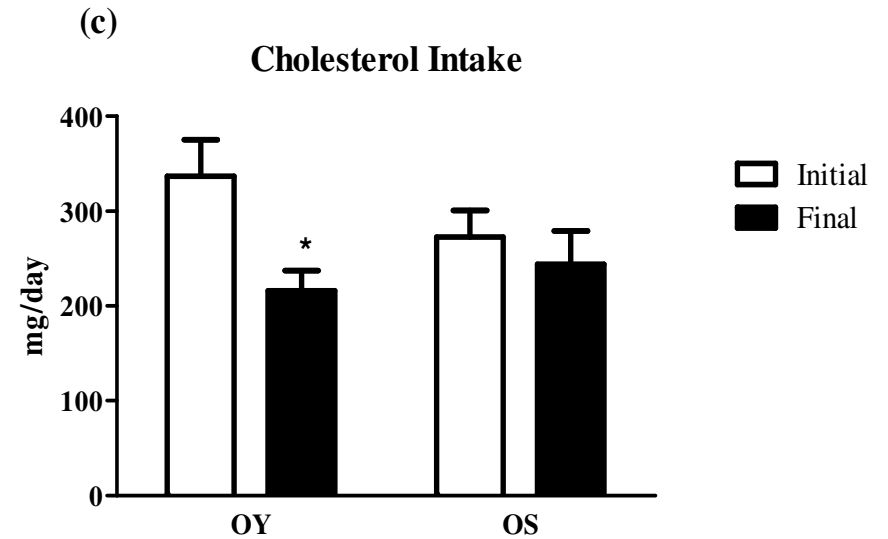
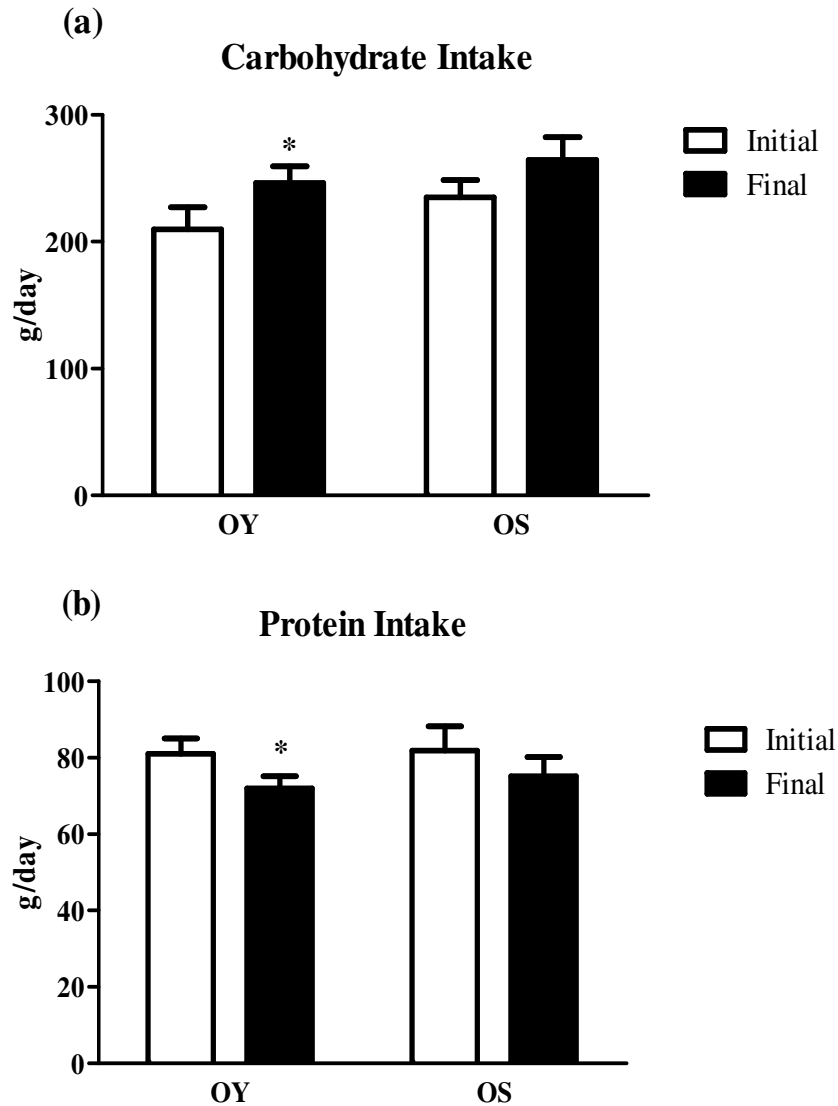


|                                      |             |             |             |             |
|--------------------------------------|-------------|-------------|-------------|-------------|
|                                      |             |             |             |             |
| <b>Vegetables<br/>(servings/day)</b> | 3.44 ± 1.76 | 4.05 ± 1.86 | 3.99 ± 2.91 | 3.40 ± 1.77 |

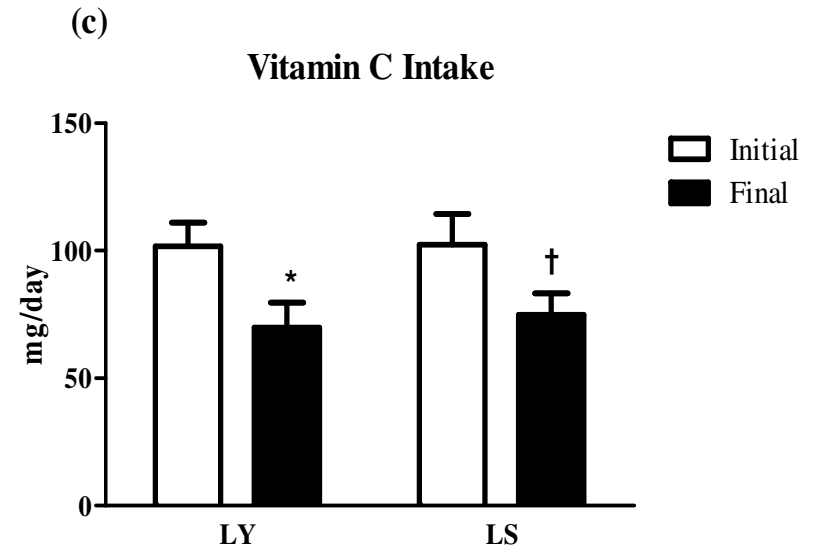
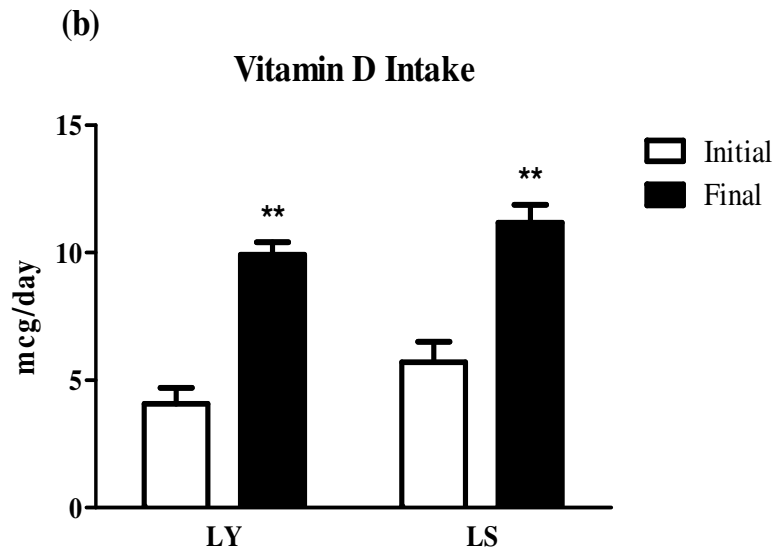
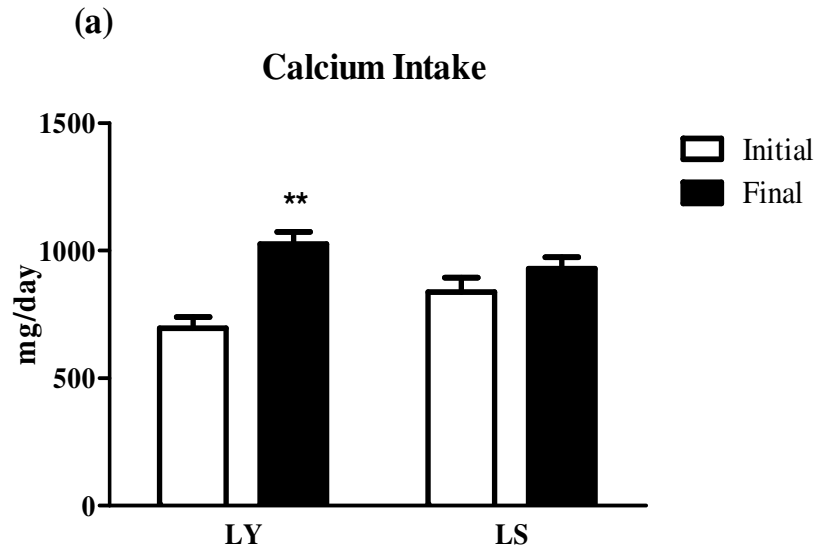
Data are mean ± SD. Within group analysis by paired t-test. \*  $P < 0.05$  from initial, †  $P < 0.10$  from initial.



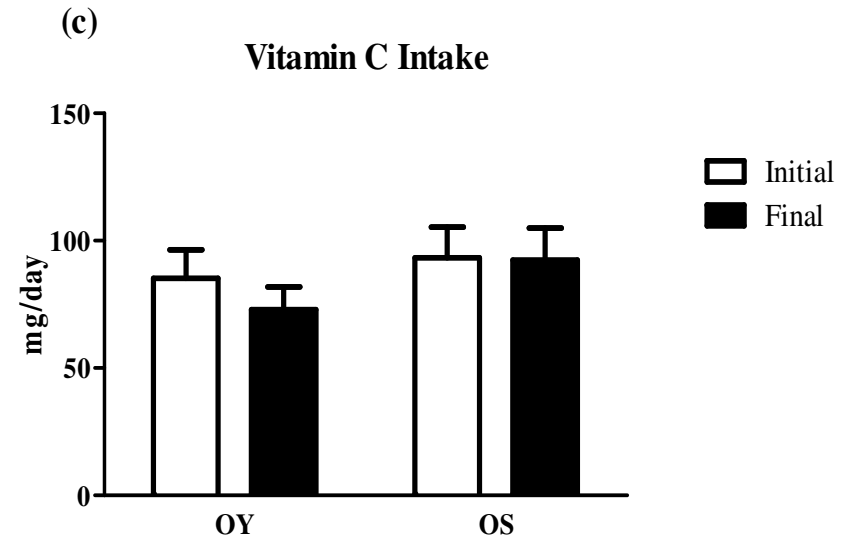
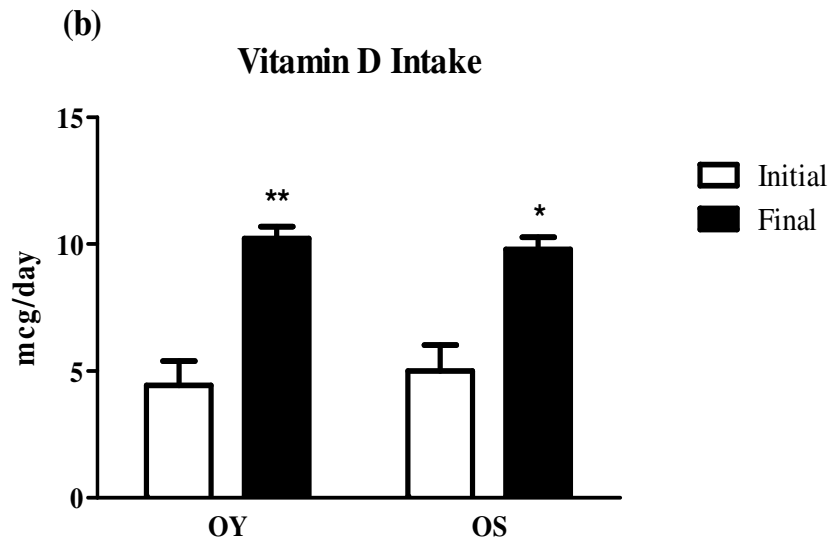
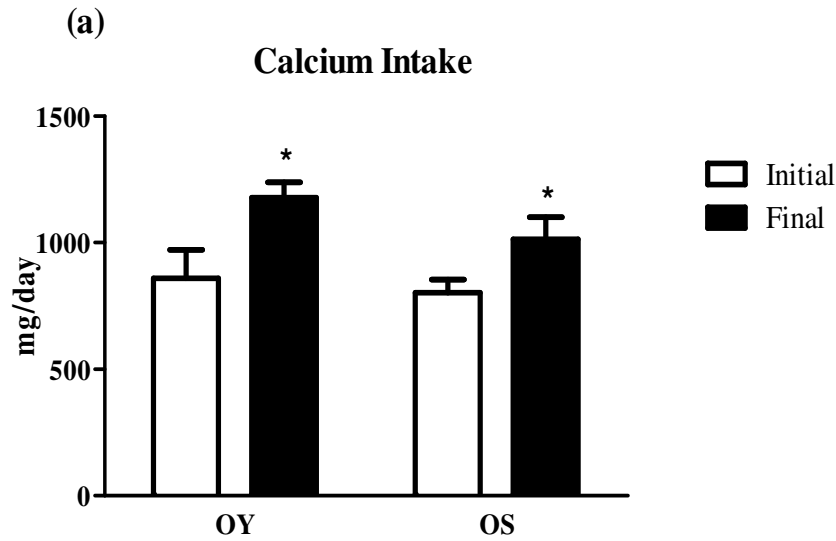
**Figure 3.1.** Changes in macronutrient intake of lean women participating in the intervention: (a) carbohydrate intake (b) protein intake (c) cholesterol intake. Dietary intake data is based on 3-day self-reported dietary records. Data are mean  $\pm$  SEM. LY = lean yogurt,  $n = 24$ ; LS = lean soy,  $n = 22$ . Between group analysis by two-way ANOVA;  $P = 0.0009$  for time effect for carbohydrate intake. Within-group analysis by paired t-test. \*  $P < 0.05$  from Initial.



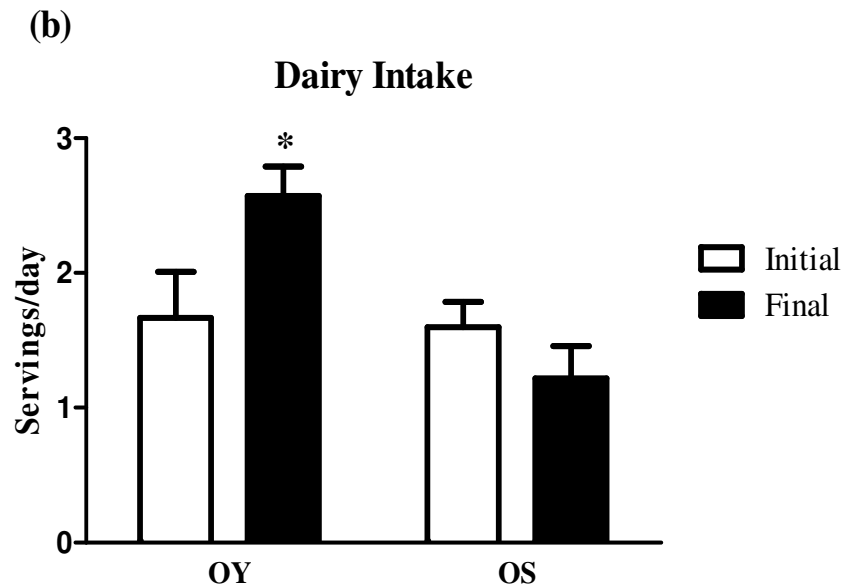
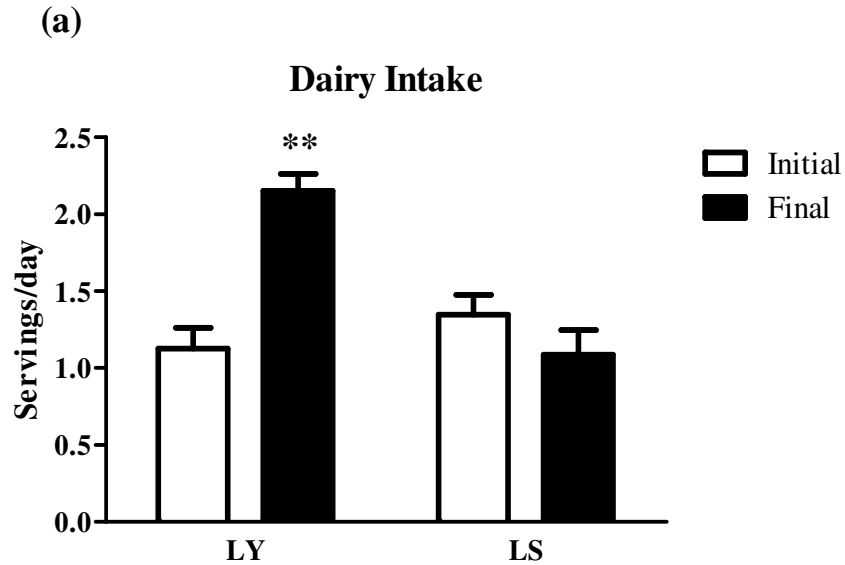
**Figure 3.2.** Changes in macronutrient intake of obese women participating in the intervention: (a) carbohydrate intake (b) protein intake (c) cholesterol intake. Dietary intake data is based on 3-day self-reported dietary records. Data are mean  $\pm$  SEM. OY = obese yogurt,  $n = 17$ ; OS = obese soy,  $n = 18$ . Between group analysis by two-way ANOVA;  $P = 0.0089$  for time effect for carbohydrate intake,  $P = 0.0106$  by paired t-test for cholesterol intake. \*  $P < 0.05$  from Initial by paired t-test.



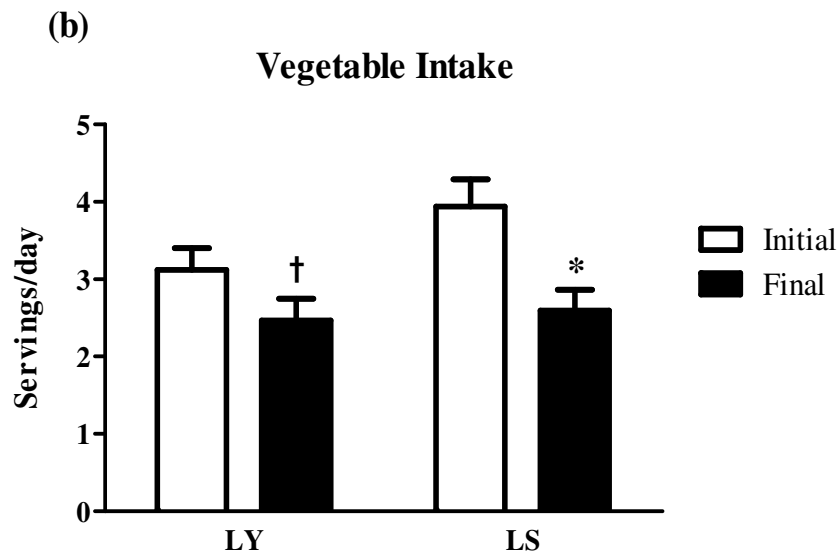
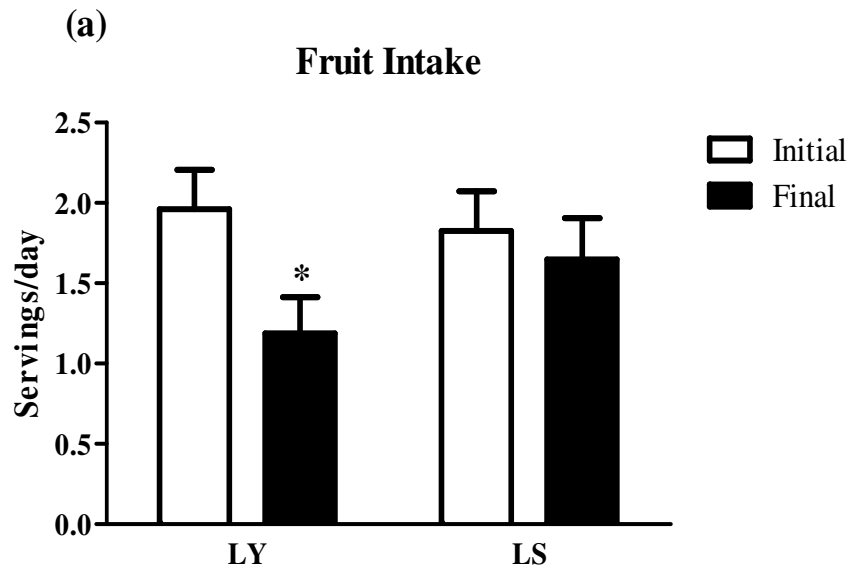
**Figure 3.3.** Changes in micronutrient intake of lean women participating in the intervention: (a) calcium intake; (b) vitamin D intake; (c) vitamin C intake. Dietary intake data is based on 3-day self-reported dietary records. Data are mean  $\pm$  SEM. LY = lean yogurt,  $n = 24$ ; LS = lean soy,  $n = 22$ . Between group analysis by two-way ANOVA;  $P < 0.0001$  for time effect for calcium intake and vitamin D intake,  $P = 0.0017$  for time effect for vitamin C intake. Within-group analysis by paired t-test. \*  $P < 0.05$  from Initial, \*\*  $P < 0.0001$  from Initial, †  $P < 0.10$  from Initial.



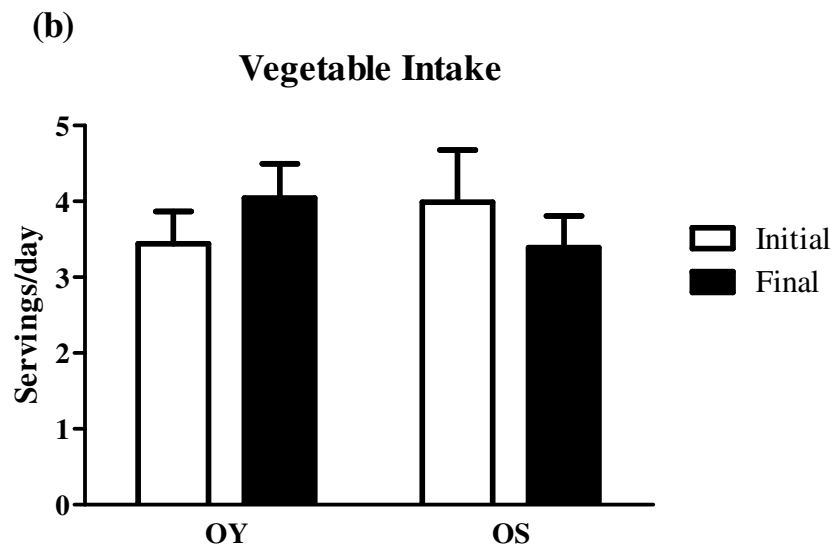
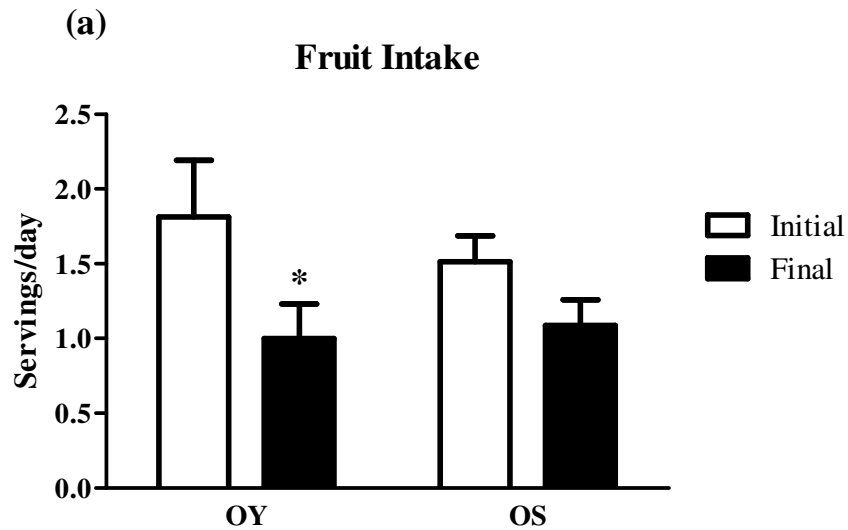
**Figure 3.4.** Changes in micronutrient intake of obese women participating in the intervention: (a) calcium intake; (b) vitamin D intake; (c) vitamin C intake. Dietary intake data is based on 3-day self-reported dietary records. Data are mean  $\pm$  SEM. OY = obese yogurt,  $n = 17$ ; OS = obese soy,  $n = 18$ . Between group analysis by two-way ANOVA;  $P = 0.0002$  for time effect for calcium intake and  $P < 0.0001$  for time effect for vitamin D intake. Within-group analysis by paired t-test; \*  $P < 0.05$  from Initial, \*\*  $P < 0.0001$  from Initial.



**Figure 3.5.** Dairy intake of (a) lean and (b) obese women participating in the intervention. Dietary intake data is based on 3-day self-reported dietary records. LY = lean yogurt,  $n = 24$ ; LS = lean soy,  $n = 22$ ; OY = obese yogurt,  $n = 17$ ; OS = obese soy,  $n = 18$ . Data are mean  $\pm$  SEM. Between group analysis by two-way ANOVA;  $P = 0.0055$  for time effect and  $P = 0.0038$  for treatment effect for lean individuals, and  $P = 0.0226$  for treatment effect for obese individuals. Within-group analysis by paired t-test; \*  $P < 0.05$  from Initial, \*\*  $P < 0.0001$  from Initial.



**Figure 3.6.** Fruit (a) and vegetable (b) intake of lean women participating in the intervention. Dietary intake data is based on 3-day self-reported dietary records. LY = lean yogurt,  $n = 24$ ; LS = lean soy,  $n = 22$ . Data are mean  $\pm$  SEM. Between group analysis by two-way ANOVA;  $P = 0.0217$  for time effect for fruit intake and  $P = 0.0001$  for time effect for vegetable intake. Within-group analysis by paired t-test; \*  $P < 0.05$  from Initial, †  $P < 0.10$  from Initial.



**Figure 3.7.** Fruit (a) and vegetable (b) intake of obese women participating in the intervention. Dietary intake data is based on 3-day self-reported dietary records. OY = obese yogurt,  $n = 17$ ; OS = obese soy,  $n = 18$ . Data are mean  $\pm$  SEM. Between group analysis by two-way ANOVA;  $P = 0.0067$  for time effect for fruit consumption,  $P > 0.05$  for vegetable consumption. Within-group analysis by paired t-test; \*  $P < 0.05$  from Initial.



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**Chapter 4:**  
**Modulation of Postprandial and Chronic Oxidative Stress by**  
**Consumption of Low-Fat Yogurt**

## 4.1 Introduction

Reactive oxygen species (ROS), reactive nitrogen species (RNS), and other oxidants are normal products of nutrient metabolism [1]. In order to quench these highly reactive oxidative species, the body has endogenous antioxidant defense systems, including enzymes such as glutathione peroxidase (GPx), catalase, and superoxide dismutase (SOD) [2, 3]. Under normal physiological conditions, these systems are sufficient to neutralize metabolic oxidative byproducts and maintain equilibrium. However in response to nutrient intake, metabolic processes such as glycolysis, fatty acid oxidation, and the mitochondrial electron transport chain are upregulated; a byproduct of this increase is an increase in the generation of ROS and RNS. This increase in oxidants overwhelms the endogenous antioxidant defense systems. When these oxidants are unable to be neutralized, they are free to interact with glucose, fatty acids, amino acids and nucleic acids, causing cellular and tissue damage [2, 4]. This imbalance between oxidant generation and neutralization is known as oxidative stress, and is associated with the development and progression of many diseases [1].

Oxidative stress can be assessed by many biomarkers, including endogenous antioxidant enzyme activity, the total antioxidant capacity of plasma, and products of oxidative damage, including oxidized sugars, lipids, proteins, and nucleic acids [2, 4]. An individual biomarker cannot provide a complete picture of oxidative stress. Instead, a combination of these markers is more useful to examine oxidative status. Some researchers consider urinary isoprostanes and 8-deoxyguanosine to be the gold standard for measures of oxidative stress due to the high degree of specificity and reliability associated with their presence [4-6]. However, urinary samples were not collected in the present study; therefore, plasma biomarkers were used to measure oxidative stress. These included malondialdehyde (MDA) as a measure of lipid peroxidation, advanced

glycation endproducts (AGEs) as a measure of formation of aldehyde-protein adducts, and total thiols (SH) as a measure of oxidative damage to proteins. The structures and significance of these biomarkers were discussed earlier. Analysis of these biomarkers will provide a partial picture of postprandial and chronic oxidative stress and the ability of yogurt to modulate these biomarkers in obese individuals.

## **4.2 Materials and methods**

### ***4.2.1 Sample collection and preparation***

The total study population consisted of 132 healthy, premenopausal women, age 21-55 y, half of who were lean (BMI 18.5-27 kg/m<sup>2</sup>; n = 66) and half of who were obese (BMI 30-40 kg/m<sup>2</sup>; n = 66). The women were free of cardiovascular disease, high blood pressure, cancer, diabetes, digestive disorders, and any other pro-inflammatory conditions. One half of the women in each group were assigned to low-fat yogurt consumption while the other half consumed a dairy-free, soy-based pudding as the control snack. A detailed discussion of the study design is presented in Chapter 2. Briefly, the women reported to the study center in the morning following a 2-week washout period during which probiotic consumption was avoided and dairy consumption was limited. They arrived fasted and consumed either the yogurt or soy pudding, followed by a high-calorie, high-fat meal. The nutritional profiles of the yogurt, pudding, and meal are elaborated in Chapter 2. The women remained in the study center for 4 h following meal consumption for hourly collection of blood samples. Following this study visit, the women consumed 12 oz. of yogurt or soy pudding daily for 9 weeks. Fasting blood samples were collected at 3 week intervals during this period. On the final day of the intervention, participants again arrived at the

study center in the fasted state, where they once again consumed the yogurt or pudding followed by the high-calorie, high-fat meal. Following collection of 4 h postprandial blood samples, the intervention was complete.

For this objective, fasting and postprandial plasma samples from 31 healthy, obese women – 15 from the obese yogurt (OY) group and 16 from the obese soy (OS) group – were used. Fasting blood samples were collected following completion of the washout period at week 2 (initial), and again at weeks 5, 8, and 11 (final). Postprandial blood samples, collected 1, 2, 3, and 4 h following consumption of the high-calorie, high-fat meal, were collected at weeks 2 and 11. All samples were collected via a catheter or single-stick draw from the antecubital vein. Samples were collected into vacuum tubes with either sodium heparin or EDTA; tubes were gently inverted 8-10 times then placed on ice. All samples were centrifuged at 1500 x g for 10 min within 30 min of collection. Plasma was collected and aliquotted into cryogenic vials, then flash-frozen in liquid nitrogen and stored at -80°C until analysis.

#### ***4.2.2 Determination of plasma malondialdehyde***

Plasma MDA is known to increase in the postprandial period and is an established biomarker for lipid peroxidation [12-15].

Briefly, EDTA plasma samples were prepared with 1% butylated hydroxytoluene to prevent further oxidation and 10 N sodium hydroxide for hydrolysis of bound MDA. Following incubation at 60°C, hydrolysis was halted by addition of 7.2% trichloroacetic acid. Samples were placed on ice, then centrifuged (14,000 x g for 10 min) and the supernatant collected. Thiobarbituric acid (TBA, 0.6%) was added to the supernatant, which reacts with MDA at high

temperatures to form a pink-colored adduct. Lastly, MDA-TBA adducts were extracted with isobutyl alcohol and placed in vials for HPLC analysis.

Analysis was conducted using a Dionex UltiMate 3000 UHPLC and an Acclaim RSLC 2.2  $\mu\text{m}$ , 2.1 x 50 mm column. The autosampler was maintained at 4°C and the column oven at 25°C. Mobile phases were (A) 25 mM potassium phosphate buffer, pH 6.5, and (B) 100% HPLC-grade methanol. The flow rate was 0.2 mL/minute and was isocratic, at 30% B. Total run time was 4 min. The MDA-TBA adducts eluted at approximately 1.3 min and were measured by fluorescence; the excitation and emission wavelengths were 553 nm and 515 nm, respectively.

Standards were generated using 1,1,3,3-tetramethoxypropane (TMP) in sodium acetate buffer (pH 3.5), ranging from 0.0943 to 6.04  $\mu\text{M}$ . Peak area under the curve (AUC) was calculated for each standard to generate a standard curve. AUC was also determined for each sample to calculate plasma MDA concentration. All samples were normalized to a control plasma value that was run daily. Intra-assay variation was 3.3%.

#### ***4.2.3 Determination of plasma total thiols***

Plasma SH were used to establish levels of protein oxidation [9, 10]. Species detected by this assay included glutathione (GSH), oxidized glutathione (GSSG), cysteine (Cys), oxidized cysteine (cystine – CysS), methionine (Met), and homocysteine (Hcy), among others, though the primary thiols detected are in the reduced form [10].

Briefly, sodium heparinized plasma samples were diluted with 0.25 M Tris-buffer EDTA (pH 8.2) and baseline absorbance was measured at 412 nm using a Beckman Coulter DU 800 spectrophotometer. 5,5'-Dithiobis-(2-nitrobenzoic acid) (DTNB) was added, which reacts with



plasma thiols and turns yellow. Samples were incubated for 15 min prior to a second absorbance reading at 412 nm. The difference between initial and final readings, when multiplied by the dilution factor and transformed by molar absorptivity, gave the SH concentration. All samples were normalized to 1 mM glutathione and standardized to a control plasma sample. Intra-assay variation was 4.1%.

#### ***4.2.4 Determination of plasma advanced glycation endproducts***

Plasma AGEs were measured to establish the concentration of covalently-modified proteins. Aldehydes react non-enzymatically with amino acids to generate aldehyde-adducted proteins [11]. Glucose is the predominate sugar that takes part in these reactions; therefore, AGE formation increases in the presence of elevated plasma glucose [11].

Briefly, EDTA plasma samples were diluted 100 times with 10mM phosphate buffered-saline (PBS), pH 7.4. Following centrifugation (10 min at 14,000 x g), the samples were transferred to a black-walled microplate. Fluorescence was measured using a Molecular Devices SpectraMax M2 microplate reader. Excitation and emission were 360 nm and 460 nm, respectively. Fluorescence readings were expressed as arbitrary units (AU). Samples were standardized to a control plasma, run daily. Intra-assay variation was approximately 4.7%.

#### ***4.2.5 Determination of plasma total proteins***

Analysis of total proteins was conducted to examine any correlations between postprandial changes in total thiols and/or AGEs and total protein content of plasma. For this assay, fasting and postprandial samples from 3 participants at week 2 were used. Briefly, EDTA plasma was diluted 100-fold with ultrapure water and combined with a working reagent (Thermo Scientific

Pierce BCA Protein Assay Kit) containing bicinchoninic acid (BCA) and 4% cupric sulfate from. Samples were incubated, cooled to room temperature, and absorbance was measured at 562 nm using a Molecular Devices SpectraMax M2 microplate reader. A standard curve was generated using 25-2000 µg/mL bovine serum albumin. Intra-assay variation was 3.5%.

#### ***4.2.6 Statistical analysis***

Statistical analysis was conducted using GraphPad Prism 5.01 software. Postprandial and fasting between-group analysis was conducted by repeated measures two-way ANOVA, with Bonferonni post-tests conducted where appropriate. Within-group analysis was conducted by paired t-test.

The Shapiro-Wilks normality test was run on all data, and data was transformed when necessary to obtain normality. One individual in the obese soy (OS) group was excluded from postprandial analyses due to a missing plasma sample, and a second individual was excluded from fasting analyses for the same reason. No data was missing from the obese yogurt (OY) group. Therefore, total sample size was  $n = 15$  in each group.

### **4.3 Results**

#### ***4.3.1 Postprandial plasma malondialdehyde***

All data for postprandial and fasting MDA was normally distributed so data transformation was unnecessary. There was a significant time effect ( $P = 0.0129$ ) and treatment effect ( $P = 0.0003$ ) for postprandial MDA (**Figure 4.1 (a)**). Additionally, despite randomization and a successful

matching of subjects ( $P < 0.0001$ ), initial fasting MDA values were significantly higher in the OY group compared with the OS group ( $0.80 \pm 0.29$  mg/dL versus  $1.24 \pm 0.27$  mg/dL;  $P = 0.0002$ ) (**Figure 4.1 (a)**). To account for this initial difference, data was evaluated on the basis of change from fasting baseline (**Figure 4.1 (b)**). By two-way ANOVA on change from fasting data, there was a statistically significant time effect ( $P = 0.0157$ ) and treatment effect ( $P = 0.0395$ ) for the postprandial change in MDA. At week 2, there was a significant time effect for MDA (change from fasting) between the OY and OS groups ( $P = 0.0186$ ). By unpaired t-test, there was also a weak trend towards a significant difference in MDA concentration (change from fasting) between the OY and OS groups postprandially at hour 2 ( $p = 0.0902$  by unpaired t-test) and a significant difference at hour 3 ( $P = 0.0352$  by unpaired t-test) at week 2 (**Figure 4.1 (b)**).

Within the OY group, there were no significant differences in postprandial MDA (change from fasting) at any time point at either week 2 or week 11, and there was also no statistically significant reduction in postprandial elevations in MDA following 9-week yogurt consumption (**Figure 4.2 (a)**). However, and unexpectedly, in the OS group there was a significant time effect ( $P = 0.0359$ ) and treatment effect ( $P = 0.0248$ ) from the initial (week 2) to final (week 11) postprandial challenges (**Figure 4.2 (b)**). When compared with values from week 2, chronic consumption of soy pudding reduced postprandial MDA change from baseline at week 11 at hour 2 ( $P = 0.0305$ ), hour 3 ( $P = 0.0029$ ), and hour 4 ( $P = 0.0086$ ).

Postprandial MDA AUC was also examined at weeks 2 and 11. Because of the statistically significant difference in baseline fasting values, AUC was calculated using the change from fasting MDA values (**Figure 4.3**). However, there were no significant time or treatment effects. There were also no significant differences in AUC between week 2 and week 11 or between the OY and OS groups at either time point by post-hoc analysis.

Because MDA can form adducts with plasma proteins, total protein content of the plasma was also measured during the postprandial period. Though differences did not reach significance, total proteins decreased slightly in the first 3 h postprandially before rising slightly at hour 4 (**Figure 4.4**). It is possible that the 4 h postprandial period was not sufficient to detect changes in plasma proteins associated with meal intake. If this were the case, it would also be interesting to examine the MDA response beyond a 4 h postprandial period.

#### ***4.3.2 Fasting plasma malondialdehyde***

There was a significant time effect ( $P = 0.0179$ ), treatment effect ( $P = 0.0346$ ), and a time-treatment interaction ( $P = 0.0470$ ) for absolute fasting MDA values (**Figure 4.5**). Because the same baseline plasma samples were used for determination of fasting and postprandial MDA, fasting OY was approximately 56% greater than OS at week 2 ( $P = 0.0002$ ). OY fasting MDA was also approximately 33% greater than OS at week 11 ( $P = 0.0127$ ). In contrast, low-fat yogurt consumption did not change fasting MDA concentrations. Interestingly, sustained consumption of soy pudding appeared to raise fasting plasma MDA; MDA concentration increased by 55% between weeks 2 and 5 ( $P = 0.0101$ ), 65% between weeks 2 and 8 ( $P = 0.0072$ ), and 27% between weeks 2 and 11 ( $P = 0.0203$ ) in the OS group (**Figure 4.5**).

Due to the significant differences in week 2 fasting values between the OY and OS groups, fasting MDA values were analyzed as the change from baseline (week 2) fasting values (**Figure 4.6**). By two-way ANOVA, there were again significant time ( $P = 0.0179$ ) and treatment effects ( $P = 0.0163$ ), and a significant time-treatment interaction ( $P = 0.0470$ ) for fasting MDA.

Additionally, fasting MDA concentrations (change from week 2) were significantly lower in the

OY group versus the OS group at week 5 ( $P = 0.0252$ ) and week 8 ( $P = 0.0245$ ). There were no changes in MDA concentration within the OY group throughout the duration of the intervention.

#### ***4.3.3 Postprandial plasma total thiols***

The raw data for fasting, postprandial, and AUC SH values were normally distributed, so data were not transformed prior to statistical analysis. The AUC of change-from-fasting SH values were not normally distributed, and attempts to normalize the data failed. Therefore, common statistical approaches may not be appropriate for this data set.

There was a significant time effect for postprandial SH concentration ( $P = 0.0028$ ) (**Figure 4.7**).

Within the OY group, there was a trend towards an increase in SH concentration (change from fasting) between hour 0 and hour 4 at week 11 ( $P = 0.0682$ ) (data not shown). Within the OS group at week 2, co-consumption of soy pudding with the high-calorie, high-fat meal led to a significant decrease in plasma SH between hour 0 and 1 ( $P = 0.0008$ ) and hour 0 and 2 ( $P = 0.0120$ ) (**Figure 4.8(a)**). At week 2, postprandial change-from-fasting plasma SH was significantly higher in the OY group than the OS group at hour 1 ( $P = 0.0072$ ) and hour 4 ( $P = 0.0418$ ) (**Figure 4.8(b)**). However, no significant differences in hourly plasma SH were observed at week 11.

There were no differences in postprandial SH AUC within the OY and OS groups or between each group at weeks 2 and 11 (**Figure 4.9**). However, there was a trend towards a larger postprandial AUC in the OY group versus the OS group at week 2 ( $P = 0.0586$  by unpaired t-test). Chronic consumption of low-fat yogurt or soy pudding did not appear to have any significant impact on the postprandial SH response.

#### ***4.3.4 Fasting plasma total thiols***

The data for fasting SH were not normally distributed; the data was transformed by the equation ( $y = y^3$ ) to obtain normality prior to statistical analysis. Following analysis by two-way ANOVA on the transformed data, there was a significant time-treatment interaction for fasting SH ( $P = 0.0167$ ) but there were no significant time or treatment effects (**Figure 4.10**). There were no differences in fasting SH within the OY group at any time point. However, there was a trend towards elevated fasting SH in the OY group at week 5 when compared with the OS group ( $P = 0.0510$ ). Within the OS group, fasting plasma thiols were significantly reduced between weeks 2 and 5 ( $P = 0.0012$ ). However, this difference did not persist, and fasting SH increased slightly (though insignificantly) from week 5 to week 8 and remained fairly stable at week 11. These latter changes did not reach significance.

#### ***4.3.5 Postprandial plasma advanced glycation endproducts***

The raw values for postprandial plasma AGEs and postprandial AGEs AUC as measured by change from fasting were normally distributed. However, unadjusted postprandial AGEs AUC and plasma fasting AGEs values were not, and attempts to normalize the data failed. Therefore, common statistical approaches may not be appropriate. Visual inspection of these data did not lead us to suspect any otherwise significant trends (**Figure 4.11**). Instead, postprandial analysis focused on the raw postprandial and change-from-fasting postprandial AUC values.

There were also no statistically significant differences in AGE concentration at any time point within each group or between the OY and OS groups (**Figure 4.11**). Postprandial change from fasting AGEs had a weak trend towards a significant time-treatment interaction ( $P = 0.0907$ ) and also towards a significant treatment effect ( $p = 0.0976$ ) (data not shown). There were no

statistically significant differences in postprandial AUC (reported as change from fasting) between weeks 2 and 11 or between the OY and OS groups at each visit (**Figure 4.12**). Finally, there were no statistically significant differences in AGE concentration at any time point within each group or between the OY and OS groups (data not shown).

#### ***4.3.6 Fasting plasma advanced glycation endproducts***

There were no statistically significant differences in fasting AGEs between groups or within groups at any time point (**Figure 4.13**).

### **4.4 Discussion**

#### ***4.4.1 Plasma malondialdehyde***

Baseline (week 2) fasting MDA concentrations were significantly higher in the OY group than the OS group, despite successful randomization. We observed large variation in MDA concentrations, which is supported by the literature; reported fasting MDA values range from < 1 mg/dL to almost 4 mg/dL in individuals with prehypertension [16-18]. Due to this variation, it is possible that this difference will no longer be statistically significant once final sample size is obtained. It is also possible that individuals in the OY group do indeed have higher baseline levels of oxidative stress. This could be due to diet, differences in health status, or perhaps differences in food intake in the day prior to arrival at the study center. However, as discussed in Chapter 2, there were no differences in any anthropometric parameters between the OY and OS groups, including BMI. There were also no statistically significant differences in baseline intake of any macronutrients, calcium, vitamins C and D, fruits, vegetables, or dairy products. Dietary

intake is discussed in detail in Chapter 3; however, a much more thorough dietary analysis would be required in order to draw any associations to oxidative stress.

Many studies on postprandial oxidative stress have been reported; however, most examine only changes associated with the 4-6 h postprandial period [15, 19, 20] Miglio, et al. conducted a trial in which 8 h postprandial blood samples were collected [21]. At this time point, certain parameters were just reaching peak plasma concentrations, including total cholesterol, TNF $\alpha$ , and IL-6. In addition, plasma triglycerides, and SH and uric acid, two measures of oxidative stress, remained significantly elevated above baseline [21]. Therefore, it is highly plausible that the oxidative effects of a high-fat meal may extend beyond 8 h. Participants in the present study reported to the study center following a 12 h fast. However, aside from limiting dairy intake and avoiding probiotics, there were no specific directives regarding what to consume in the day prior to the visit. For most participants, Visit 2 took place on a Saturday morning; as Friday night is often a night for socializing and other activities, consumption of alcohol or of a high-calorie, high-fat meal may have occurred within 12-14 h of arrival at the study center. Therefore, the remaining oxidative impacts of such a meal may have been detected in some of our baseline blood samples. Clearly more evidence is needed on the duration of postprandial changes in oxidative stress.

Due to the difference in week 2 fasting MDA, all MDA data were evaluated as change from baseline. By this analysis, low-fat yogurt consumption appeared to inhibit postprandial increases in MDA. At week 2 in the OY group, MDA slightly increased beginning 1 h postprandially and remained elevated above baseline values for 4 h. However, none of these differences were statistically significant. At week 11, postprandial MDA showed little change over fasting concentrations and actually decreased to below baseline concentrations at the 4 h time point in



the OY group. However, this decrease was not significantly different from the fasting values. In addition, postprandial 4 h MDA AUC did not change from week 2 to week 11 in the OY group.

As mentioned above, fasting MDA was significantly higher in the OY group than the OS group at week 2. When compared to the OS group, OY fasting MDA concentrations were also higher at week 11. When fasting MDA values were evaluated as change from baseline, MDA was found to be significantly higher in the OS group than the OY group at weeks 5 and 8; though MDA was also higher in the OS group at week 11, this difference did not reach significance. Indeed, this is reflective of the observed increase in fasting MDA in the OS group, while fasting MDA remained unchanged in the OY group. Therefore, short-term soy pudding consumption may increase fasting levels of lipid peroxidation in obese women, though the effect may not be sustained. This is in agreement with the results of a previous intervention, in which 4-week soy consumption slightly increased biomarkers of inflammation in overweight or obese subjects [22].

Interestingly, MDA was significantly higher at hours 2, 3, and 4 postprandially in the OS group at week 2 compared with week 11 despite the fact that fasting MDA at week 11 was significantly higher than that at week 2. In fact, increases in postprandial MDA at week 11 in the OS group were negligible. This suggests that sustained intake of soy pudding may reduce the postprandial MDA response. However, the elevation in fasting MDA observed in the OS group indicates that overall MDA exposure may actually be increased by daily soy pudding consumption.

Fasting MDA in the OY group did not change from week 2 (initial) to week 11 (final). Therefore, it appears that while sustained yogurt consumption did not reduce fasting MDA concentrations in obese women, it also did not increase their exposure to oxidative stress.

Previous studies show that co-consumption of calcium, milk proteins and/or whole dairy products with a high-fat meal attenuates the postprandial response [19, 23-25]. In addition, calcium is known to interfere with fat absorption [26]; a reduction in postprandial fat absorption would tend to lead to a reduction in postprandial elevations in lipids and, therefore, lipid peroxidation. Data from the present study supports an acute effect of yogurt in the inhibition of postprandial MDA.

#### ***4.4.2 Plasma total thiols***

Low-fat yogurt did not appear to impact postprandial or fasting SH. Interestingly, co-consumption of soy pudding with the high-calorie, high-fat meal depleted SH at 1 and 2 h postprandially at week 2 and SH was also significantly lower in the OS group at hour 4 as well. Therefore, soy pudding may have a short-term postprandial impact on plasma SH but this appears to be temporary.

Interpreting plasma SH concentrations is difficult, as there are numerous variables involved for which we did not account in the present study. The total thiols assay used for this analysis does not discriminate between protein and non-protein thiols or between each individual thiol. Miglio, et al. measured plasma SH following intake of a high-fat meal and found that though peak SH concentration was reached at 4 h postprandially, concentrations remained elevated above baseline for the entirety of the 8 h postprandial period [21]. It is possible that co-consumption of the yogurt or soy pudding in the present study delayed the peak plasma protein and SH elevations that are expected in response to excess energy intake. It is also possible that both the yogurt and soy pudding were successful in reducing postprandial elevations in SH. A second study using a non-dairy, non-soy control would be necessary to examine this hypothesis.

There are also discrepancies in the literature regarding the meaning of changes in plasma SH. There are natural cyclic variations in plasma thiols throughout the day, though these cycles are not uniform for each thiol. Cysteine (Cys) tends to increase throughout the day, with lowest concentrations typically occurring very early in the morning [27]. Oxidized cysteine (cystine – CysS) fluctuates on a 12 h cycle rather than a 24 h cycle, with maximal values at approximately noon and midnight [27]. Glutathione (GSH) is on a cycle similar to Cys but delayed by about 6 h, while oxidized glutathione (GSSG) does not appear to fluctuate in such a fashion [27]. The highest concentration of GSH is measured just after waking; this may be suggestive of a restoration of the antioxidant capacity of plasma during the overnight fast. It also indicates that the oxidative burden inflicted by a high-calorie, high-fat meal may be better handled by the body when the meal is consumed in the morning. This would be an interesting topic to explore in future research.

It has also been suggested that, following depletion of plasma SH, more thiols (in particular GSH) are released into the bloodstream to restore antioxidant balance. In particular, Cys is preferentially oxidized in the plasma in the postprandial state [21]. This reduction in Cys is counteracted by an increase in GSH release [21]. In fact, an increase in oxidative stress upregulates genes associated with GSH synthesis [28]. Therefore, an increase in plasma SH may represent increased antioxidant capacity of the plasma in response to increased oxidative stress.

Because of the natural daily variation in SH, as well as the ability of the body to maintain tight control over plasma thiol concentrations, changes in fasting thiols were expected to be minimal. This was the case, as the only significant difference was a reduction in fasting SH at week 5 in the OS group. However, this reduction was short-lived. It is possible that daily intake of the soy pudding increased oxidative stress in this group and that, following more sustained consumption,

the body adapted by increasing plasma SH. Fasting SH also slightly, but insignificantly, increased in the OY group. This may be representative of an increased antioxidant capacity as a result of the yogurt. However, it also may be indicative of the body compensating for yogurt-induced oxidative stress by increasing plasma SH. More research is needed to clarify the mechanisms behind these changes. It would also be prudent to examine changes in individual thiol species in relation to yogurt and soy pudding consumption.

#### ***4.4.3 Plasma advanced glycation endproducts***

AGEs are formed by the non-enzymatic reaction of aldehydes with exposed amino groups on lipids (including plasma lipoproteins), proteins (such as collagen), and nucleic acids [29-32]. The result is the formation of covalently-modified adducts. Endogenously, glucose is the most common aldehyde involved in advanced glycation endproduct (AGE) formation [29]. Therefore, increased concentrations of AGEs are associated with elevations in plasma glucose in cases of impaired glucose metabolism or in the postprandial period [11]. AGEs also increase in the postprandial period in response to AGEs provided by the diet, particularly in foods that are fried, grilled, or broiled [31]. Therefore, the high-calorie, high-fat meal in the present study was hypothesized to increase plasma AGEs in the postprandial period.

Interestingly, there were no significant changes in postprandial AGE concentration following co-consumption of either the yogurt or soy pudding with the high-calorie, high-fat meal. There were also no changes in fasting AGEs as a result of the intervention. It is possible that a 4 h postprandial examination was not sufficient to detect changes in plasma AGEs. It is also possible that the components of the meal exerted opposing effects on AGEs. For example, milk consumption is known to minimize postprandial elevations in glucose, which would tend to

reduce postprandial AGE formation [15, 19]. However, the meal used in the present study likely contained a high concentration of AGEs. In addition, the low-fat yogurt and soy pudding contributed large amounts of carbohydrates, which may have exacerbated the postprandial glucose response, blunting any benefits of the other components of the yogurt that may have helped to reduce postprandial hyperglycemia.

Though carbohydrate intake increased in the OY group, as discussed in Chapter 3, there was no increase in fasting AGE concentration. This suggests that perhaps the daily low-fat yogurt consumption was able to minimize the increase in AGE formation that would be expected with increased carbohydrate intake. It would be interesting to examine the long-term effects of consumption of low-fat yogurt versus a non-dairy high-carbohydrate snack to determine whether yogurt provides a long-term benefit to plasma AGE concentration. It would also be interesting to conduct an intervention similar to the present study but using a lower carbohydrate yogurt.

Despite the high sugar content of low-fat yogurt in the present study, consumption did not have any negative impacts on AGEs or oxidative stress in obese women. This is important, as many individuals do not consume the requisite amounts of calcium, vitamin D, or dairy products [33, 34]. In particular, there is an association between BMI and dairy intake, such that individuals with higher BMIs tend to consume less dairy and calcium [35]. However, research supports an association between dairy intake and numerous beneficial health outcomes, including weight maintenance, additional weight loss when paired with a calorie-restricted diet, and reduced incidence of high blood pressure, type-2 diabetes, and other metabolic disturbances [35-40]. Therefore, low-fat yogurt would be a beneficial addition to the diet. Participants in the present study showed no increases in BMI or waist circumference with daily low-fat yogurt intake. More importantly, while low-fat yogurt did not lower fasting or postprandial oxidative stress, it also

did not have a negative impact on these parameters. Therefore, low-fat yogurt could be added to the diet to increase calcium and dairy intake without negatively impacting BMI or oxidative stress in obese women.

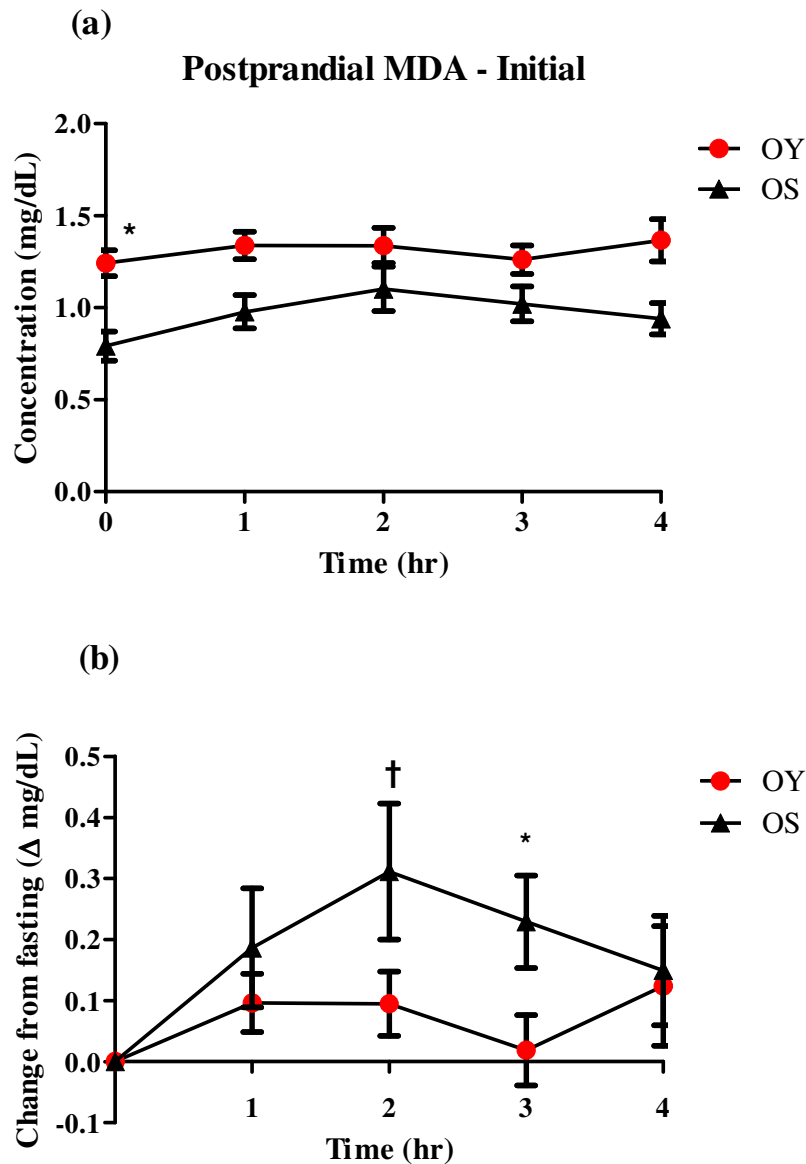
#### ***4.4.4 Strengths and limitations***

It is important to note that the examination of oxidative stress conducted in the present study is not representative of overall oxidative status. There are numerous biomarkers of oxidative stress, including urinary isoprostanes, 8-deoxyguanosine (a measure of DNA oxidative damage), uric acid, oxidized LDL, and the endogenous antioxidant enzymes, including catalase, GPx, and SOD [2, 21, 41, 42]. Taken together, all of these biomarkers would be more representative of overall oxidative stress than the three examined in the present study. Therefore, though soy pudding appeared to have moderate beneficial impacts on postprandial lipid peroxidation and low-fat yogurt neither favorably nor unfavorably altered postprandial and fasting oxidative stress, it is important to consider that the outcomes of the present study do not provide a comprehensive picture of oxidative stress. Nevertheless, these outcomes do provide a basis for future study.

A strength of the present study is the use of a commercially-available low-fat yogurt that is representative of most marketed low-fat yogurts. Because chronic consumption of this low-fat yogurt did not unfavorably alter oxidative stress levels, it could serve as an addition to the diet in order to increase calcium, Vitamin D, and dairy intake. However, the high carbohydrate content of this yogurt may have masked the potential benefits of regular consumption. Previous studies link dairy intake with many beneficial health outcomes, including reductions in oxidative stress [43, 44]. This was partly confirmed by the present study, as the intervention appeared to inhibit fasting and postprandial increases in MDA and AGEs and decreases in SH.

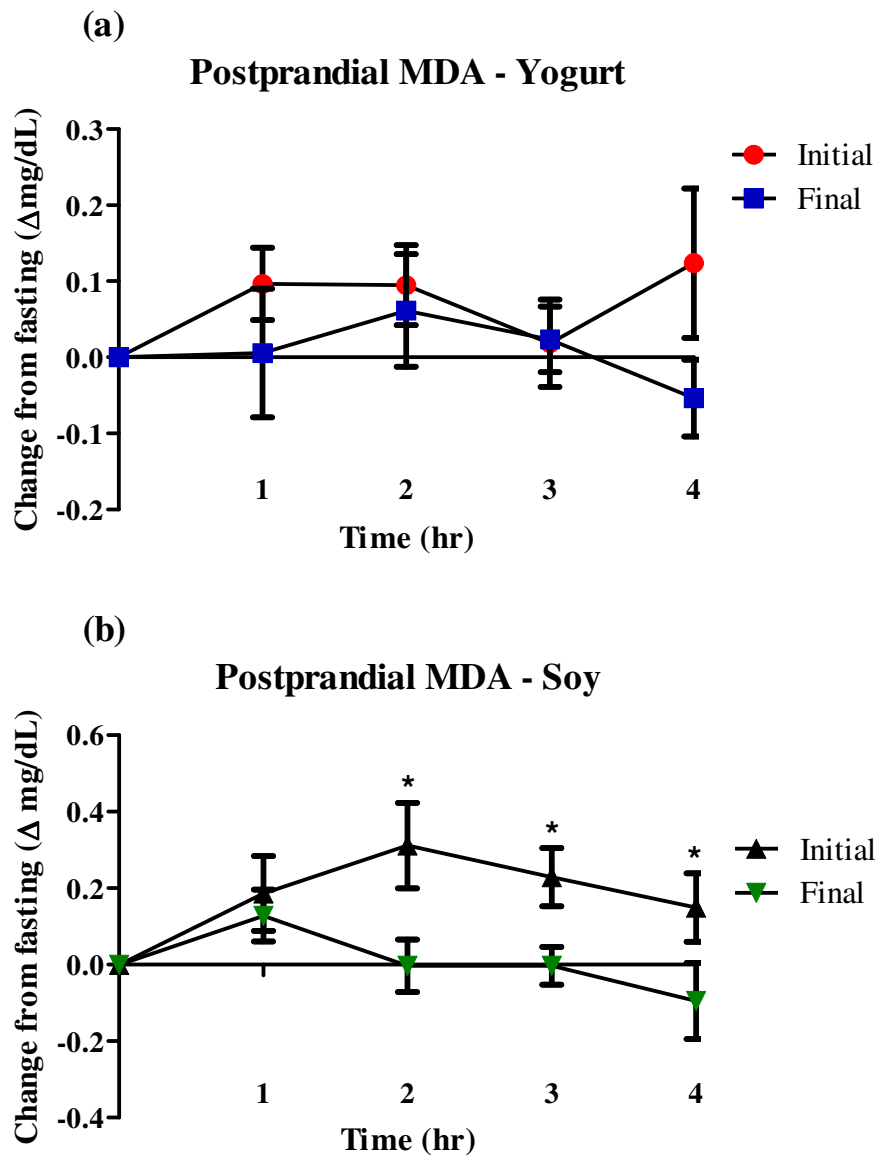
Probiotics are also known to impact intestinal health, and may have indirect antioxidant activity, by increasing synthesis of antioxidant enzymes [45-47]. The intestinal microbiota profile may also be altered in association with obesity and other metabolic disturbances; this is reviewed in detail elsewhere [48]. Though probiotic intake was not examined in the present study, it seems plausible that they may affect biomarkers relevant to this study; this should be examined further in relation to yogurt consumption. However, the yogurt used in the present study was fairly low in probiotics in comparison to other commercial yogurts. Nestel, et al. found 3-week intake of a fermented dairy diet to be associated with decreases in certain inflammatory biomarkers [36]. Therefore, consumption of a higher-probiotic yogurt may provide additional health benefits.

A limitation of the present study is that we did not account for where the women were in their menstrual cycles. Due to the antioxidant capabilities of estrogen, baseline oxidative stress levels fluctuate throughout the cycle [49]. Indeed, TBA-reactive substances were found to be highest during the luteal phase of the cycle. Future studies should ensure that women begin and end the intervention in the same phase of their cycles to eliminate the potential confounding effects of these natural fluctuations.

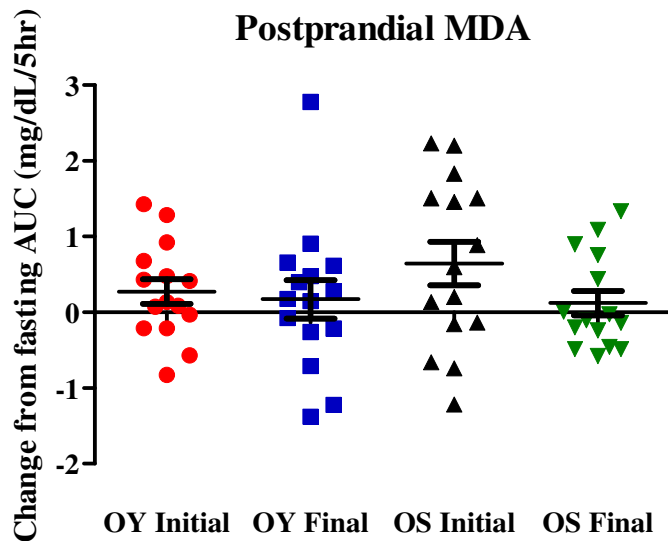


**Figure 4.1.** Initial postprandial changes in plasma MDA in obese women following co-consumption of low-fat yogurt or soy pudding with a high-calorie, high-fat meal. OY = obese yogurt (n = 15); OS = obese soy (n = 15). (a) Mean group MDA values in OY and OS groups. Data are mean  $\pm$  SEM. Between-group analysis by two-way repeated measures ANOVA;  $P = 0.0129$  for time effect,  $P = 0.0003$  for treatment effect, and  $P = 0.1331$  for time-treatment interaction. \* $P < 0.05$  from OS by unpaired t-test. (b) Mean individual MDA changes from fasting values in OY and OS groups. Data are mean  $\pm$  SEM. Between-group analysis by two-way repeated measures ANOVA;  $P = 0.0186$  for time effect,  $P > 0.05$  for treatment effect and time-treatment interaction. Within-group analysis by paired t-test; \*  $P = 0.05$  from OS, †  $P < 0.10$  from OS by unpaired t-test.

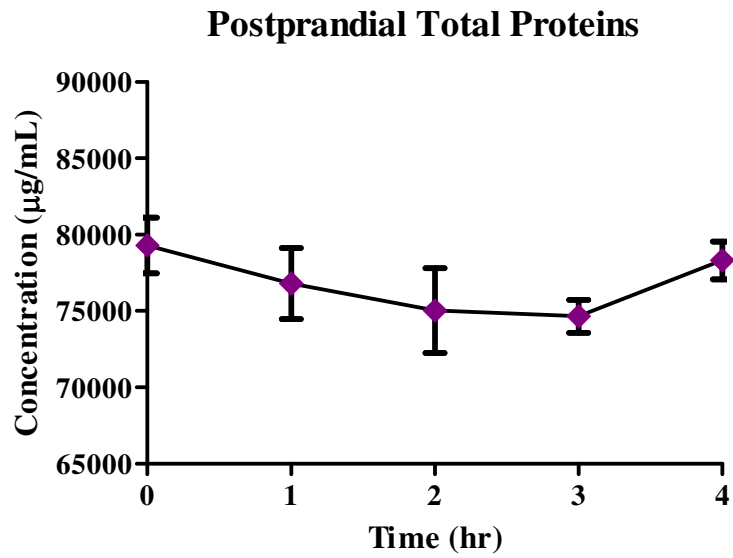




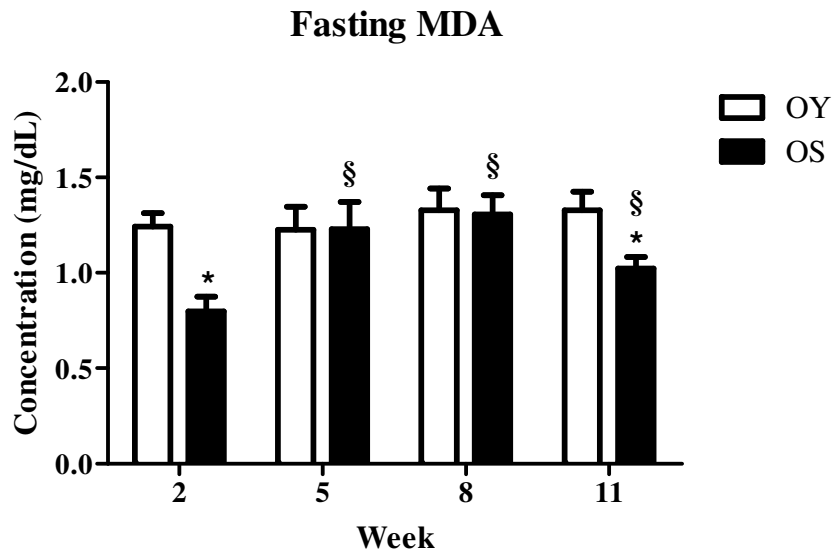
**Figure 4.2.** Postprandial changes in plasma MDA in obese women following co-consumption of yogurt or soy pudding and a high-calorie, high-fat meal prior to (initial) and following (final) 9-week daily 12 oz. low-fat yogurt or soy pudding consumption. OY = obese yogurt (n = 15); OS = obese soy (n = 15). (a) Initial (week 2) and final (week 11) postprandial changes in MDA in the OY group. Data are mean  $\pm$  SEM.  $P > 0.05$  by two-way repeated-measures ANOVA. (b) Initial (week 2) and final (week 11) postprandial changes in MDA in the OS group. Data are mean  $\pm$  SEM. Analysis by two-way repeated-measures ANOVA;  $P = 0.0359$  for time effect,  $P = 0.0248$  for treatment effect, and  $P = 0.0677$  for their interaction. Within-group analysis by paired t-test. \*  $P < 0.05$  from fasting.



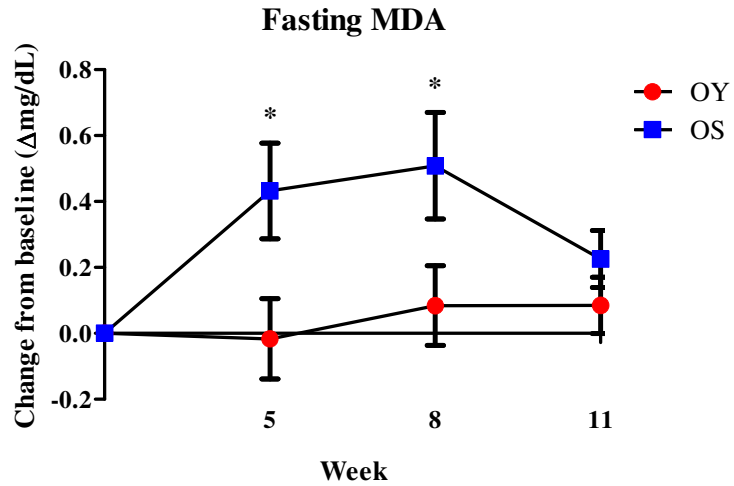
**Figure 4.3.** Postprandial plasma MDA change from fasting area under the curve (AUC) in obese women following co-consumption of low-fat yogurt or soy pudding and a high-calorie, high-fat meal prior to (initial) and following (final) 9-week daily intake of 12 oz. of low-fat yogurt or soy pudding. OY = obese yogurt (n = 15); OS = obese soy (n = 15). Data are mean  $\pm$  SEM. Between-group analysis by two-way repeated measures ANOVA;  $P > 0.05$  for time effect, treatment effect, and their interaction.



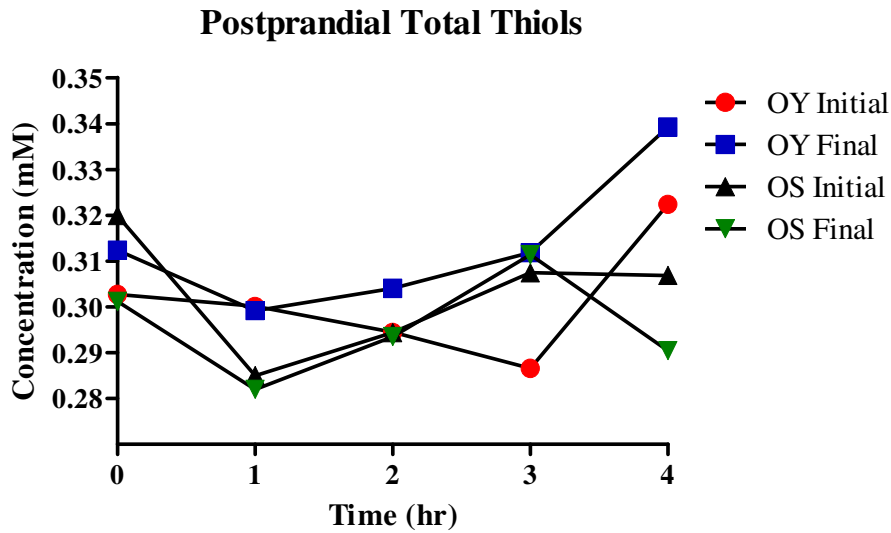
**Figure 4.4.** Initial postprandial total plasma proteins in obese women following co-consumption of low-fat yogurt and a high-calorie, high-fat meal.  $n = 3$ . Data are mean  $\pm$  SEM. By one-way ANOVA,  $P > 0.05$ .



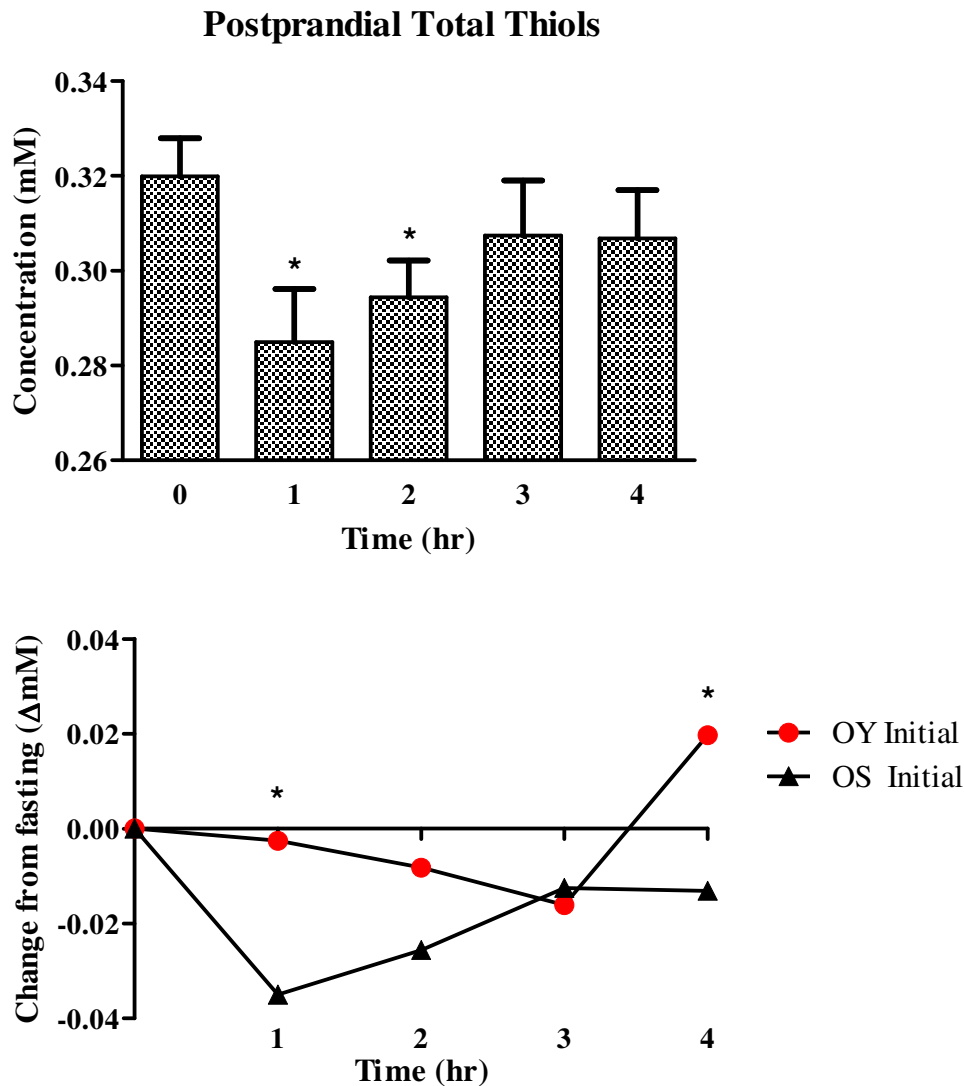
**Figure 4.5.** Fasting plasma MDA concentrations in obese women prior to (week 2), during (weeks 5 and 8), and after (week 11) 9-week daily consumption of low-fat yogurt or soy pudding. OY = obese yogurt (n = 15); OS = obese soy (n = 15). Data are mean  $\pm$  SEM. By two-way, repeated measures ANOVA,  $P = 0.0179$  for time effect,  $P = 0.0346$  for treatment effect, and  $P = 0.0470$  for their interaction. \*  $P < 0.05$  from OY by unpaired t-test and §  $P < 0.05$  from baseline by paired t-test.



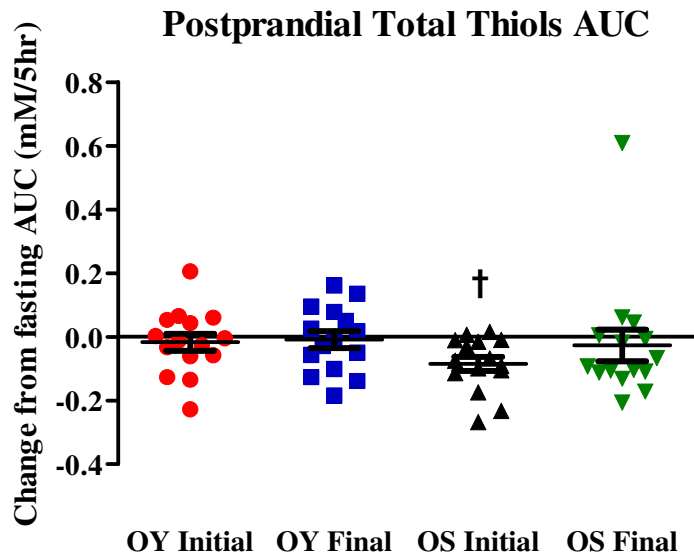
**Figure 4.6.** Change in fasting plasma MDA from week 2 in obese women during (weeks 5 and 8), and after (week 11) 9-week daily consumption of 12 oz. of low-fat yogurt or soy pudding. OY = obese yogurt (n = 15); OS = obese soy (n = 15). Data are mean  $\pm$  SEM. Between group analysis by two-way repeated measures ANOVA;  $P = 0.0179$  for time effect,  $P = 0.0163$  for treatment effect, and  $P = 0.0470$  for their interaction. \*  $P < 0.05$  from OY by paired t-test.



**Figure 4.7.** Postprandial SH concentration in obese women following co-consumption of low-fat yogurt or soy pudding and a high-calorie, high-fat meal prior to (initial) and following (final) 9-week daily 12 oz. low-fat yogurt or soy pudding consumption. OY = obese yogurt (n = 15); OS = obese soy (n = 15). Data are mean  $\pm$  SEM. Between-group analysis by two-way repeated measures ANOVA;  $P = 0.0028$  for time effect,  $P > 0.05$  for treatment effect and time-treatment interaction.

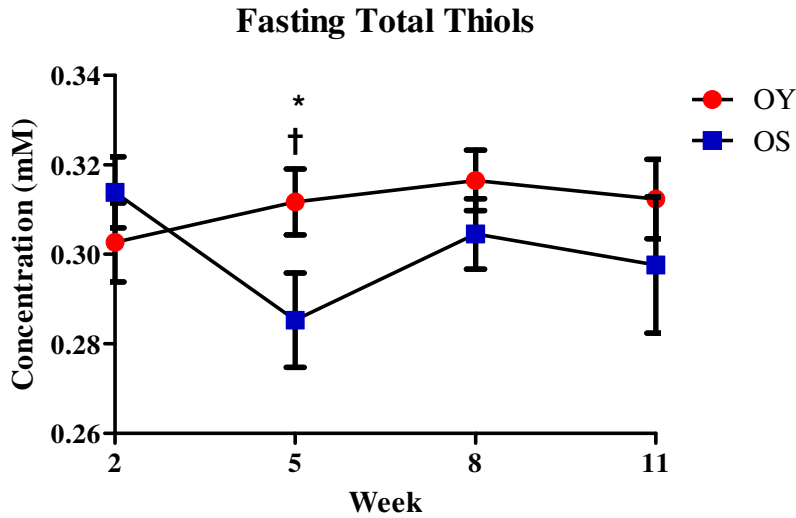


**Figure 4.8.** Initial postprandial plasma SH concentrations in obese women. OY = obese yogurt (n = 15), OS = obese soy (n = 15). (a) Postprandial SH following co-consumption of soy pudding and a high-calorie, high-fat meal. Data are mean  $\pm$  SEM. \*  $P < 0.05$  from fasting by paired t-test. (b) Postprandial plasma SH in obese women following co-consumption of low-fat yogurt or soy pudding and a high-calorie, high-fat meal. Data are mean  $\pm$  SEM. Between-group analysis by two-way repeated measures ANOVA; \*  $P < 0.05$  from OS by unpaired t-test.

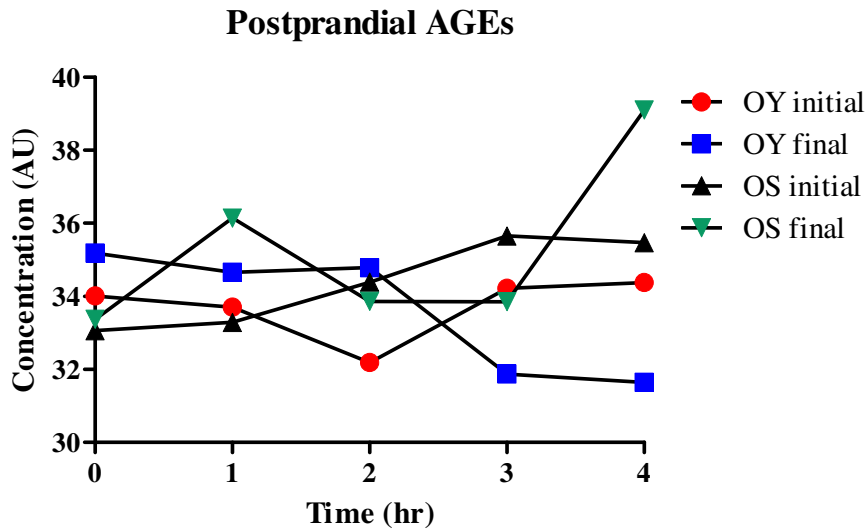


**Figure 4.9.** Postprandial SH AUC in obese women following co-consumption of soy pudding and a high-calorie, high-fat meal following a 2-week washout period in which all probiotic-containing foods were avoided and dairy consumption was limited to < 4 servings/day. OY = obese yogurt (n = 15); OS = obese soy (n = 15). Data are mean  $\pm$  SEM. Between-group analysis by two-way repeated measures ANOVA;  $P > 0.05$  for treatment effect, time effect, and their interaction. †  $P < 0.10$  from OY Initial by unpaired t-test.

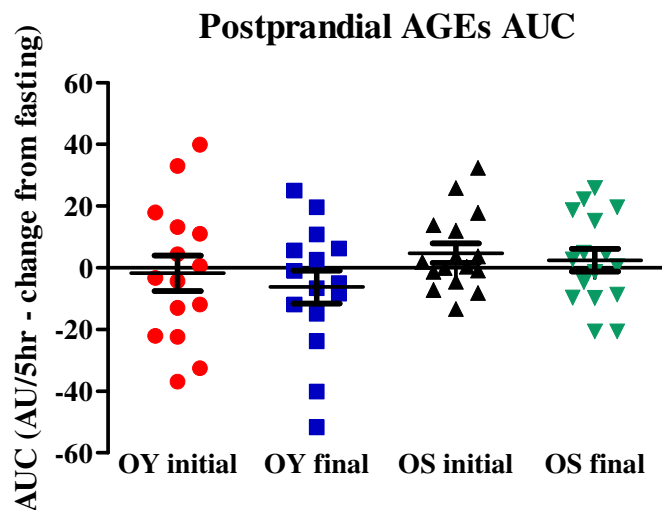




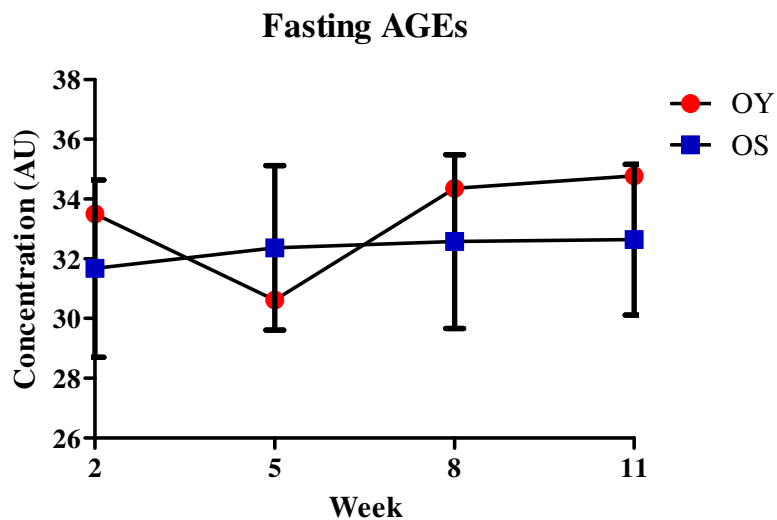
**Figure 4.10.** Fasting SH concentration in obese women prior to (week 2), during (weeks 5 and 8), and after (week 11) 9-week daily consumption of low-fat yogurt or soy pudding. OY = obese yogurt (n = 15); OS = obese soy (n = 15). Data are mean  $\pm$  SEM. Between-group analysis by two-way repeated measures ANOVA;  $P > 0.05$  for time effect and treatment effect,  $P = 0.0167$  for time-treatment interaction. \*  $P < 0.05$  (OS week 5 from OS week 2) by paired t-test, †  $P < 0.10$  from OS by unpaired t-test.



**Figure 4.11.** Postprandial plasma AGEs in obese women following co-consumption of low-fat yogurt or soy pudding and a high-calorie, high-fat meal prior to (initial) and following (final) 9-week daily intake of 12 oz. of low-fat yogurt or soy pudding. OY = obese yogurt (n =15); OS = obese soy (n = 15). Between-group analysis by two-way repeated measures ANOVA;  $P > 0.05$  for time effect, treatment effect, and their interaction.



**Figure 4.12.** Change from fasting area under the curve (AUC) of postprandial plasma AGEs in obese women following co-consumption of low-fat yogurt or soy pudding and a high-calorie, high-fat meal prior to (initial) and following (final) 9-week daily intake of 12 oz. of low-fat yogurt or soy pudding. OY = obese yogurt (n = 15); OS = obese soy (n = 15). Data are mean  $\pm$  SEM. Between-group analysis by two-way repeated measures ANOVA;  $P > 0.05$  for time effect, treatment effect, and their interaction.



**Figure 4.13.** Fasting plasma AGEs in obese women prior to (week 2), during (weeks 5 and 8), and after (week 11) 9-week daily consumption of low-fat yogurt or soy pudding. OY = obese yogurt (n = 15); OS = obese soy (n = 15). Data are mean  $\pm$  SEM. Between-group analysis by two-way repeated measures ANOVA;  $P > 0.05$  for time effect, treatment effect, and their interaction.

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## **Chapter 5:**

### **Conclusions and Future Directions**

## 5.1 Summary of Key Findings

- Daily intake of 12 oz. of low-fat yogurt or soy pudding did not lead to any changes in weight, BMI, waist circumference, or blood pressure in lean and obese women.
- Daily consumption of 12 oz. of low-fat yogurt increased carbohydrate intake by ~15% in lean and obese women. Soy pudding consumption increased carbohydrate intake in lean women only by ~15%. Low-fat yogurt consumption also significantly decreased protein and cholesterol intake in obese women.
- Daily low-fat yogurt consumption increased vitamin D and calcium intake in lean and obese women. Daily consumption of fortified soy pudding also increased vitamin D intake in lean and obese women and calcium intake in lean women. However, vitamin D intake in lean and obese women in both groups still fell well below the RDA.
- Daily low-fat yogurt consumption increased intake of dairy products by ~1 serving/day in lean and obese women. However, intake still fell below the recommended 3 servings/day.
- Daily low-fat yogurt and soy pudding consumption decreased vitamin C intake in lean women. Fruit intake also decreased in the LY and OY groups. Vegetable intake decreased in the LY and LS groups.
- Co-consumption of low-fat yogurt with a high-calorie, high-fat meal resulted in no significant changes in MDA, SH, or AGEs in the postprandial period, suggesting the low-fat yogurt may attenuate the postprandial response in obese women. However, 9-week daily consumption of low-fat yogurt did not further attenuate this response. Likewise, daily yogurt intake did not alter baseline levels of MDA, SH, or AGEs in obese women.
- Co-consumption of soy pudding with a high-calorie, high-fat meal did not attenuate the postprandial MDA response at week 2. However, at week 11, postprandial MDA was

significantly decreased compared to week 2 at 2, 3, and 4 h postprandially. Conversely, 9-week daily intake of soy pudding increased baseline levels of MDA in obese women.

In summary, addition of low-fat yogurt to the diet of lean and obese women increased the intake of calcium and vitamin D, two nutrients which are not consumed in adequate amounts by many women [1]. Low-fat yogurt also increased dairy intake by ~1 serving/day in lean and obese women. Though the recommendations for vitamin D and dairy intake were still not met as a result of this intervention, low-fat yogurt could be added to the diet in concert with other dairy products and foods containing vitamin D to help meet recommendations. However, nutritional counseling should be provided to participants to avoid reductions in fruit and vegetable consumption as a result of the increased dairy intake.

Furthermore, the addition of low-fat yogurt to the diet did not alter BMI, waist circumference, or blood pressure, parameters that are of particular importance to obese women. Co-consumption of low-fat yogurt and a high-calorie, high-fat meal led to suppression of postprandial increases in MDA and AGEs and depletions in SH in obese women at both the initial and final study visits. Sustained intake of soy pudding resulted in small reductions in postprandial MDA from week 2 to week 11; however daily soy pudding intake increased baseline MDA, suggesting that total exposure to MDA was likely unchanged. Conversely, sustained low-fat yogurt consumption showed no effect on chronic levels of oxidative stress in obese women; fasting MDA, SH, and AGEs were unaffected by daily intake. Therefore, addition of low-fat yogurt to the diet of obese women could be employed as a strategy to increase vitamin D, calcium, and dairy intake without any unfavorable changes in body composition, blood pressure, and postprandial or chronic levels of oxidative stress.

## 5.2 Future Directions

Previous epidemiological and intervention studies support an association between adequate dairy, calcium, and vitamin D intake and numerous favorable health outcomes. In addition, dairy intake decreases oxidative stress in overweight and obese individuals [2, 3]. However, much remains unknown regarding this association. For example, the impact of consumption of individual dairy products on oxidative stress has not been elucidated. Furthermore, though dairy products contain calcium, vitamin D, protein, whey, casein, numerous bioactive peptides, and probiotics, the effects of these components, alone and in tandem, are unknown. Furthermore, the mechanisms responsible for observed reductions in oxidative stress are not well understood.

Future studies should examine the impact of other types of low-fat yogurt on postprandial and chronic oxidative stress, particularly those products lower in carbohydrates and higher in probiotics. In the present study, fruit and vegetable intake decreased in some of the study groups as a result of the intervention. Moreover, the addition of low-fat yogurt to the diet raised dairy intake by ~1 serving per day and increased vitamin D intake; however, neither of these increases were sufficient to reach recommendations. Therefore, intervention studies should also be conducted to examine the impacts of low-fat yogurt intake as a part of a diet rich in fruits and vegetables. Studies should also incorporate the consumption of low-fat yogurt with other dairy products and/or foods rich in vitamin D. Finally, future studies should include more comprehensive measurements of oxidative stress, including not only the markers used in this study but also the examination of urinary isoprostanes and 8-deoxyguanosine, as well as endogenous antioxidant enzyme activity.

### 5.3 References

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