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EFFECT OF LOW GLYCININ SOYMILK ON BODY COMPOSITION, BIOMARKERS OF
INFLAMMATION AND OXIDATIVE STRESS AND GUT MICROBIOTA IN
OVERWEIGHT MEN

BY

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THESIS

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ABSTRACT

Several studies suggest that consumption of soy protein has positive effects on preventing obesity. Preliminary *in vitro* studies with β -conglycinin showed significant reduction in lipid accumulation and inflammatory parameters compared with glycinin. The objective of this study was to compare the effect of low glycinin soymilk (LGS) (48% β -conglycinin) with conventional soymilk (S) (28% β -conglycinin) and bovine milk (M) (0% β -conglycinin) on body composition, serum lipids, biomarkers of oxidative stress and inflammation and gut microbiota in overweight men. A randomized, double-blind study was conducted with 64 overweight men (BMI > 25; 18-45 years old). Subjects consumed 500 mL of each beverage daily for 3 months. Fasting blood samples, fecal samples, dual x ray absorptiometry scans, anthropometrics, lipid profile, plasma and serum biomarker analyses were executed at baseline and after 3 months. Glycemic index measurements were also performed. Total energy, total fat and total protein content were standardized among treatments. LGS and S contained similar levels of total isoflavones. Energy intake ($p = 0.40$), moderate physical activity ($p = 0.21$) and strenuous physical activity ($p = 0.49$) did not change during the study period. Oxidized low density lipoprotein (LDL) was dramatically decreased after LGS consumption (-61%) in comparison to S (-36%, $p = 0.0007$) and M (-12%, $p = 0.002$). Serum antioxidant capacity increased significantly in LGS (18%, $p = 0.001$) and S (28%, $p < 0.001$) compared with M (-40%). The decrease in plasma interleukin-6 after 3 months of LGS (-22%, $p = 0.025$) and S (-26%, $p = 0.014$) consumption was significantly different from M. LGS consumption significantly increased plasma adiponectin (14%) compared with S (4%, $p = 0.039$) and M (-8%, $p = 0.036$). No effects were detected in BMI ($p = 0.721$), waist-hip ratio (WHR) ($p = 0.454$), weight ($p = 0.836$), triglycerides ($p = 0.947$), total cholesterol ($p = 0.320$), plasma leptin ($p = 0.655$), fatty acid synthase (FAS) ($p = 0.976$), tumor necrosis

factor alpha (TNF α) (0.61) and C-reactive protein (CRP) ($p = 0.959$) among treatments. LGS did not alter bone mineral density (BMD) or t-scores ($p > 0.05$). Changes in total phyla of microorganisms ($p = 0.36$), *Lactobacillus* ($p = 0.06$) and *Bacteroides* ($p = 0.27$) were not significant among treatments. However, universal microbiota increased after three months with all treatments. *Bifidobacteria* changes among treatments were short to significant ($p = 0.06$), showing a decrease with both soymilks consumption however general *Actinobacteria* increased with S ($p = 0.06$) and LGS ($p = 0.07$). Relative abundance of *Bacteroidetes* increased with soymilk consumption (LGS: $30\% \pm 11$, $p < 0.0001$; S: $29\% \pm 10$, $p = 0.0001$). Relative abundance of *Firmicutes* decreased after three months of LGS ($-4\% \pm 2$, $p < 0.0001$) and S ($-6\% \pm 2$, $p < 0.0001$) consumption. Dietary intakes of protein, fat, carbohydrates, dietary fiber, calcium, iron, phosphorus, potassium, caffeine, vitamins D, B₁₂, K, and E were not influenced by LGS, S or M ($p > 0.05$). However, dietary sodium ($p = 0.02$) and vitamin C ($p = 0.0002$) intakes were reduced with LGS consumption. LGS, S and M are low glycemic index products (41 ± 7 , 40 ± 10 and 29 ± 6 respectively). In conclusion, 3 months of LGS consumption, which is a low glycemic index food, inhibited a modest but significant accumulation of body fat, reduced inflammation and oxidative stress, and promoted positive intestinal microbiota composition in overweight men. Stronger effects of LGS consumption may be observed in combination of a low caloric diet and moderate physical activity.

To God, my parents, my soul sisters and to my love Julio, thank you for blessing my life.

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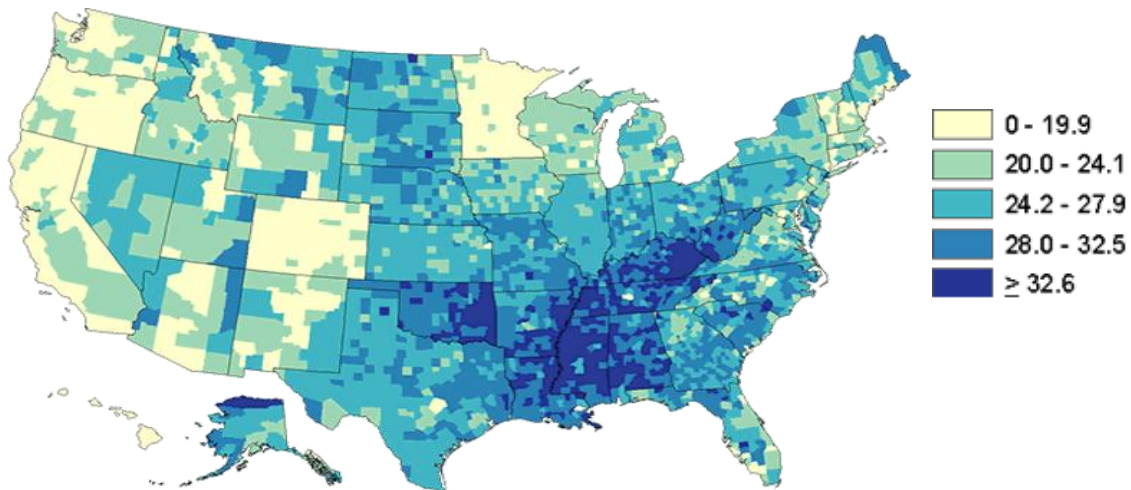
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I. INTRODUCTION

According to Center for Disease Control and Prevention (CDC, 2011) the population of the United States is obesogenic, and obesity is one of the most important health epidemic issues. An obesogenic environment includes multiple stimuli that increase food intake, physical inactivity and non-healthy food consumption. Physical inactivity is one of the major factors increasing the percentages of obesity of the country. Several regions in United States (US) have more than 32% of the adult population physically inactive (Figure 1)

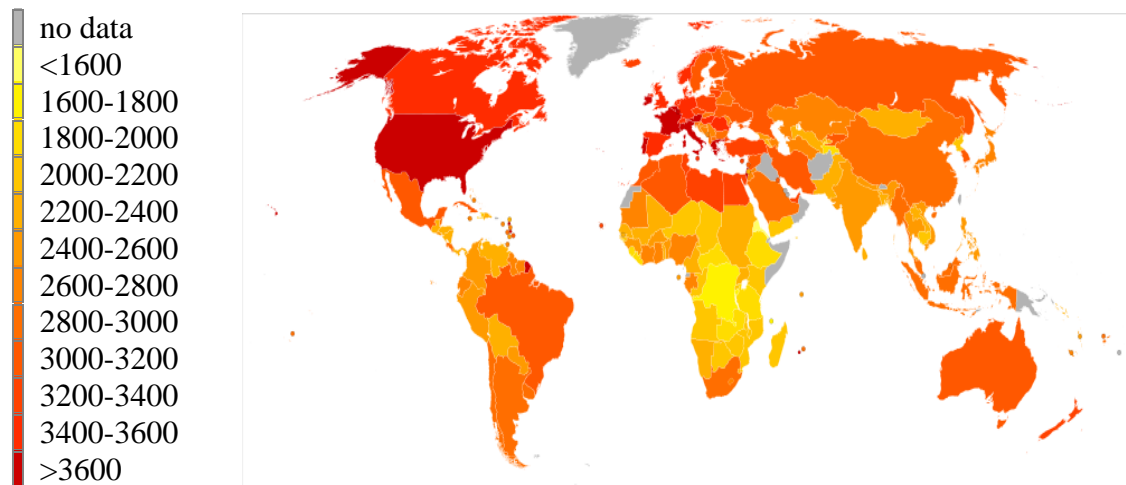


Diabetes Data & Trends-CDC, 2011 (Public domain)

Figure 1. Age-adjusted estimates of the percentage of physically inactive adults (2008)

High caloric intake is the complementary factor promoting obesity in the American population. In 2003, the average energy intake in the US and some European countries exceeded 3600 kilocalories per person per day, which is significantly higher than the world average (2800 kcal/person/day) (CDC,2011) (Figure 2). The situation has worsened with the high availability and variety of unhealthy, energy-dense foods. Two thirds of the US population is either

overweight or obese. Wang *et al.*, (2008) projected 90% of the American adult population will be overweight or obese for 2030 if this trend does not change.



FAO statistical yearbook, 2004
(Unrestricted use permission)

Figure 2. Map of energy consumption (kcal/person/day) per country in 2001-2003.

Scientists all over the world are trying to find solutions to reduce obesity and obesity-related conditions as well as ameliorate the economic impact on the US health care system. It's evident, that the simple recommendation to eat less and exercise more has not been adopted by many. There is a need for research and development of new strategies that meet the comfort, taste and nutritional needs of consumers at a low cost.

The industry of soy products has been involved in developing innovative strategies to try to counteract obesity and its side effects. Whole soy, soy isoflavones, and soy protein and peptides have been related to many health benefits, for instance; weight lost, reduced body fat, improved lipid profiles (Konig *et al.*, 2008), alleviating menopause symptoms (Williamson-Highes *et al.*, 2006), preventing osteoporosis (Zhang *et al.*, 2005), managing blood pressure

(Altorf-van der Kuil *et al.*, 2010), lowering cardiovascular risk (Richard *et al.*, 2010), and reducing inflammation and oxidative stress (Valsecchi *et al.*, 2011), among others. There are also studies that contradict such findings, as Balk *et al.* (2005) that suggested small soy effects on lipids did not support the effect of soy on endocrine function, menstrual cycles and bone health.

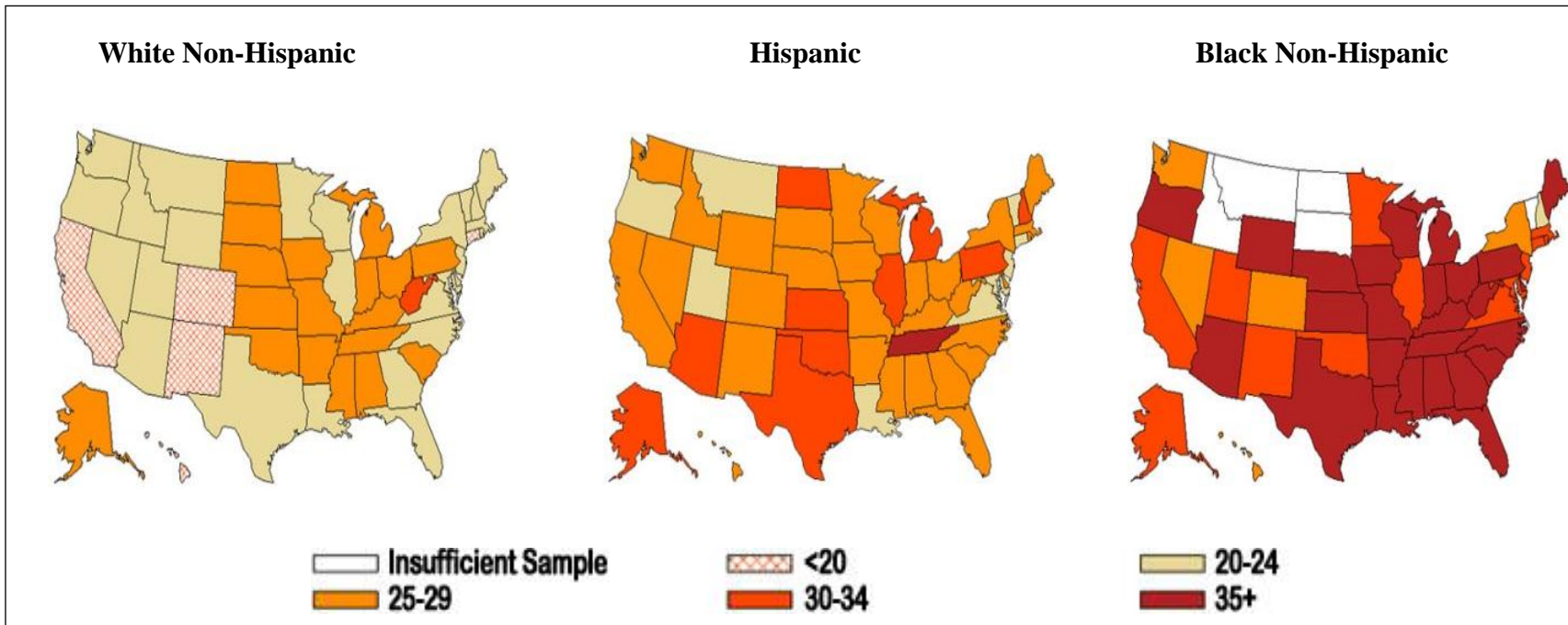
The general objective of the present study was to compare the effect of low glycinin soymilk on body composition, serum lipids, adipokines, biomarkers of oxidative stress, inflammation, glycemic response and gut microbiota composition with conventional soymilk and bovine milk in overweight men. The final aim is to increase knowledge of the benefits of soy bioactive compounds and to provide new healthy food alternatives for the overweight and obese population.

II. LITERATURE REVIEW

1. Obesity and weight management

Currently, the US Dietary Guidelines for adults (2005) define a BMI of 18.5 kg/m² up to 24.9 kg/m² as healthy weight, body mass index (BMI) of 25 kg/m² up to 29.9 kg/m² as overweight and BMI of 30 kg/m² or greater as obesity. BMI is calculated dividing the weight in kilograms by the height in meters squared. According to the CDC, BMI is the most reliable indicator of body fat. Two-thirds of Americans have a BMI > 25 (CDC, 2011) and the prevalence of obesity differs from region to region due to many demographic and intrinsic factors such as age, race-ethnicity, gender, socioeconomic status, genetics, among others.

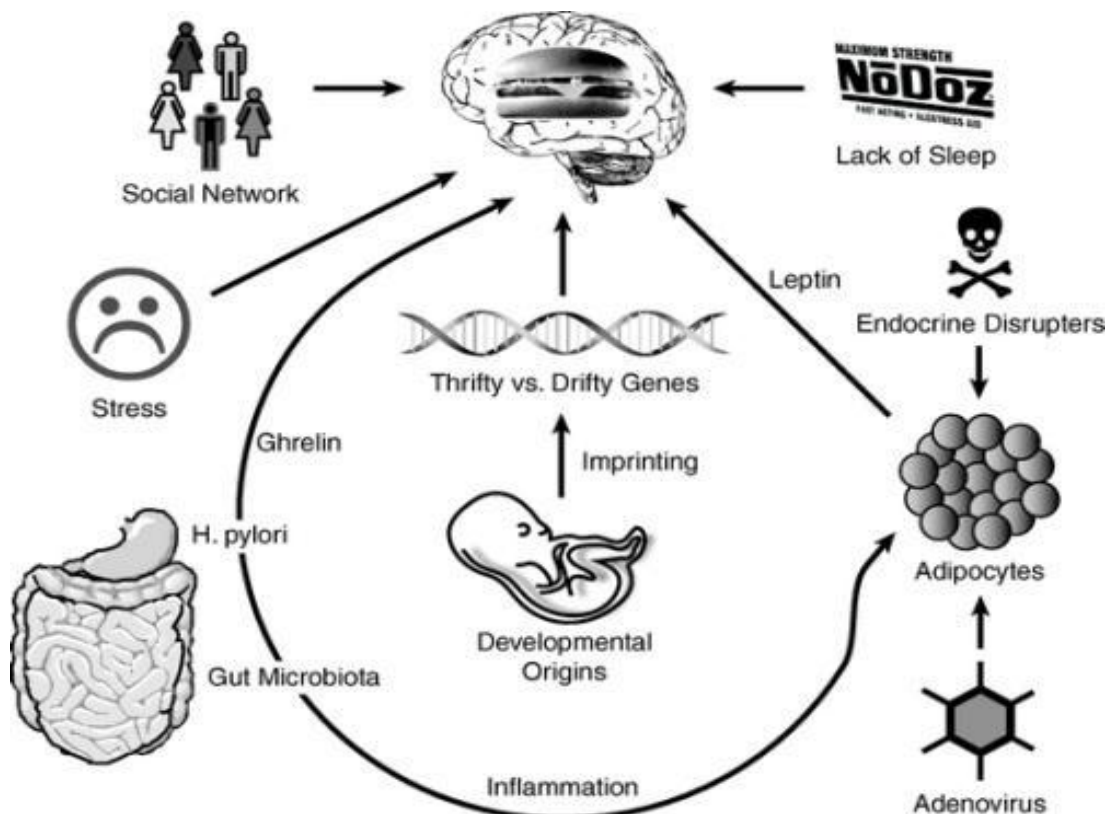
The prevalence of obesity in different ethnic groups can be observed in Figure 3. The black non-Hispanic population has a higher prevalence of obesity than Hispanic and white-non Hispanic populations. Socio-economic status has an impact on obesity prevalence as well. High obesity prevalence in low income groups may be due to consuming energy-dense foods products to cover their caloric needs and these products are usually of low cost (Drewnowski and Specter (2004). Baum and Ruhm (2007) reported that age is directly related to increments in weight and socio-economic status is inversely related to weight gain. Obesity and its related consequences elevate the risk of almost every cause of mortality (Ogden *et al.*, 2007).



CDC, 2011

Figure 3. Obesity among ethnic groups in US (2006-2008). (Public domain)

The reasons for this epidemic problem are extensive, environmental, physiological and motivational obstacles hamper the efforts in the reduction or maintenance of weight (VanWormer *et al.*, 2008). A recent publication of the University of Texas Southwestern Medical Center summarized the most popular obesity influencing factors in the last years of research (Zinn, 2010). This comprehensive analysis gives special importance to the lack of sleep hours, psychosocial stress and gut microbiota composition (Figure 4). According to Zinn (2010), several studies agree with an inverse correlation of sleep hour and BMI. People sleeping less than 8 h per night increases nocturnal snacking, tiredness to do physical activity, gut ghrelin and decrease in leptin levels.



Zinn, 2010. (Permission by Wolters Kluwer Health group)

Figure 4. Unconventional hypotheses for the etiology of the obesity epidemic

Genetic factors are also associated with obesity and numerous genes have been related with body weight, for example, melanocortin 4 receptor, β -adrenergic receptor 2 and 3, hormone-sensitive lipase, mitochondrial uncoupling proteins 1, 2 and 3 and FTO (Sabin *et al.*, 2011). However the genetic influence in obesity cannot be isolated analyzed without a deep understanding in the obesogenic environment underlying the genetic predisposition.

Genetics have been widely blamed for the increment in body fat and weight; however, genetics only explains 30-70% of changes in BMI (Loos and Bouchard, 2003) and the activation of such genes requires singular conditions. For instance, Jacobson *et al.* (2009) affirmed that FTO gene variants associated with obesity is activated only with physical inactivity. Obesity is a multifactorial disease and the current and future efforts to find alternatives of solution must define the detailed conditions to better understand this this epidemic health concern.

2. Soybean

Soybean is botanically classified into the *Rosaceae* order, *Leguminosae* or *Papilloceae* family, *Papilionoidae* subfamily, *Glycine* genus and *Glycine max* cultivar (Mateos-Aparicio *et al.*, 2008). The rich nutrient content of soybean has promoted extensive research projects and has increased the diversification of soybean products. A very detailed description of the total macronutrient, minerals, lipids and minerals content of soybean and soymilk is shown in Table 1. Other soy based products vary in their nutritional profiles however most of them are characterized for the high protein content, and depending of their processing, a high percent of dietary fiber. Soybean contains several biologically active compounds such as isoflavones, saponins, peptides, and proteins with functional properties (Dia *et al.*, 2008).

Table 1. Nutritional content of soybean and soymilk

Nutrients/100 grams	Units	Mature raw soybean	Soymilk, light, unsweetened, fortified
Proximate Analysis			
Water	g	8.54	92.03
Energy	kcal	446	34
Energy	kJ	1866	140
Protein	g	36.49	2.62
Total lipid (fat)	g	19.94	0.85
Ash	g	4.87	0.64
Carbohydrate, by difference	g	30.16	3.85
Fiber, total dietary	g	9.3	0.6
Sugars, total	g	7.33	0.41
Minerals			
Calcium, Ca	mg	277	123
Iron, Fe	mg	15.70	0.46
Magnesium, Mg	mg	280	13
Phosphorus, P	mg	704	103
Potassium, K	mg	1797	117
Sodium, Na	mg	2	63
Zinc, Zn	mg	4.89	0.10
Vitamins			
Vitamin C, total ascorbic acid	mg	6.0	0.0
Folate, total	mcg	375	7
Vitamin B-12	mcg	0.00	0.23
Vitamin A, IU	IU	22	206
Vitamin D	IU	0	41
Lipids			
Fatty acids, total saturated	g	2.884	0.078
Fatty acids, total monounsaturated	g	4.404	0.263
Fatty acids, total polyunsaturated	g	11.255	0.558
Cholesterol	mg	0	0

USDA, 2010 (Public domain)

2.1. Soy proteins

Whole soy contains a significant amount of total protein which varies depending on the variety but is usually around 40% of its total chemical composition. Soy proteins are considered of very high quality by its amino acid composition and has been associated with multiple health benefits. The Food and Drug Administration (FDA) published a recommendation to include 25 g of soy protein per day in a diet low in saturated fat and cholesterol may be helpful to improve lipid profile. Soy protein is constituted by 90% storage globulins, with glycinin (11S) and β -conglycinin (7S) proteins as the major storage proteins in soy (Ferreira *et al.*, 2011).

2.2. β -conglycinin

The basic β -conglycinin molecule contains subunits of 27 kD and 16 kD chains linked by disulfide bonding acting as biotic and abiotic stress protectors in the soybean plants (Shutov *et al.*, 2010). β -conglycinin is around 30% of the total protein profile in soybean, and glycinin is usually present in higher amount than β -conglycinin (Mo *et al.*, 2011). β -conglycinin is a relatively stable molecule. A complex study developed by Hou and Chang (2004) determined that β -conglycinin did not present significant changes in all structural characteristics after 18 months under three conditions: mild adverse (57% RH/20°C), cold (4°C) and uncontrolled environmental conditions.

The consumption of β -conglycinin has been investigated for its nutraceutical potential in different obesity-related diseases *in vivo* and *in vitro* studies. Soy β -conglycinin have been related with decreases in very low density lipoprotein (VLDL) and triglycerides, increments in adiponectin (Tachibana *et al.*, 2010), inhibition of fatty acid synthase (FAS) (Martinez-Villaluenga *et al.*, 2010), reduction of cholesterol (Lovati *et al.*, 2000), reduction of fat accumulation and inflammation (Martinez-Villaluenga *et al.*, 2009), inhibition of leukemia cells

growth (Wang *et al.*, 2008), prevention of hypertension (Yang *et al.*, 2004), prevention of hyperglycemia, hyperinsulinemia and hyperglycemia (Moriyama *et al.*, 2004), maintenance of body fat ratio (Baba *et al.*, 2004), and prevention of obesity (Kohno *et al.*, 2006) among others.

3. Adipose tissue, inflammation and oxidative stress.

Adipose tissue is not a fat storage system exclusively. It also secretes hormones of high importance in regulation and metabolism (Baudrand *et al.*, 2010). Obesity causes increases in the number and size of adipocytes and promotes inflammation due to the presence and accumulation of macrophages. These macrophages activate and secrete inflammatory cytokines such as Interleukin-6 and TNF α (Tesauro *et al.*, 2011).

Adipose tissue is located in visceral and subcutaneous depots and obesity accelerates its accumulation in these specific depots, throughout the body and other organs such as the heart, the kidneys and blood vessels. This expansion of adipose tissue is accompanied by adipokine secretion affecting organ functions and systemic metabolism (Ouchi *et al.*, 2011). A detailed list of adipokines secreted by adipose tissue is shown in Table 2.

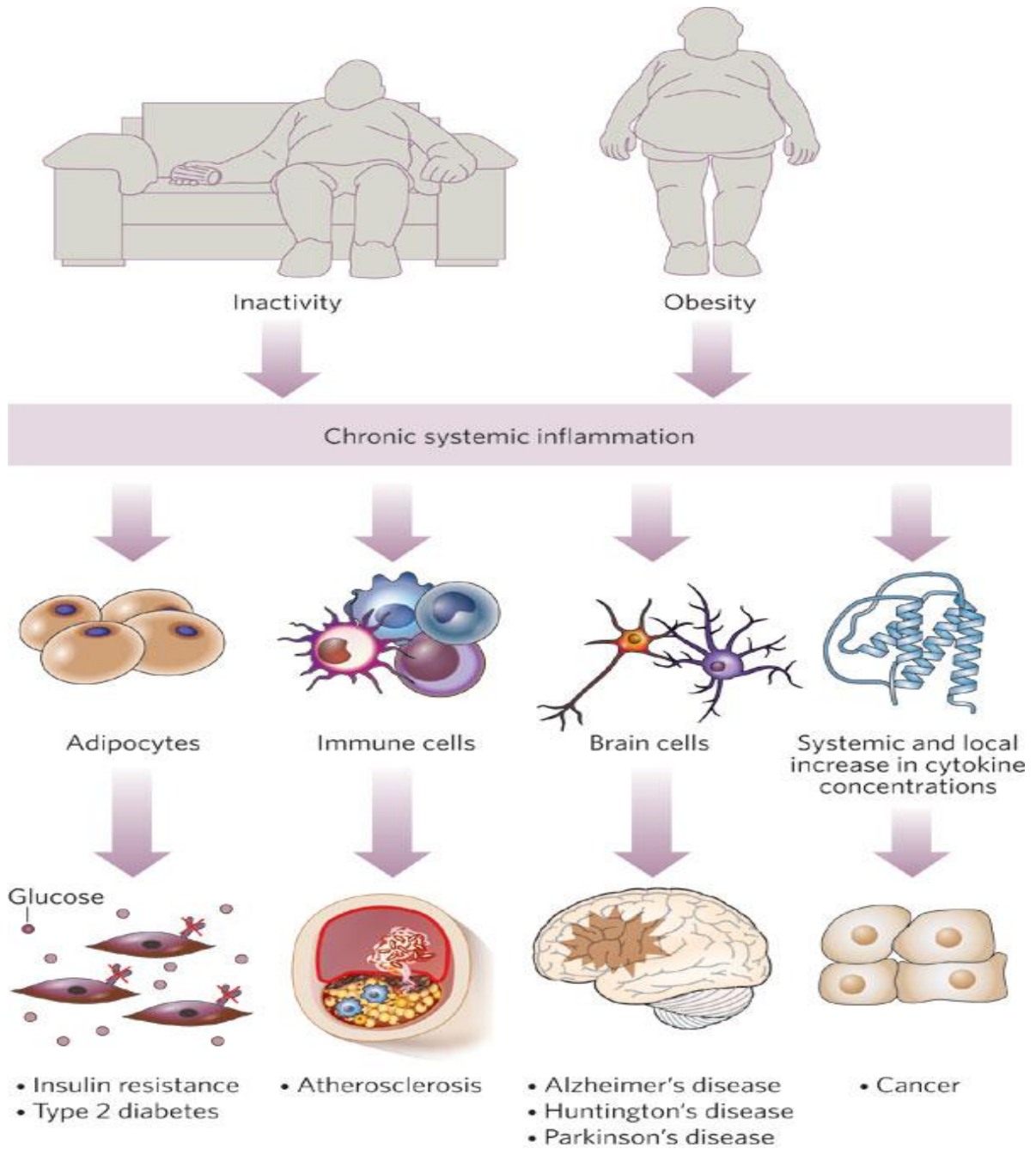
Obesity is also defined as a low-grade chronic systemic inflammation state worsened by physical inactivity and excessive high energy intake. This inflammatory state is related to the development of many chronic diseases. Examples of the variety of obesity consequences in human health are shown in Figure 5. The obesity-related chronic systemic inflammation is usually accompanied by marked physical inactivity. Inflammatory responses are also associated with atherosclerosis, neurodegenerative diseases, type 2 diabetes, insulin resistance, and cancer (Handschin and Spiegelman, 2008).

Table 2. Sources and functions of key adipokines

Adipokine	Primary source(s)	Binding partner or receptor	Function
Leptin	Adipocytes	Leptin receptor	Appetite control through the central nervous system
Resistin	Peripheral blood mononuclear cells (human), adipocytes (rodent)	Unknown	Promotes insulin resistance and inflammation through IL-6 and TNF secretion from macrophages
RBP4	Liver, adipocytes, macrophages	Retinol (vitamin A), transthyretin	Implicated in systemic insulin resistance
Lipocalin 2	Adipocytes, macrophages	Unknown	Promotes insulin resistance and inflammation through TNF secretion from adipocytes
ANGPTL2	Adipocytes, other cells	Unknown	Local and vascular inflammation
TNF	Stromal vascular fraction cells, adipocytes	TNF receptor	Inflammation, antagonism of insulin signalling
IL-6	Adipocytes, stromal vascular fraction cells, liver, muscle	IL-6 receptor	Changes with source and target tissue
IL-18	Stromal vascular fraction cells	IL-18 receptor, IL-18 binding protein	Broad-spectrum inflammation
CCL2	Adipocytes, stromal vascular fraction cells	CCR2	Monocyte recruitment
CXCL5	Stromal vascular fraction cells (macrophages)	CXCR2	Antagonism of insulin signalling through the JAK-STAT pathway
NAMPT	Adipocytes, macrophages, other cells	Unknown	Monocyte chemotactic activity
Adiponectin	Adipocytes	Adiponectin receptors 1 and 2, T-cadherin, calreticulin-CD91	Insulin sensitizer, anti-inflammatory
SFRP5	Adipocytes	WNT5a	Suppression of pro-inflammatory WNT signalling

ANGPTL2, angiopoietin-like protein 2; CCL2, CC-chemokine ligand 2; CXCL5, CXC-chemokine ligand 5; IL, interleukin; JAK, Janus kinase; NAMPT, nicotinamide phosphoribosyltransferase; RBP4, retinol-binding protein 4; SFRP5, secreted frizzled-related protein 5; STAT, signal transducer and activator of transcription; TNF, tumour necrosis factor.

Ouchi *et al.*, 2011. (Permission by Elsevier group)

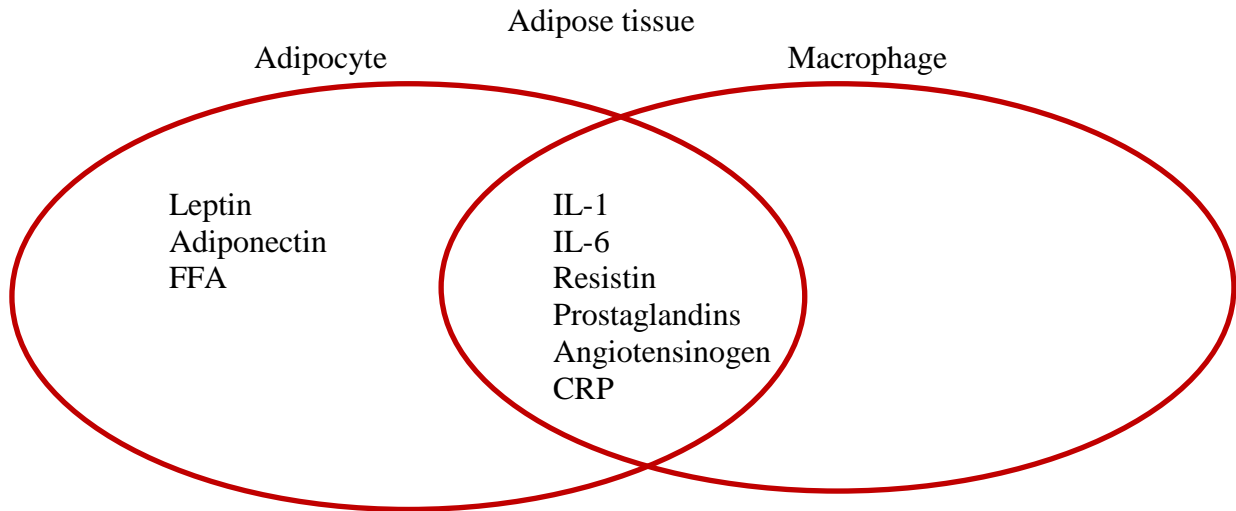


Handschin and Spiegelman, 2008. (Permission by Nature publishing group)

Figure 5. Consequences of obesity and inflammatory

Inflammatory predictors of type 2 diabetes and cardiovascular disease in adults such as C-reactive protein (CRP), interleukin-6 and biomarkers of oxidative stress such as oxidized-LDL have been under investigation however the influence of different variables on these parameters remain partially unclear. Extreme and middle obesity stages have been recently related with high oxidative stress and inflammation (Norris *et al.*, 2011). Correlations between increments in BMI and oxidative stress have been reported (Tunc *et al.*, 2010). According to Mellissas *et al.* (2006) oxidative stress may be a key element in obesity pathogenesis. Circulatory levels of oxidized LDL are higher in obese individuals than normal and directly correlate with adiposity; however, it's independent of body fat composition. This phenomenon may be the result of the maturity acceleration of macrophages promoted by oxidized-LDL (Kelly *et al.*, 2010).

In an effort to define the adipokine profile of obese and normal individuals, many researchers have quantified levels of adiponectin, leptin, IL-6, tumor necrosis alpha (TNF α); however the results are still variable. According to Kuo and Helpern (2011) interleukin-6 directly correlates with BMI in women, but adiponectin and TNF α is not affected by obesity. Leptin and adiponectin are adipocyte-derived hormones that respond to adiposity increments, subcutaneous and visceral respectively. Leptin (16k-Da) and adiponectin (30 kD) increase fatty acid oxidation and decrease triglycerides (Dyck, 2009). Leptin is a regulator of food intake and energy storage dynamic and adiponectin, which is almost exclusively produced by adipose tissue, exerts for its strong anti-inflammatory, antiatherogenic, and insulin-sensitizing effect (Ouchi and Walsh, 2006). A complete list of cytokines secreted by adipocytes and/or macrophages in human adipose tissue is shown in Figure 6.



Based on: Wang and Nakayama, 2010

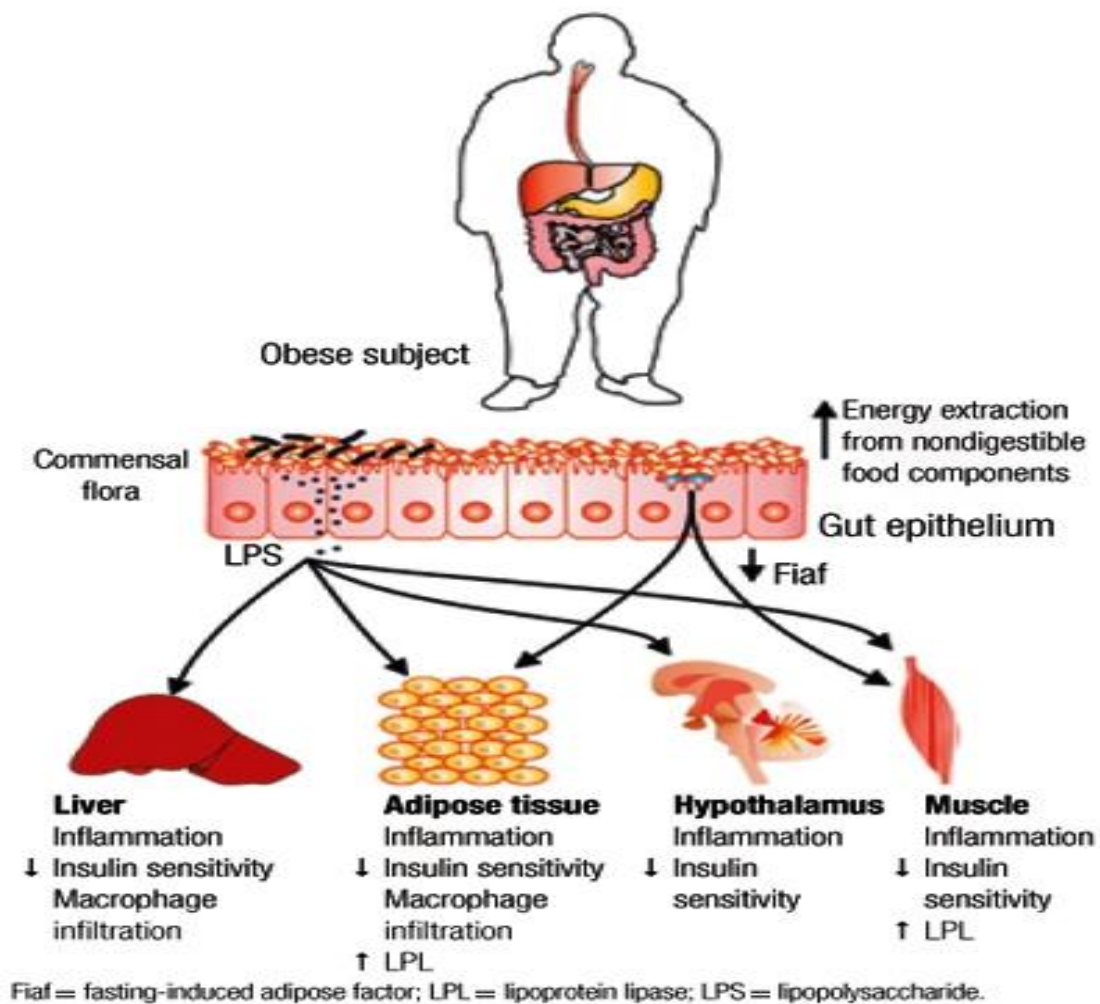
Figure 6. Cytokines from human tissue

4. Gut microbiota composition

4.1. Intestinal microbiota composition and obesity

The human gut is a complex consortium of microorganisms called microbiota whose quantification and characterization is not absolutely defined. It is believed that collectively, the genome of the gut microbiota contain 100 times more genes than the human genome (Cani and Delzenne, 2007). One of the essential roles of intestinal microbiota is the metabolism of dietary components (Atkinson *et al.* 2005). According to Serino *et al.* (2009), variations in nutrient intake produce changes in intestinal microbiota and metabolic processes, unbalancing the gram-positive – gram-negative ratio as well as changes in biomarkers of inflammation. It has been recognized that gut microbiota may play an important role in human health due to the fact that gut microbiota is different among obese and normal individuals. These advances in science motivate to study its different changes to consider a potential gut microbiota manipulation in pro of humanity. According to Cani and Delzenne (2007), gut microbiota change metabolism by three mechanisms: a) gut microbiota increases the capacity to harvest energy from the diet, b) gut

microbiota controls triacylglycerol fate and c) gut microbiota modulates the increment in plasma lipopolysaccharide levels. Similar theories but not exactly the same was proposed by Tsukumo *et al.* (2009). In this study the researchers explained the link of gut microbiota and obesity through three mechanisms (Figure 7); a) microbiota increases energy extraction from indigestible dietary polysaccharides, b) microbiota modulates plasma lipopolysaccharides which increases inflammation, obesity and diabetes, c) induce regulation of the host genes that modulate how energy is expended and stored.



Tsukumo *et al.*, 2009 (Permission by Creative Commons)

Figure 7. Schematic view of the possible mechanism linking gut flora to obesity

4.2. Microbiota changes and weight reduction

Recent findings have tried to explain the relationship between human microbiota, obesity and obesity-associated diseases. Backhed *et al.* (2004) concluded that mice gut microbiota as a whole promotes adiposity by the increment in energy harvest from diet and fat storage in adipose tissue and liver. Ley (2010) found an association between genetic obesity and altered gut microbiota composition which can impact host metabolism, produce effects in inflammation, insulin resistance and deposition of energy in fat stores. Others authors believe that the diet is the modifying factor in gut microbiota composition (Turnbaug *et al.*, 2008; Nadal *et al.*, 2009).

Obesity also has an effect at the phyla level in the microbiota, reducing bacterial diversity due to abnormal energy input and altering metabolic pathways as well (Turnbaugh *et al.*, 2009). Other specific studies have underlined the impact of calorie restriction and physical activity on gut microbiota during weight loss. This study also determined that weight loss is associated with an increase in *Bacteroidetes* and *Lactobacillus* and to a decrease in *Clostridium* and *Bifidobacteria* (Santacruz *et al.*, 2009). In the other hand, western diet has been related with the increase in *Firmicutes* and decrease of relative abundance of *Bacteroidetes* (Turnbaugh *et al.*, 2008). In addition, high fat diets have also been analyzed and according to Amar *et al.* (2008) high fat intake is directly related with plasma lipopolisaccaride (LPS) levels in men which normally come from gram-negative bacteria. LPS is known as the new inflammatory factor from microbiota increasing obesity and insulin resistance.

Type 2 diabetes is also related with different microbiota composition, diabetic subject's microbiota contain less *Firmucites* and more *Bacteroidetes* and *Proteobacteria* than non-diabetic subjects, being *Bacteroides-Prevotella* higher in non-diabetic and *Clostridia* and *C. coccoides-E.rectale* higher in diabetics (Larsen *et al.*, 2010). In a separate investigation developed by Wu

et al. (2010) it was clarified that microbial diversity is not associated with diabetes but rather microbiota composition. They also determined that diabetic subjects presented lower proportion of *Bacteroides vulgatus* and *Bifidobacteria* than non-diabetic subjects.

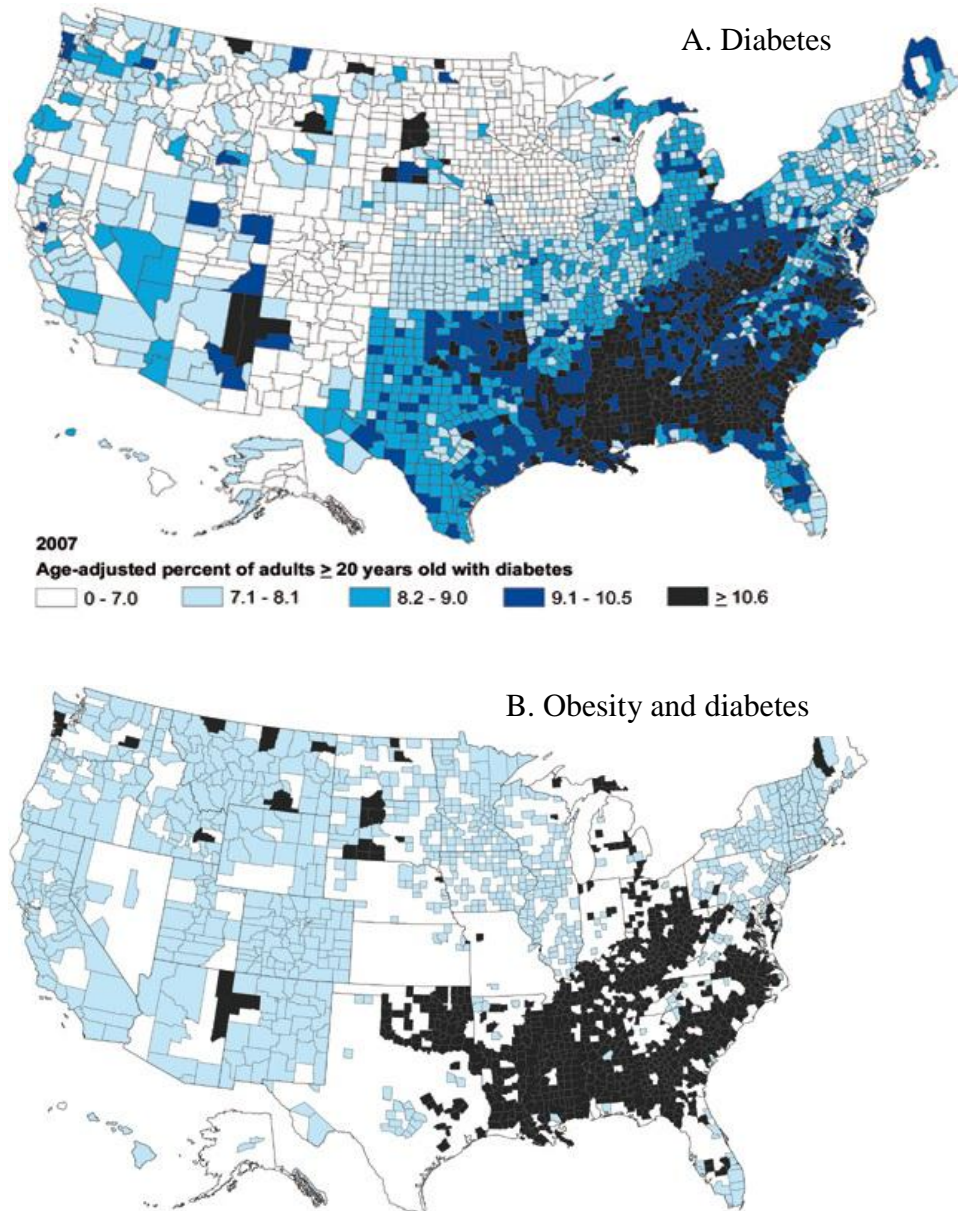
The clearest agreement among many studies is that obese microbiota is characterized for low abundance of *Bacteroidetes* and a high abundance of *Firmicutes*. According to the findings that obesity affect gut microbiota diversity, an intentional manipulation of the gut microbiota community may be a potential therapeutic option for the overweight and obese population (Ley *et al.*, 2005). All these advances have partially clarified some relationships between obesity and the intestinal microflora, however deeper analyses about microbial adaptation and mutualistic community assembly with different diets and in different environments are still needed.

5. Glycemic index and glyceimic response

Obesity and diabetes are two health concerns that often have overlapping incidence. In Figure 8 we can graphically differentiate how many of the obese population also have type 2 diabetes. In this regard, governmental institutions and the food industry have tried to educate to the consumer about the content and properties of food products to make smart choices to reduce health risks. Glycemic index (GI) is a tool used to classify foods based on the glyceimic response and is defined as the glucose response area under the curve after 25-50 g of carbohydrate consumption. Low glyceimic index products may be tested with lower than 50 g carbohydrate consumption (Cocate *et al.*, 2011). The Food and Agriculture Organization (FAO) of the World Health Organization (WHO) defined the GI in 1998 as the incremental area under the blood glucose response curve of a 50 g carbohydrate of food expressed as a percent of the response to 50 g of carbohydrate from a standard food in same subject (FAO/WHO, 1998). The American Diabetes Association classifies as low GI, medium GI and high GI foods with GI's of 55 or less,

56-69 and more than 70 respectively. Several studies have claimed successful maintenance of glucose concentrations with the consumption of low GI foods (Stevenson *et al.*, 2005). High GI produces a higher postprandial blood glucose response after 2 h of consumption (Foster-Powell *et al.*, 2002). This reaction may be due to slower digestion and absorption rates (Brand-Miller *et al.*, 2004). Isken *et al.*, 2010) reported that only long term (not short term) consumption of high GI foods results in obesity, insulin resistance and metabolic complications. Contradictory, Cocate *et al.* (2011) failed to confirm different glycemc responses between low and high GI foods. The determination of GI is still controversial due the variable responses observed in the same type of products. Measurement methods, food ingredients and food processing conditions must be take into consideration when compared GIs. For instance, larger degrees of processing yield higher GI responses (Fernandes *et al.*, 2005). Ripeness and storage time have a marked influence in glycemc index measurements (American Diabetes Association, 2011). The methodology used also may influence the variances in results, for instance, capillary blood samples are more reliable than venous blood samples to determine GI (Wolever *et al.*, 2003). Low GI diets have shown improvements in glycemc control, reduction in serum lipids, cardiovascular risk and diabetes risk (Jenkins *et al.*, 2002). The effects that a low GI diet has on obese individuals are also debated. Low GI diets have been related to increments in satiety (Ludwig, 2000). The consumption of low GI products in order to prevent the development of diabetes or other obesity-related issues may be achieved by replacing a) energy from carbohydrates with energy from proteins, b) energy from carbohydrates with energy from fat, or c) replace high GI with low GI foods (Brand-Miller, 2004). International tables of GI of foods have been published and used by the scientific community as instruments to evaluate the relationship between glycemc index and human health; however new reliable information is

needed for update of such tables. Glycemic loads are also related to lower risk of cardiovascular disease and are calculated multiplying the GI of a food by the available carbohydrate present in that food divided by 100 (Lacombe and Ganji, 2010) .



A. Diabetes map 2007. B. Diabetes and obesity incidence 2007. Blues are counties in the top 2 quintiles of both diabetes and obesity. Blacks are in the bottom 2 quintiles of both diabetes and obesity. CDC, 2011 (Public domain)

Figure 8. Obesity and diabetes prevalence in US adult population in 2007.

III. RESEARCH SIGNIFICANCE

Heart disease and stroke, in association with obesity, will be the leading causes of death in the world for 2020. In addition, obesity is one of the most important contributors of premature mortality and is associated with several metabolic disorders resulting in high levels of serum lipids and abnormalities in biomarkers of inflammation, oxidative stress which in turn are also associated with adiposity and weight gain. It is also important to keep in mind that oxidized LDL (ox-LDL) plays a key role in the development of atherosclerosis and cardiovascular disease and contribute to the development of obesity-related diseases. Additionally, elevated levels of interleukins and C-reactive proteins are result of chronic inflammation. An approach to mitigate the incidence of obesity and metabolic disorders should include exercise and replacing high energy foods with healthy foods containing bioactive compounds with positive impacts in gut bacteria composition, glycemic response, lipid metabolism, inflammation and oxidative stress. Soybean [*Glycine max* (L.) Merr.] is a rich source of proteins and bioactive compounds which have been related to the prevention fat accumulation and cardiovascular diseases. The ratio of β -conglycinin and glycinin present in soy may produce different positive effects in human health. Thus, β -conglycinin has received particular attention as potential agent to improve health of overweight individuals. The effect of consuming whole soy foods made from low glycinin soybean enriched in β -conglycinins is unknown. Recent studies have tried to explain the role of soy proteins in health factors such as bone density, glycemic response and intestinal microbiota; however the results are still inconclusive. The complex mixture of protein and isoflavones present in soy have been studied, showing positive effects in bone health with consumption of soy isoflavones and soy proteins however other studies contradict such results. Nutritional intake and physical activity changes were of important consideration in the present analysis, in order to

determine all possible influencing factors. Also, gut microbiota changes were studied due to the unclear influence of the consumption of soy-based products in gastrointestinal function and health. Given this background, it was hypothesized that the consumption of low glycinin soybean could be beneficial for oxidative stress, inflammation, gut microbiota and body composition in overweight individuals.

IV. HYPOTHESIS AND OBJECTIVES

1. Hypothesis

Low glycinin soymilk modulates body fat accumulation, serum lipids and biomarkers of oxidative stress and inflammation and benefits microbiota composition in overweight men.

2. Main objective

To compare the effect of a low glycinin soymilk in body fat accumulation, serum lipids, biomarkers of oxidative stress, inflammation, glycemic response and gut microbiota with conventional soymilk and bovine milk in overweight men.

3. Specific objectives

- Compare the changes in body fat accumulation and anthropometric parameters in overweight men after three months of consumption of low glycinin soymilk, conventional soymilk and bovine milk.
- Determine the effect of low glycinin soymilk on bone mineral density and nutrient intake in overweight men.
- Analyze serum lipids, antioxidant capacity and biomarkers of inflammation and oxidative stress after three months of consumption of low glycinin soymilk, conventional soymilk and bovine milk.
- Compare the changes in gut microbiota composition in overweight men after three months of consumption of low glycinin soymilk, conventional soymilk and bovine milk.
- Determine the glycemic response of two soy milks as compared to bovine milk.

V. MATERIALS AND METHODS

1. Subjects

Participants included otherwise healthy overweight men, age 18-45 years with body mass index (BMI) (25-44), non-vegetarian, non-athletes, and non-smokers, recruited voluntarily from the campus of the University of Illinois, Urbana-Champaign using fliers. They participated in an initial orientation meeting on the procedures of the study and signed a consent form.

Age, BMI, weight, waist-hip ratio, body fat composition were measured at baseline to organize subject whitening groups using a stratified randomization. There were not statistical differences among groups in any baseline anthropometric characteristics ($P > 0.05$). Based on BMI, these individuals are considered as pre-obese subjects according to the WHO criteria. They agreed to maintain their regular diet and physical activity during the study. Subjects were asked to avoid consumption of dietary supplements, antibiotics and to inform investigators about any new disease condition or new medication.

2. Soymilk and bovine milk products

The low glycinin soybean/high β -conglycinin seeds were provided by The Monsanto Co. (Saint Louis, MO). The corresponding whole soybean milk powders, including the one from traditional soybean seeds were manufactured by Archer Daniels Midland (Decatur, IL). The liquid preparation and packaging of the soymilk beverages and bovine's milk were conducted using an Ultra High Temperature procedure (Tetra Pak Co., Danton, TX).

The process compresses a preheating at 175 °F for 1 second, homogenization at 1500 psi and pasteurization at 285°F for 3 seconds. Milk was then cooled at 90°F and packaged in Tetra Brik material by Tetra Therm Aceptic System by Tetra Pak (Figure 9). This high thermal process allowed a shelf life of the product of 12 months without refrigeration and 24 months under

refrigeration temperatures. Final products were condemned for 2 weeks and microbiologically analyses were performed by Tetra Pak to ensure safety status of the samples previous to the shipping to University of Illinois.

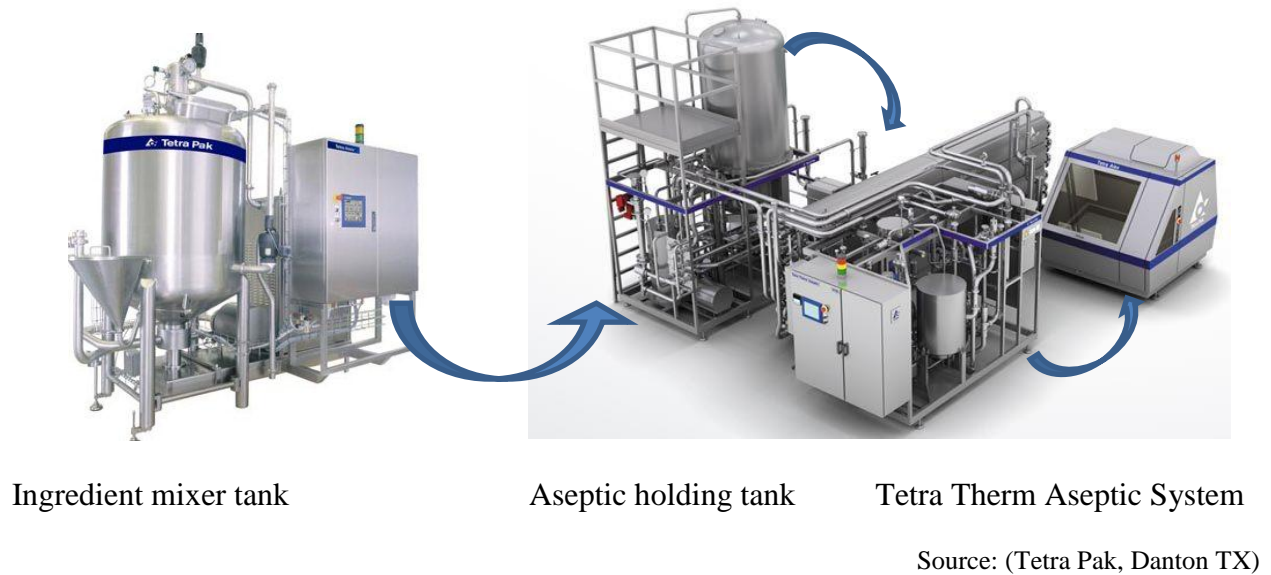


Figure 9. UHT equipment for Tetra Pak milk samples processing

3. Study design

The study was a randomized, double-blind trial. It was conducted in accordance with good clinical practice guidelines in compliance of the declaration of Helsinki and approval of the Institutional Review Board of the University of Illinois at Urbana- Champaign (IRB #09454). Eighty one volunteers who met the inclusion criteria were randomly distributed into three groups to consume 500 mL per day of low glycinin (high β -conglycinin) soymilk (LGS), conventional soymilk (S) or bovine milk (M).

The originally enrolled participants (n = 81), were staggered from June to December 2009 and 64 subjects completed the study. The study comprised one week of washout period in which subjects were requested to avoid the consumption of any food product containing soy; a complete list of food products to avoid was provided. Consumption of any other fluid milk intake

was prohibited. The washout period was followed by three months of soymilk or bovine's milk consumption according to the experimental protocol.

The test products were provided to participants to take home or at work in 250 mL Tetra Pak containers that looked exactly the same, with instructions to consume two portions (500 mL) per day. Compliance of product consumption was determined monthly by counting the number of clipped cardboard codes returned monthly to investigators and by a signed declaration of the number of beverage packages consumed.

4. Body composition and anthropometrics

4.1 Anthropometric measurements

Body weight, height, waist and hip circumferences were measured in triplicate at baseline, 1, 2 and 3 months. Body weight was measured without shoes and wearing medical clothes using an electronic scale (Tanita, model BWB-627A), height was measured with a regular stadiometer and waist and hip circumference on a horizontal plane with a Nontretch anthropometric tape. The waist circumference was measured at a level midway between the lowest rib and the iliac crest and the hip circumference at the level of the great trochanter. All anthropometrics were measured by the same person three times during each meeting.

4.2. Body fat and lean composition

Subjects changed into medical clothing or wore light-weight clothing and removed all jewelry and other clothing except underwear. Dual Energy X-ray Absorptiometry (DXA) scan of total body fat, left arm, right arm, left leg, right leg and trunk fat and lean composition were measured at baseline and after three months of milk consumption. All DXA scans were performed by an Illinois state licensed X-ray technologist and analyzed by the same investigator trained in scan analysis by Hologic. Total body scan time lasted approximately 5 min. Scan

acquisition of total body was performed with a single beam whole-body scanner (Illinois: Holistic 4500A, software version 11.1.3). Individual scans were analyzed by the same person according to standard manufacturer's procedure. The suprailiac trunk region (trunk percent fat) was defined superiorly as one-third the distance from the superior aspect of the iliac crest to the knee joint, from the superior iliac crests and inferiorly as a horizontal line at the level of the superior iliac crest (Figure 10). Each scan was reviewed for quality control by one of the investigators and was judged technically satisfactory if the external calibration step phantom and the skeletal outline of the subject lay within the scan region and without significant movement artifact. Any subject with more of 300 pounds of weight was recruited following the DXA scan restriction.



Figure 10. DXA scan zones

4.3. Bone mineral density

Bone mineral density (BMD) was also measured with a Dual Energy X-ray Absorptiometer (DXA) scans (Illinois: Holistic 4500A, software version 11.1.3) at baseline and at the end of the study. The technique is based on the relative tissue absorbance of X-radiation at two energy

levels and is very stable, and DXA estimated bone mineral content (BMC) and bone mineral density (BMD). This estimate is made by comparing the energy levels passed through the men with the energy levels passed through calibrated samples of bone, soft tissue and air. Quality control for DXA measures was verified by scanning a Hologic calibration phantom of known mineral content before testing. Using the tissue calibration bar, energy levels were determined for equivalent lean and fat tissues. With this calibration, the fat mass, lean mass and percent fat were estimated. Individual scans were analyzed by the same person according to standard manufacturer's procedures.

Precision for DXA bone mineral density measures is 1-1.5% CV% calculated from duplicate scans of young adults. BMD changes were measured as an increase or decrease in g/cm^2 . Comparison of BMD T-scores after three months was also conducted. The BMD-T score was calculated from the BMD of the patient (BMD_p) and the mean of a healthy adult (BMD_μ) and standard deviation (BMD_σ) of healthy young adult of the same age and ethnicity using the following formula: $\text{BMD-T-scores} = (\text{BMD}_p) - (\text{BMD}_\mu) / (\text{BMD}_\sigma)$.

5. Dietary and physical activity assessment

5.1. Five-day diet record

A five-day dietary record was used to assess monthly nutrient intake during the period of the study. Participants were trained on portion size and on proper management of personal dietary records. Serving sizes were reported according to USDA guidelines for the US population (USDA, 1996). Consumption data were filled out at home by the subjects and received monthly upon an interview with investigators. Dietary records were analyzed with the Food Processor SQL, Nutrition Analysis and Fitness Software (version 10.5.0, ESHA Research) and were revised by a second investigator.

5.2. Seven-day physical activity recall (SDPAR)

A seven-day physical activity recall (SDPAR) was conducted by trained interviewers every month. Subjects were asked to list the frequency, length and intensity of physical activity seven days prior to the interview. Data collected provided number of daily hours of physical activity with moderate, mild and strenuous intensity as well as the sleep hours. Moderate physical activity included fast walking, tennis, easy bicycling, volleyball, softball, easy swimming, dancing. Mild physical activity included yoga, easy walking, and bowling. Strenuous physical activity included running, jogging, hockey, football, soccer, basketball, karate, vigorous swimming, long distance bicycling. Changes in hours per day of mild-moderate and strenuous physical activity were analyzed throughout the study.

6. Lipid profile, adipokines, oxidative stress, inflammation and assessment

6.1. Blood samples collection

Fasting blood specimens (12 h) were obtained by venipuncture of the anti-cubital vein at baseline, 1, 2 and 3 months of milk consumption. Samples for blood plasma were collected in BD vacutainers-K₂ EDTA and for serum in BD Vacutainers SST™ (BD Diagnostics, Franklin Lakes, NJ). After centrifugation at 3000 g for 15 min at 4 °C, the resulting plasma and serum were aliquoted into cryovials and stored at -80 °C until analyses.

6.2. Serum lipids and fatty acid synthase (FAS)

Serum lipids were measured in serum samples at baseline and 3 months and the relative differences were calculated. Serum total cholesterol was measured with a fluorometric assay from Cayman Chemical (Ann Arbor, MI). This assay is based on an enzyme-coupled reaction that detect both free cholesterol and cholesteryl ester which are hydrolyzed by cholesterol esterase into cholesterol, which is then hydrolyzed by cholesterol oxidase to yield hydrogen

peroxidase and ketone products. Hydrogen peroxidase is detected using ADHP (10-acetyl-3,7-dihydroxyphenoxazinw). Fluorescence was read with 565-580 nm of excitation and 585-595 nm of emission wavelength. Cholesterol concentrations were documented as μM of cholesterol using the formula $\text{Cholesterol } (\mu\text{M}) = [(\text{sample adjusted fluorescence} - \text{y-intercept})/\text{slope}] * \text{sample dilution} * 0.001$. The assay had an intra-assay and inter-assay coefficient of variation of 6.4% and 3.4% respectively.

Serum triglycerides were measured with a Triglyceride assay kit from Cayman Chemical (Ann Arbor, MI). This tool was sensitive to detect serum or plasma triglycerides and uses an enzymatic hydrolysis of the triglycerides by lipase to glycerol and free fatty acids. The glycerol released is subsequently measured by a coupled enzymatic reaction. The glycerol formed is phosphorylated to glycerol-3-phosphate in a reaction catalyzed by glycerol kinase. Then the glycerol-3-phosphate is oxidized by glycerol phosphate oxidase producing dihydroxyacetone phosphate and hydrogen peroxide. Subsequent reaction produce a brilliant purple detected and quantified by spectrophotometry at 550 nm. Triglycerides concentrations were calculated as $\text{mg/dL of human serum} = [(\text{corrected absorbance} - \text{y-intercept})/\text{slope}]$. The assay had an intra-assay and inter-assay coefficient of variation of 1.34% and 3.17% respectively.

Plasma fatty acid synthase (FAS) was analyzed using a FAS-detectionTM human ELISA from FASgenTM (Baltimore MD) following the standard manufacturer procedures. Briefly, this double sandwich ELISA assay is used to detect soluble FAS in human serum. FAS antigen and Biotinylated FAS detection antibody were used. Horseradish peroxidase-labeled Streptavidin was used for detection and a FAS standard curved for quantification (Figure 11). Two different quality control standards were used and the failure of one of them produced the repetition of the entire plate. Results were obtained in ng/mL of serum and absorbance was read at 450 nm

Plates were read in a multimode microplate reader Biotek Synergy 2 and the concentration calculations with the software Biotek Gen5™ (Winooski, VT).

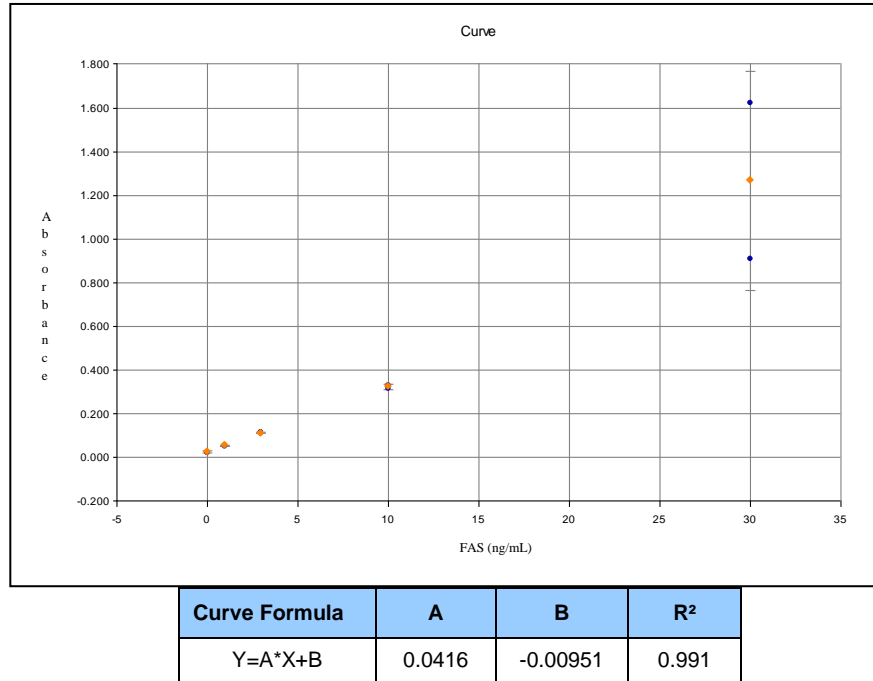
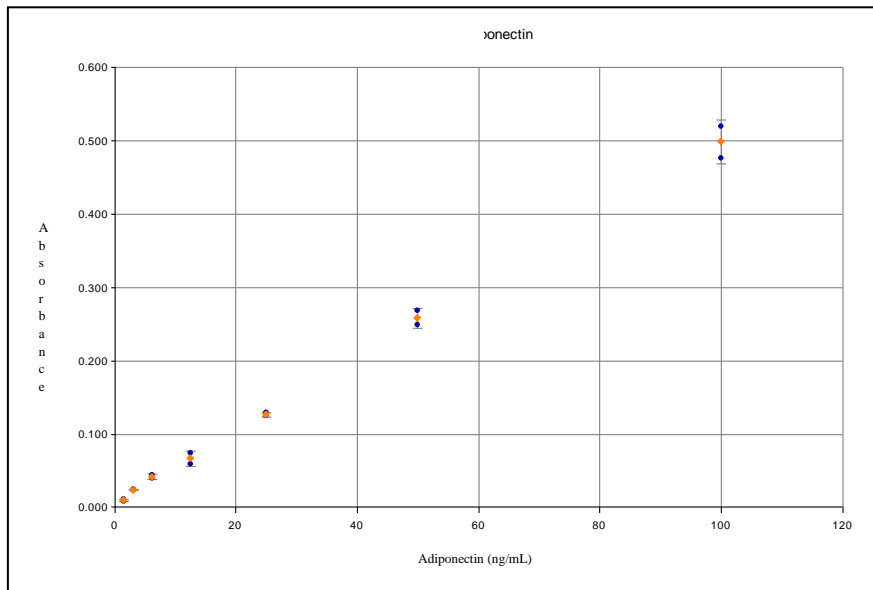


Figure 11. Example of fatty acid synthase (FAS) standard curve

6.3. Plasma adiponectin and leptin

Plasma adiponectin and leptin were determined by a human adiponectin enzyme-linked immunosorbent assay (ELISA) and human leptin ELISA, both from Millipore (St Charles, MO). Plates were read in a multimode microplate reader Biotek Synergy 2 and the calculations of concentrations were conducted with the software Biotek Gen5™ (Winooski, VT). Adiponectin sandwich ELISA used a monoclonal anti-human adiponectin antibodies and a secondary biotinylated monoclonal anti-human antibody. Quantification of the immobilized antibody-enzyme conjugates was done by measurement of horseradish peroxidase activity under the presence of 3,3',5,5'-tetramethylbenzidine. Adiponectin absorbance was read at 450 nm and 590

nm and compared against a standard curve (Figure 12). The assay had an intra-assay and inter-assay coefficient of variation of 1.8% and 6.2% respectively. Leptin assay was performed under the same principle but using a polyclonal rabbit anti-human antibody and secondary biotinylated monoclonal antibody. Leptin absorbance was read at 450 nm and 590 nm and presented an intra-assay and inter-assay coefficient of variation of 1.9% and 1.3% respectively.



Curve Formula	A	B	C	D	E	R ²
$Y = (A-D)/(1+(X/C)^B)^E + D$	0.0118	1.18	1.54E+04	1.16	211	1

Figure 12. Example of plasma adiponectin standard curves

6.4. Serum oxidized-LDL

Serum oxidized-LDL (ox-LDL) was measured using enzyme-linked immunosorbent assay ELISA from Cayman Chemical (Ann Arbor, MI). To determine the levels of ox-LDL a double-antibody sandwich technique was used. The 96 microwell plate was coated with human ox-LDL-β2GPI antibody. The bound β2GP was detected using horseradish peroxidase (HRP)-

labeled monoclonal antibody. The concentration of ox-LDL-β2GPI was determined measuring the enzymatic activity of the HRP using tetramethylbenzidine (TMB). The concentration of plasma oxi-LDL was determined by spectrophotometry (450 nm) equivalent to the amount of bound conjugate compared against the standard curve (Figure 13). The assay had an intra-assay and inter-assay coefficient of variation of 1.34% and 3.17% respectively.

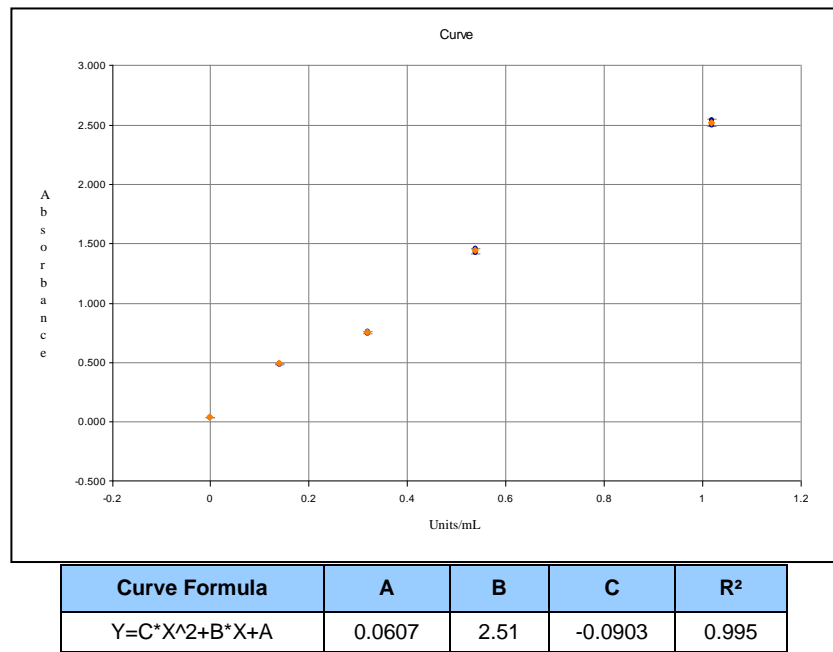


Figure 13. Example of oxidized-LDL standard curve

6.5. Serum antioxidant capacity

Serum anti-oxidant capacity biomarkers of inflammation and oxidative stress were measured only at baseline and at the end of the study. Serum antioxidant capacity was determined using the Oxygen Radical Absorbance Capacity (ORAC) procedure described by Prior *et al.* (2003). Briefly, aliquots of 20 μL sample, Trolox standard dissolved in 75 mM phosphate buffer pH 7.4 or 75 mM phosphate buffer pH 7.4 blank were added to a 96-well black walled plate. This was followed by the addition of 120 μL 17 nM fluorescein. The plate was

then incubated for 15 min at 37 °C and then 60 µL of 153 mM AAPH were added. The plates were read in a fluorescent plate reader, FLx800tbi (Bio-Tek, Winooski, VT), at 37 °C, sensitivity 60, read every 2 min for 120 min with excitation 485 and emission 582 nm. Results were expressed as mmol Trolox equivalents as compared with the standard curve (Figure 14). Plates were read in a multimode microplate reader Biotek Synergy 2 and the calculations of concentrations were conducted with the software Biotek Gen5™ (Winooski, VT).

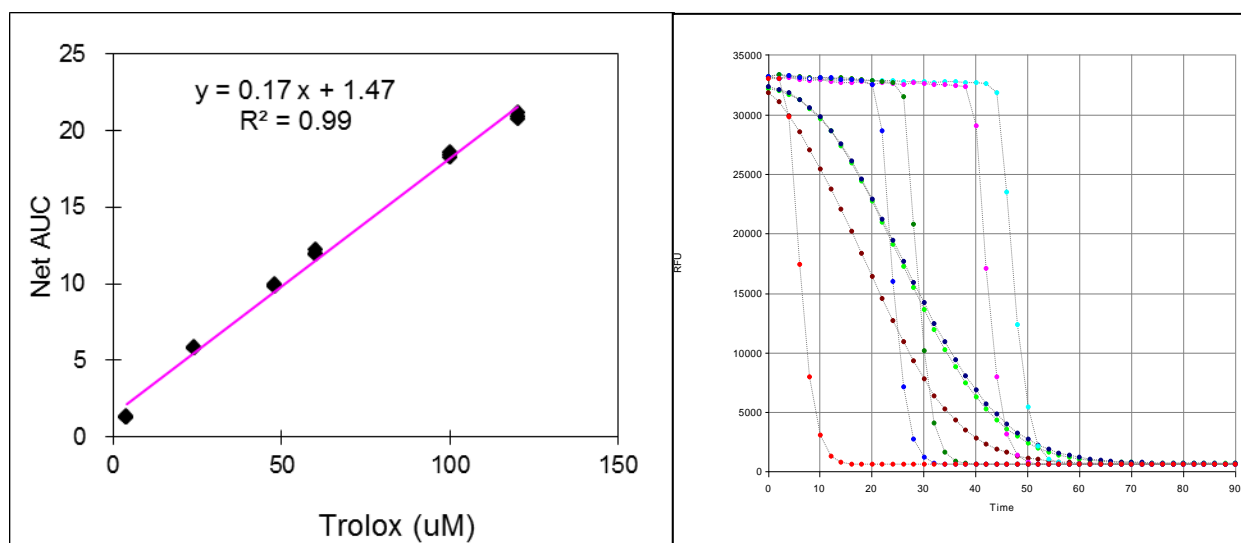


Figure 14. Example of Trolox equivalents standard curve

6.6. Plasma interleukin-6, C-reactive protein and tumor necrosis factor alpha

Plasma interleukin-6 (IL-6) was measured with an interleukin-6 (human) immunometric assay EIAs and changes in plasma tumor necrosis factor alpha (TNF α) were determined by an ACE™ immunometric assay EIAs, both from Cayman Chemical (Ann Arbor, MI). The immunometric assay is based in a double-antibody sandwich technique. This techniques uses an acetylcholinesterase:fab conjugate (AChE) to bind IL-6 molecules. The sandwich of antibodies were immobilized with Ellman's reagent and washed to determine the concentration by

measuring the enzymatic activity of AChE. The concentration was determined by spectrophotometry by comparison of the bound conjugates against a standard curve (Figure 15).

Interleukin-6 EIA assay had an intra-assay and inter-assay coefficient of variation of 4.6% and 15.6% respectively and the absorbance was read at 405 nm. EIA permits Il-6 measurements within the range of 0-250 pg/mL. Changes in plasma C-reactive protein (CRP) was also determined by a C-reactive protein (human) ELISA technique from Cayman Chemical (Ann Arbor, MI) following the double sandwich ELISA principle. The assay had an intra-assay coefficient of variation of 3.0% and the absorbance was read 450 nm. Plates were also read in a multimode microplate reader Biotek Synergy 2 and the calculations of concentrations were conducted with the software Biotek Gen5™ (Winooski, VT).

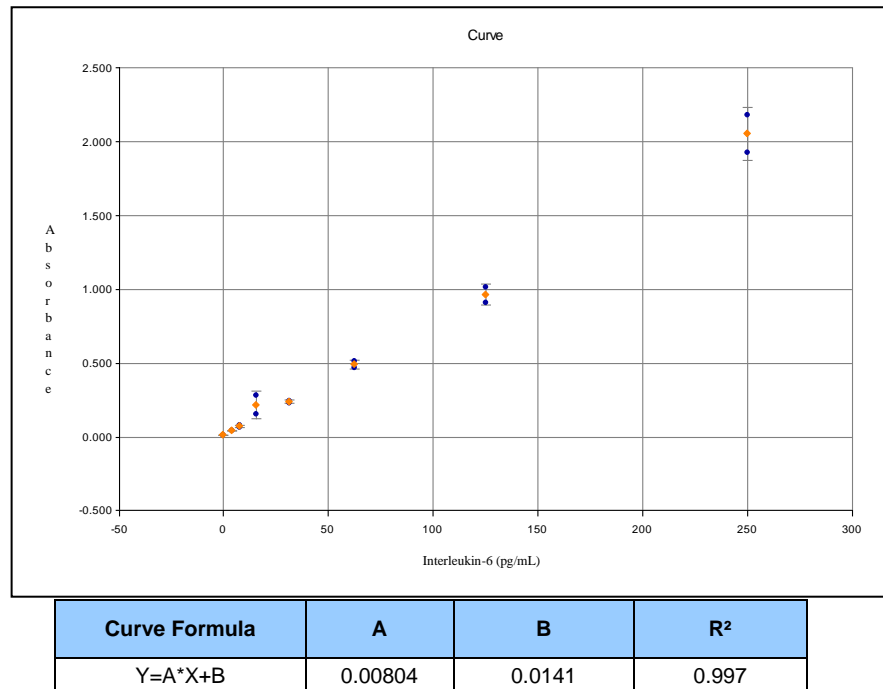


Figure 15. Example of interleukin-6 standard curve

7. Fecal microbiota composition quantification

7.1. Fecal material preparation

Fecal material collection was deposited by the participants in a commode specimen collection system (Fisher Scientific, Hanover Park, IL) and placed in a Styrofoam box and kept on ice by participant. Three fecal samples were collected from three different drop-offs and frozen within two hours of defecation at baseline and after 3 months (Figure 16). Four total samples were collected from each separate drop-off into tubes (3-200 mg and 1 -1 g tube; Sarstedt 80.734.001 and 80.623.022 respectively). DNA was isolated from 200 mg of colonic contents and purified by QIAmpDNA Stool Kits (Qiagen, Germantown, MD), in combination with glass beads beating. The primers used for qPCR are listed in Table 3.

7.2. General quantification of microbiota using q-PCR

PCR was performed in Applied Biosystems 7900HT Fast Real-Time PCR 1 and qPCR with reactionµSystem (Applied Biosystems). A master mix containing 5 µl of 2X Power SYBR Green PCR Master mix (Applied Biosystems), bovine serum albumin with a final concentration of 1 µg BSA /µL (AµEngland Bio Labs, Ipswich, MA), 0.5 µM of each primer and 2 µL of 1:100 diluted DNA sample was prepared. The PCR conditions were 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. A dissociation step was included to analyze the melting profile of the amplified products.

Standard curves (10^1 to 10^6 16S rRNA gene copies per reaction) were generated using purified 2.1TOPO-TA plasmids (Invitrogen, CA) containing the 16S rRNA gene of *Lactobacillus rhamnosus* (used for *Lactobacillus* and universal quantitation), *Bifidobacterium longum*, and *Bacteroides fragilis*. Results are presented as number of 16S rRNA gene copies per g colon contents (dry basis). The accuracy of the methodology used for this kind of studies have

been subject of criticism, however q-PCR have been defined as a powerful technique to study the diverse and complex human fecal microbiota matrix (Mariat *et al.*, 2009).

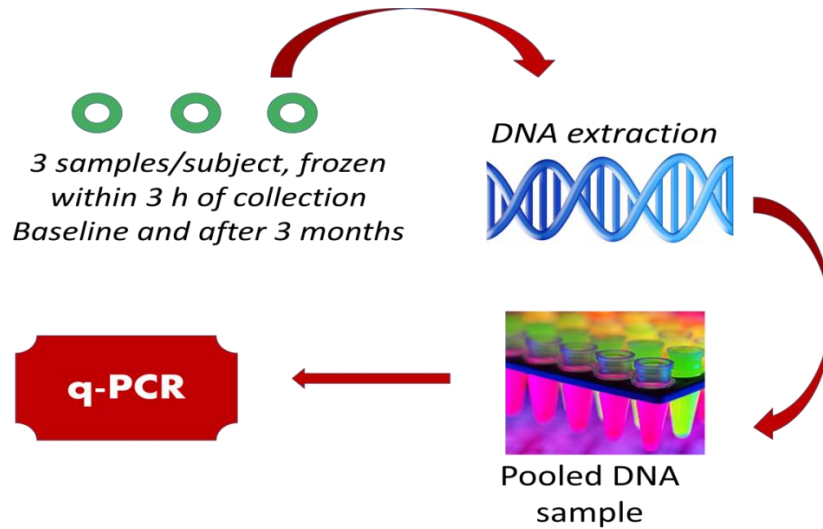


Figure 16. General microbiota analysis diagram

Table 3. Primers used for quantification of Total Bacteria, *Bacteroides*, *Bifidobacterium* and *Lactobacillus*

Target group	Primer	Sequence (5'-3')
Total bacteria	Uni331F	TCCTACGGGAGGCAGCAGT
	Uin797R	GGACTACCAGGGTATCTATCCTGTT
<i>Bacteroides</i>	AllBac296F	GAG AGG AAG GTC CCC CAC
	AllBac2112R	CGC TAC TTG GCT GGT TCA G
<i>Bifidobacterium</i>	Bif164F	GGGTGGTAATGCCGGATG
	Bif662R	CCACCGTTACACCGGGAA
<i>Lactobacillus</i>	LacF	AGCAGTAGGGAATCTTCCA
	LacR	CACCGCTACACATGGAG

8. Genera identification by pyrosequencing

8.1. DNA extraction

Fecal samples were homogenized and 200 mg aseptically suspended in 500 µl RLT buffer (Qiagen, Valencia, CA) (with β- mercaptoethanol). A sterile 5 mm steel bead (Qiagen, Valencia, CA) and 500 µl volume of sterile 0.1 mm glass beads (Scientific Industries, Inc., NY, USA) were added for complete bacterial lyses in a Qiagen TissueLyser (Qiagen, Valencia, CA), run at 30 Hz for 5 min. Samples were centrifuged and 100 µl of 100% ethanol added to a 100 µl aliquot of the sample supernatant. This mixture was added to a DNA spin column, and DNA recovery protocols were followed as instructed in the Qiagen DNA Stool Kit (Qiagen, Valencia, CA) starting at step 5 of the Protocol. DNA was eluted from the column with 50 µl water and samples were diluted accordingly to a final nominal concentration of 20 ng/µl. DNA samples were quantified using a Nanodrop spectrophotometer (Nyxor Biotech, Paris, France).

8.2. Massively parallel bacterial tag encoded EFL amplicon pyrosequencing (bTEFAP)

Bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) was performed using Gray28F 5' TTTGATCNTGGCTCAG and Gray519r 5' GTNTTACNGCGGCKGCTG as described in previous studies (Dowd *et al.*, 2008; Callaway *et al.*, 2009; Wolcott *et al.* 2009; Smith *et al.*, 2010). Initial generation of the sequencing library used a one-step PCR with a total of 30 cycles, a mixture of Hot Start and HotStar high fidelity taq polymerases, and amplicons originating and extending from the 28F for bacterial diversity. Tag-encoded FLX amplicon pyrosequencing analyses utilized Roche 454 FLX instrument with Titanium reagents, titanium procedures performed at the Research and Testing Laboratory (Lubbock, TX) based upon RTL protocols (Down *et al.*, 2008).

8.3. Bacterial diversity

Following sequencing, all failed sequence reads, low quality sequence ends and tags and primers were removed and sequences collections depleted of any non-bacterial ribosome sequences and chimeras using B2C2 (Gontcharova 2010) as has been described previously (Wolcott *et al.* 2009; Smith *et al.*, 2010; Ishak *et al.*, 2011; Dowd *et al.*, 2008). To determine the identity of bacteria in the remaining sequences, sequences were denoised, assembled into clusters and queried using a distributed BLASTn .NET algorithm (Dowd *et al.*, 2005) against a database of high quality 16S bacterial sequences derived from NCBI (01-01-11 or current version). Database sequences were characterized as high quality based upon similar criteria utilized by RDP ver 9 (Cole *et al.* 2009). Using a .NET and C# analysis pipeline the resulting BLASTn outputs were compiled and validated using taxonomic distance methods, and data reduction analysis were performed as described in the standard method (Dowd *et al.* 2005; Gontcharova *et al.*, 2010; Handl *et al.*, 2011; Guerrero *et al.*, 2009; Sen *et al.*, 2009).

8.4 Bacterial identification

Based upon the above BLASTn derived sequence identity (percent of total length query sequence which aligns with a given database sequence) and using taxonomic distance methods the bacteria were classified at the appropriate taxonomic levels based upon the following criteria. Sequences with identity scores, to known or well characterized 16S sequences, greater than 97% identity (<3% divergence) were resolved at the species level, between 95% and 97% at the genus level, between 90% and 95% at the family and between 85% and 90% at the order level, 80 and 85% at the class and 77% to 80% at phyla. After resolving based upon these parameters, the percentage of each bacterial and Fungal ID was individually analyzed for each sample providing relative abundance information within and among the individual samples based upon relative

numbers of reads within each. Evaluations presented at each taxonomic level, including percentage compilations represent all sequences resolved to their primary identification or their closest relative (Stephenson *et al.*, 2010; Dowd *et al.*, 2008; Andreotti *et al.*, 2011).

9. Glycemic response analysis

9.1. Subjects and study design

Subjects from previous study IRB#09454 were invited to participate in a glucose response analysis under IRB amended on November 10, 2010. Participants were healthy overweight men, age 18-45 years with body mass index (BMI) (25-44), non-vegetarian, non-athletes, and non-smokers, recruited voluntarily from the campus of the University of Illinois, Urbana-Champaign. Twenty four subjects were recruited with a mean age of 31 (23-45) years with a mean BMI of 29 (26-38) and with normal serum triglycerides levels (<200 mg/dL). Subjects were asked to watch a video with the procedures to be performed in the study and sign a new informed consent. From the 24 subjects enrolled, 19 subjects finished the entire study and were considered for the analysis. The main reasons for withdrawal were inconveniences on work schedules and weather conditions. Participants were asked to participate in three repetitions of the glycaemic curve assessments on three different days (at least one week apart). Subjects were asked to fast for 12 h prior each meeting and each subject consumed a different treatment per repetition (low glycinin soymilk (LGS), conventional soymilk (S) or bovine milk (M)). They also participated in a BMI evaluation, one day diet record and a demographic interview in every repetition.

9.2. Glucose curve assessment

Subjects received instructions of how to use the glucometer (One Touch Ultra) and were asked to wash and dry their hand before each prick. Subjects consumed within 10-15 min 250

mL of milk containing same amount of carbohydrates, fat, energy and protein and were compared with the bovine milk response Table 4. Capillary finger-stick blood samples (1 μ L/sample) were taken in the fasting state (0 min) and 15, 30, 45, 60, 90, and 120 min after the start of the consumption of milk. Glucose levels were measured using a calibrated *One Touch Ultra*[®] glucometer. According to Solnica *et al.*, (2003) One Touch glucometers have acceptable performance and standard deviation from laboratory analysis <10%. The positive area under the curve (AUC) changes in blood glucose was computed by the trapezoidal method. The AUC was calculated for all variables measured by using the trapezoidal method (GRAPHPAD PRISM, version 4.00 (GraphPad Software, San Diego CA). Incremental AUC (iAUC) was considered as the AUC above baseline.

The glucose curve shapes were classified following the definition of Tschritter *et al.* (2003). When plasma glucose increases to the maximum after 30-90 min and decreased until 120 min is a “monophasic” plasma glucose curve. If glucose shapes reaches a nadir after an initial increase again > 0.25 mmol/L (4.5 mg/dL) until 120 min is classify as “biphasic” plasma glucose curve. If three glucose peaks are observed (>0.25 mmol/L minimum increment) was considered “triphasic”.

Table 4. Nutrient composition of 250 mL of treatments

Content in 250 mL	S	LGS	M
Calories (Cal)	105 \pm 0.5	103 \pm 0	93 \pm 0.1
Fat (g)	3.5 \pm 0.2	3.5 \pm 0	3.6 \pm 0
Carbohydrates (g)	11 \pm 0.5	11 \pm 0.6	8 \pm 0
Dietary Fiber (%)	<1	<1	<1
Protein (g)	7 \pm 0.2	7 \pm 0.1	7 \pm 0.1
Calcium (mg)	330 \pm 3	350 \pm 2.5	223 \pm 2

*Data are Means \pm STD.

10. Statistical analysis

Statistical analyses were conducted using SAS version 9.2. (SAS institute, Cary, NC). Relative differences, as percentage changes from baseline, were calculated for body fat, body lean mass, total cholesterol, serum triglycerides, serum ox-LDL, CRP, plasma interleukin-6, serum antioxidant capacity, plasma leptin, and plasma adiponectin. Calculation of relative differences was made by means of the following equation:

$$\text{Relative difference (\%)} = [(\text{month 3-baseline}) * 100] / \text{baseline}]$$

Data normality was analyzed with box plots, stem leaf diagrams, residual plots and Shapiro-Wilks tests. Not-normally distributed data were transformed to meet the assumption of normality for further analysis. Subject characteristics at baseline, energy intake and physical activity were analyzed by one-way ANOVA test. Changes in body fat accumulation, body lean mass, and biochemical parameters were analyzed with a mixed procedure with nested random effect model considering the influence of age, initial BMI and physical activity. Treatment effects per variable were compared using SAS contrast statements. Weight changes and nutrient intake were analyzed by a general linear model (GLM) using analysis of variance for repeated measurements.

Mixed model with random effect (subjects) was also used for results of BMD, BMD T-scores and q-PCR microbiota composition and pyrosequencing results. Influence of age, initial BMI, kilocalorie intake and physical activity were considered for bone density analyses. Influence of initial BMI, changes in body fat, plasma adiponectin, oxidized LDL, physical activity, interleukin-6 and antioxidant capacity were considered for intestinal microbiota

analyses. Treatment effects per variable were compared using SAS contrast statements. T-tests were performed to determine the changes from baseline within treatments in microbiota composition data. All statistics are presented as means \pm SEM and two sided *P*-values < 0.05 were considered significant.

Glycemic index analyses were analyzed with Multivariate analysis of variance (MANOVA) considering the effect of subjects, treatments, initial fasting glucose, type of curve, BMI, age, serum triglycerides and dietary intake of energy, protein, carbohydrates and fat. Glycemic response per treatment throughout the time was analyzed using analysis of variance for repeated measurements. All statistics are presented as means \pm SEM and two sided *P*-values < 0.05 were considered significant. Power analyses for the entire study were performed with the program SPSS version 15.0 for Windows.

VI. RESULTS

1. Chemical composition of soymilk and bovine milk treatments

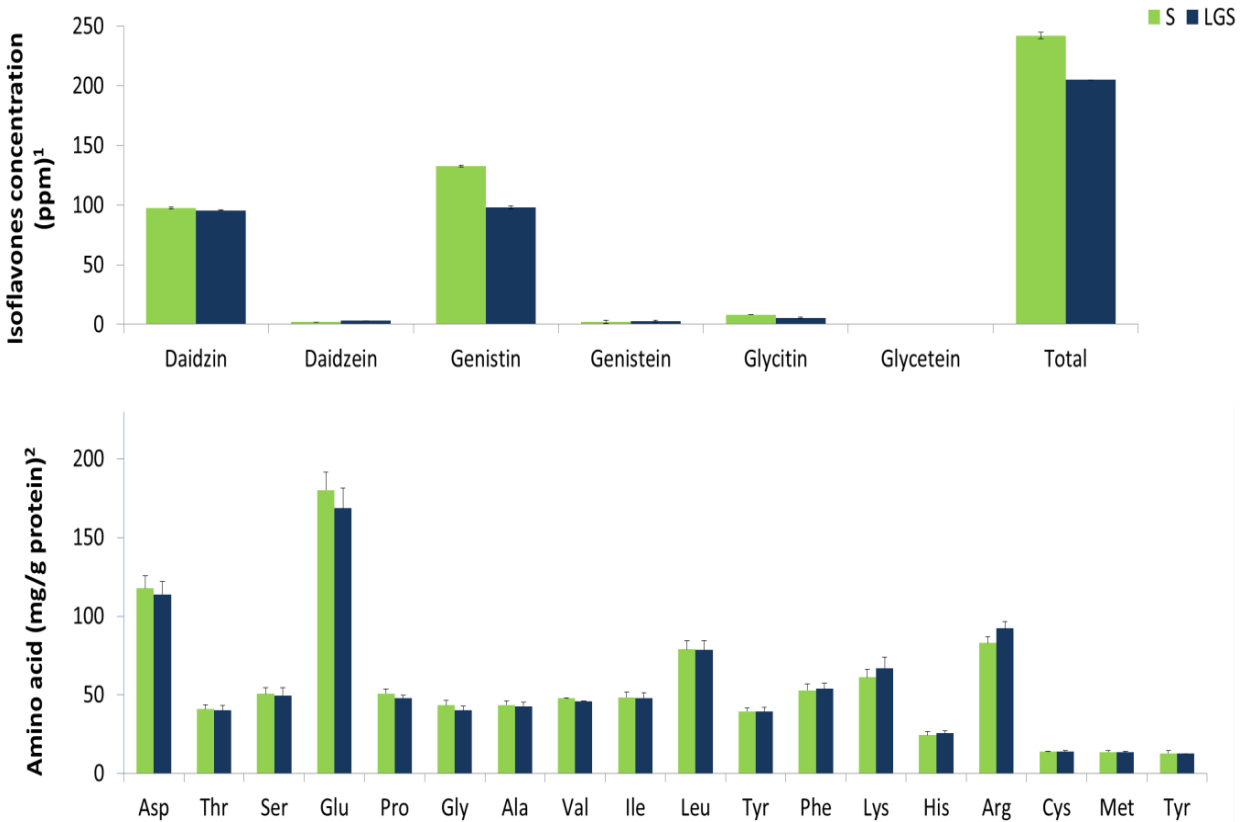
Chemical analyses of the test products revealed no significant differences in total energy, protein (14 g) and fat (7g) among soymilks and bovine's milk (Table 5), but different concentration of β -conglycinin (LGS, 49.5%; S, 26.5%; and M, 0% of total protein) and glycinin (LGS, 6.0%; S, 38.7 %; and M, 0% of total protein) as measured by SDS-PAGE of the soymilk powders used to make the liquid products.

The total isoflavones content was similar in the two soymilk products ($P > 0.05$) (Figure 17), and as expected bovine's milk contained less than 1 ppm of isoflavones. In addition, the concentration of amino acids between the two soymilks (Figure 17) was not significantly different ($P > 0.05$), eliminating any influence of specific amino acids.

Table 5. Chemical composition of S, LGS and M

Content in 500 mL	S	LGS	M
Calories (Cal)	209 \pm 0.5	206 \pm 0	186 \pm 0.1
Fat (g)	7 \pm 0.2	7 \pm 0	7 \pm 0
Carbohydrates (g)	23 \pm 0.5	23 \pm 0.5	17 \pm 0.2
Dietary Fiber (%)	<1	<1	<1
Protein (g)	14 \pm 0.2	14 \pm 0.1	14 \pm 0.1
Calcium (mg)	660 \pm 3	699 \pm 2.5	446 \pm 2

*Data are Means \pm STD per 500 mL of milk.



A. Data are means \pm SD ($P > 0.05$). Total isoflavones concentration in bovine's milk was < 1 ppm. B. Data are means \pm SD, ($P > 0.05$). Differences in Thr, Ala, Gly, Ile, Leu, Tyr, Phe, Cys and Met between S and GLS were < 1 mg/g.

Figure 17. Isoflavones and amino acid concentrations of LGS and S.

2. Compliance

Subject compliance with consumption of test materials was high (95% of packages consumed per month on average) and there were no compliance differences among treatments. The caloric intake of the participants remained constant during the study. Energy consumption was estimated, on average, as 9908 kJ (2368 kcal) per day. Likewise, physical activity did not change during the period of study in any of the groups ($P = 0.90$). No adverse events were reported by the subjects. The attrition rate was mainly due to return of the subjects to their home towns upon the University's summer recess (Table 6).

Table 6. Withdrawal reasons IRB 09454

Withdrawal reasons	Subjects
Change of domicile	10
Dislike the treatment flavor	2
Other	1
Did not explained reasons	4

*Original recruitment: 81 – 17 drops = Final # subject = 64

3. Baseline characteristics

Participants characteristics at baseline did not differ significantly among groups (Table 7). Subjects were 32 ± 7 years, weighed 96.3 ± 15.2 kilograms, BMI of 29 ± 4 , $27.6 \pm 5\%$ body fat and 0.94 ± 0.06 waist to hip ratio. The homogeneity of the baseline status of the participants provides the basis to make a reliable comparison among treatments in the target parameters.

Table 7. Subject characteristics in the different treatment groups at baseline

Characteristic	Low glycinin soymilk (LGS) n = 19	Conventional soymilk (S) n = 23	Bovine's milk (M) n = 22
Age (years)	33 ± 6	32 ± 8	32 ± 6
Body weight (Kg)	95.8 ± 11.1	96.2 ± 17.3	97.0 ± 17.2
BMI	29 ± 3	29 ± 4	30 ± 4
Total Body Fat (%)	28 ± 3.5	27 ± 6.0	28 ± 5.6
Waist-Hip ratio	0.95 ± 0.05	0.94 ± 0.07	0.94 ± 0.06

*Data are means \pm SD ($P > 0.05$)

4. Anthropometrics and body composition

4.1 Anthropometrics

Changes from baseline in anthropometric parameters and body composition are presented in Table 8. LGS, S and M waist-hip ratio changes after three months were 0.33 ± 0.45 ; -0.16 ± 0.50 and 0.82 ± 0.35 respectively; however, these changes were not significant among treatment comparison ($P = 0.454$). Body mass index and weight changed in the same proportion, due to their direct relationship. The weight of the participants after LGS, S and M consumption were 0.71 ± 0.61 Kg, 0.29 ± 0.29 Kg and 1.30 ± 0.52 Kg respectively, however did not differ significantly among treatments ($P = 0.836$ for weigh and $P = 0.721$ for BMI).

4.2 Body fat and lean composition

After three month of consumption, total lean composition changes were not significant ($P = 0.201$) for LGS ($2.49 \pm 0.72\%$), S ($1.10 \pm 0.52\%$) and M ($1.60 \pm 0.47\%$) (Figure 18). On closer evaluation, lean changes in left arm, right arm, trunk, left leg and right leg were also non-significant for all treatments ($P > 0.05$) (Table 8). Relative total body fat accumulation was slightly less in LGS ($1.66\% \pm 1.40$) compared to S ($1.90\% \pm 0.67$) ($P = 0.015$), or M ($3.85\% \pm 0.68$) ($P = 0.011$) (Table 8). Tendencies to accumulate less fat in different parts of the body in subjects consuming LGS beverage, were not significantly different, except for less fat accumulation in the right leg of subjects in the LGS group (Figure 19). Although this difference was statistically significant, its biological meaning needs to be determined by further studies. However, these results support *in vitro* and *in vivo* observations on the effect of β -conglycinin on fat accumulation. Martinez-Villaluenga *et al.* (2008) observed that alcalase hydrolysates from 45% β -conglycinin soybean exhibited higher inhibition of lipid accumulation than hydrolysates from conventional soybean (25% β -conglycinin) and bovine milk in 3T3-L1 adipocytes (37.5%,

29.3% and 4%, respectively, $P < 0.0001$). Kohno *et al.* (2006) also concluded that consumption of β -conglycinin during 3 months reduced total body fat in pre-obese and obese subjects; however, no changes were observed in abdominal fat. This observation agrees with the results in our study in regards to trunk composition and waist-hip ratio. Furthermore, Moriyama *et al.* (2004) observed that β -conglycinin significantly reduced body weight in normal and obese male mice compared with casein. Additionally, Baba *et al.* (2004) determined that the daily consumption of 5 g of β -conglycinin maintained body fat ratio in healthy women. However, in our study there were not significant changes in body weight ($P > 0.05$).

Although changes in body weight were not significant, there was a tendency of maintaining a higher total lean body in the LGS group which could explain the lesser accumulation of body fat. Studies in the literature report that the inclusion of soy protein in the diet can have an impact on weight loss or reduction of body fat after three months (Allison *et al.*, 2003); produce a mild effect on body composition in post-menopausal women after six months (Liu *et al.*, 2010); significantly reduce weight and fat composition in diabetic patients after twelve months (Li *et al.*, 2005); and combined with low-fat diet, can improve body composition, preserving muscle mass in pre-obese and obese people after six months (Deibert *et al.*, 2004). In contrast, Anderson *et al.* (2005), Zemel *et al.* (2009) and St-Onge *et al.* (2007) did not find effect on weight reduction or body composition in obese people after consumption of soy protein.

The reduction of fat accumulation in the present study was modest and had no impact on body weight. This was probably due to the short duration of the study and that the intake of total soy protein per day was only 14 g/day (5.94 g/day of β -conglycinin in LGS, 3.18 g/day in S and 0 g/day in M).

Table 8. Percent change from baseline (% relative differences) at the end of the study in anthropometric parameters and body composition after consumption of LGS, S or M^{1,2}

Anthropometric parameters	LGS (%)	S (%)	M (%)	P-value
Waist-Hip ratio	0.33 ± 0.45 ^a	-0.16 ± 0.50 ^a	0.82 ± 0.35 ^a	0.454
Body Weight	0.71 ± 0.61 ^a	0.29 ± 0.46 ^a	1.30 ± 0.52 ^a	0.836
BMI	0.71 ± 0.61 ^a	0.29 ± 0.46 ^a	1.30 ± 0.52 ^a	0.721
Total body fat	1.66 ± 1.40 ^b	1.90 ± 0.67 ^a	3.85 ± 0.68 ^a	0.017
Left arm fat	5.37 ± 1.97 ^a	4.75 ± 1.88 ^a	5.58 ± 2.09 ^a	0.892
Right arm fat	-0.47 ± 1.93 ^a	4.34 ± 2.37 ^a	4.68 ± 1.58 ^a	0.672
Trunk fat	3.76 ± 2.17 ^a	3.86 ± 1.90 ^a	6.63 ± 1.95 ^a	0.740
Left leg fat	4.26 ± 1.43 ^a	3.43 ± 1.27 ^a	6.85 ± 1.24 ^a	0.124
Right leg fat	2.76 ± 1.62 ^{ab}	5.31 ± 1.71 ^{ac}	7.29 ± 1.52 ^c	0.023
Total body lean mass	2.49 ± 0.72 ^a	1.10 ± 0.52 ^a	1.60 ± 0.47 ^a	0.201
Left arm lean	0.37 ± 0.79 ^a	-2.10 ± 0.83 ^a	-0.88 ± 1.01 ^a	0.871
Right arm lean	0.76 ± 1.37 ^a	0.11 ± 1.21 ^a	1.56 ± 0.94 ^a	0.659
Trunk lean	0.75 ± 0.81 ^a	0.52 ± 0.87 ^a	-0.12 ± 0.98 ^a	0.135
Left leg lean	-0.87 ± 0.68 ^a	-1.61 ± 0.64 ^a	-1.17 ± 0.61 ^a	0.912
Right leg lean	-0.40 ± 0.66 ^a	-1.12 ± 0.58 ^a	-0.28 ± 0.74 ^a	0.899

¹ Values are Means ± SEM of relative differences from baseline, n = 64.

² Different letters in rows are statistically different ($P < 0.05$).

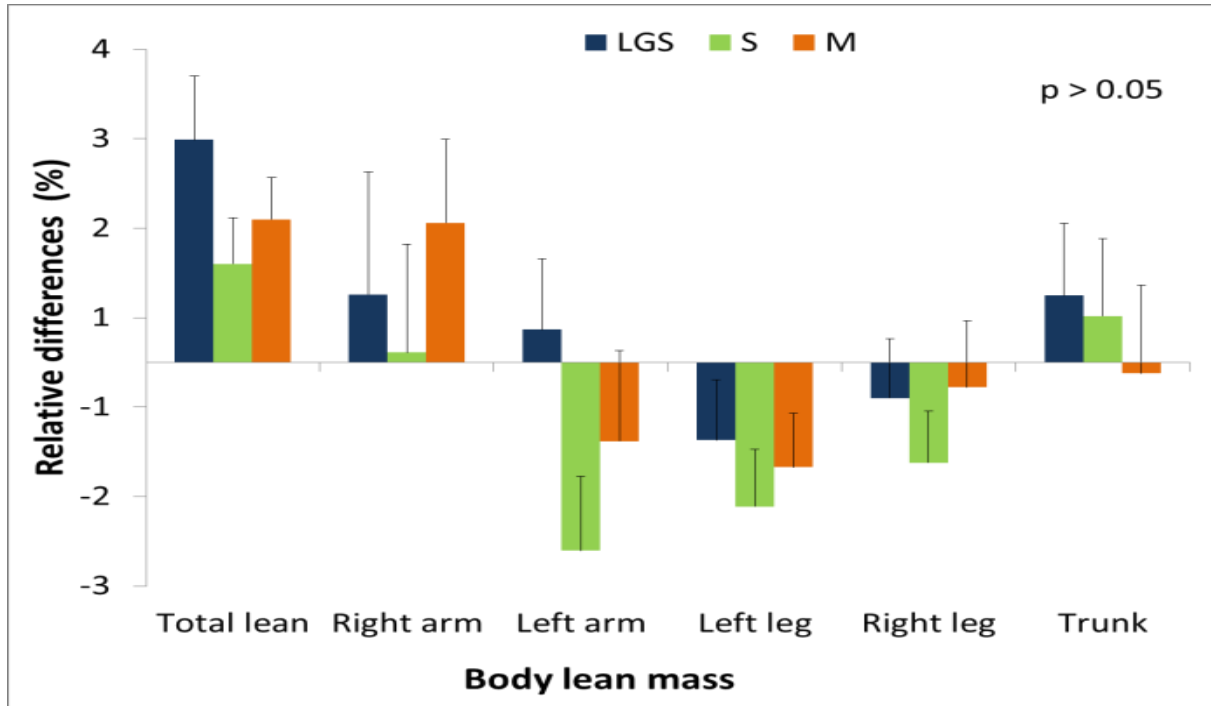


Figure 18. Body lean composition changes after three months of LGS, S and M consumption

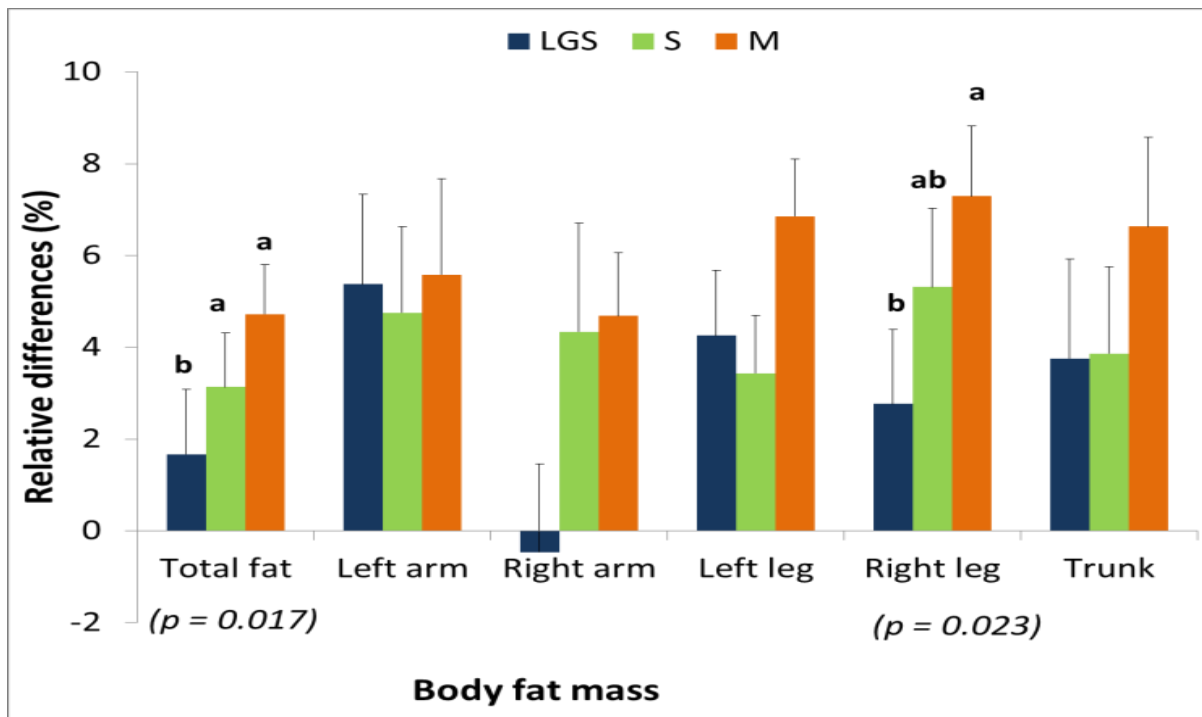


Figure 19. Body fat composition changes after three months of LGS, S and M consumption

4.3 Bone mineral density

The effect of protein beverages on BMD is presented in Table 9. Total BMD, left arm, right arm, left leg, left ribs, right ribs, thoracic-spine (T-spine), and lumbar-spine (L-spine) did not have significant changes in BMD after three months of consumption of LGS, S or M ($P>0.05$). However, in the right leg, we detected that the consumption of S produced a slight reduction in BMD ($-0.014 \pm 0.011 \text{ g/cm}^2$) as compared to LGS ($-0.042 \pm 0.010 \text{ g/cm}^2$) and M ($-0.019 \pm 0.009 \text{ g/cm}^2$), $P=0.003$. BMD in the pelvis showed a slight decrease with the consumption of M (-0.022 ± 0.020) compared with a slight increase in the LGS (0.013 ± 0.011) and S (0.001 ± 0.013) groups ($P=0.032$) (Figure 20). On average, total bone mineral density for the three treatments did not decrease more than 0.005 g/cm^2 which represents, a minimum biological relevant impact in the short term.

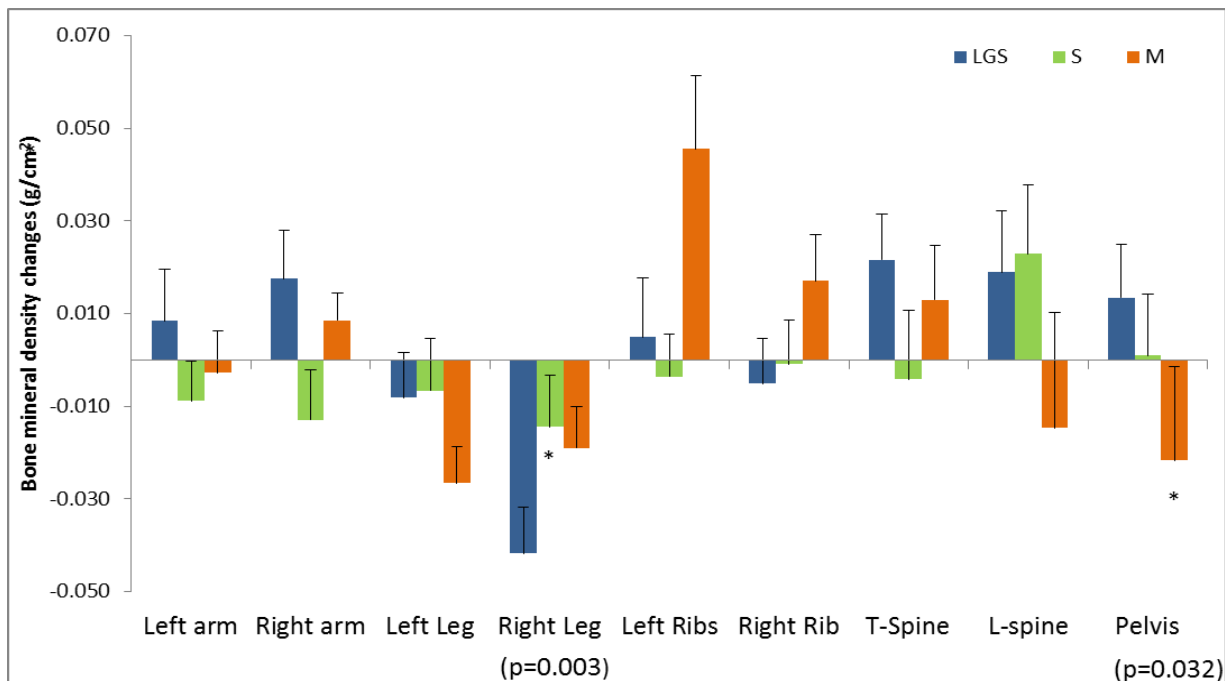


Figure 20. Bone mineral density changes after three months of LGS, S and M consumption

No subject in the present study had osteoporosis (BMD t-score <-2.5) and ten subjects presented osteopenia problems at baseline and after three months (BMD t-score -1.0 to -2.5) according to WHO (1994). The changes in BMD t-scores within each treatment were small but significant ($P<0.0001$), however, the relative difference from baseline among groups (Figure 21) were not significant ($P=0.094$). Figures 22.a, 22.b and 22.c show that most subjects had a healthy bone condition (BMD t-score > -1.0) and anyone changed from a healthy condition to osteopenia condition which allowed us to conclude that BMD t-scores were not affected either by the type of milk consumed. On average, the participants consuming LGS (-0.05 ± 0.02), S (-0.01 ± 0.04) and M (-0.02 ± 0.03) showed healthier BMD t-scores (Figure 22) compared with the study developed by Lee *et al.* (2010), where normal and pre-diabetic men had BMD t-scores of -1.34 ± 1.42 and -1.33 ± 1.30 respectively. More research is required with a longer term of consumption to better understand the effects of different soy protein profile on bone mineral density.

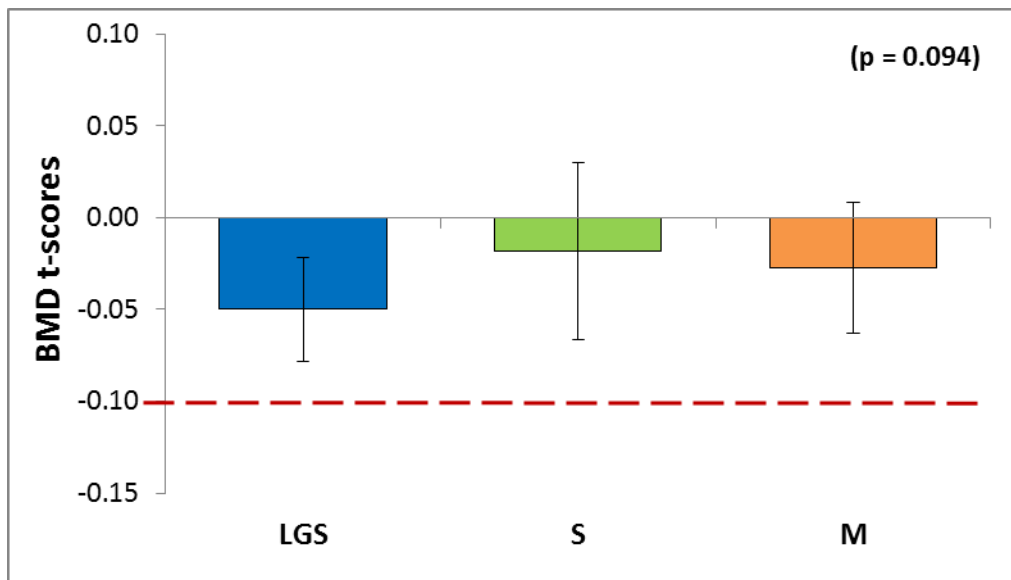
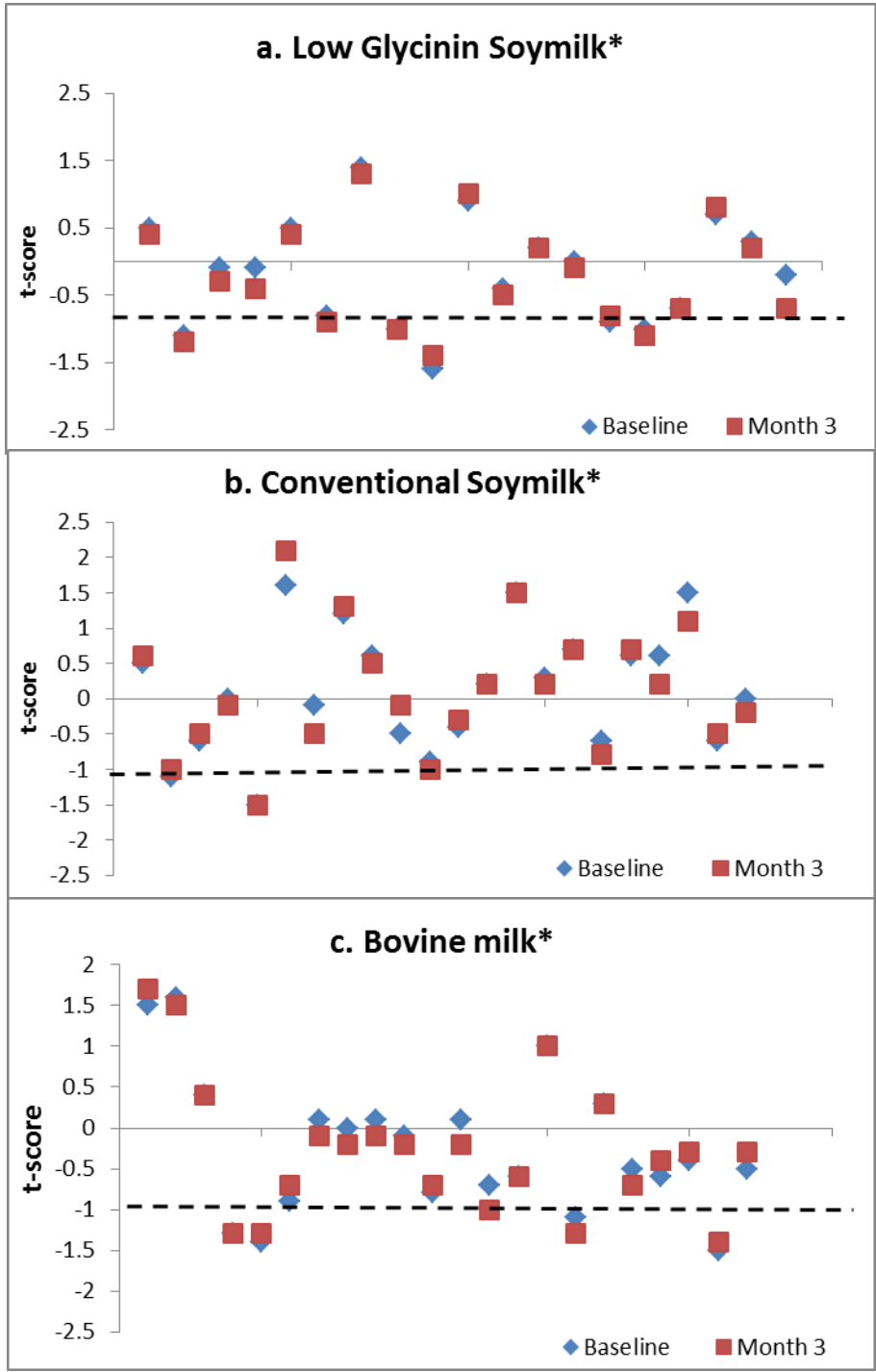


Figure 21. T-score changes after three months of LGS, S and M consumption



*Changes from baseline to month 3 are significant (p<0.0001)

Figure 22. T-scores at baseline and after three months of consumption of LGS, S and M.

Table 9. Bone mineral density changes after three months of consumption of LGS, S and M.

BMD	Low glycinin soymilk		Conventional soymilk		Bovine milk		Among treatments
	g/cm ²	SEM	g/cm ²	SEM	g/cm ²	SEM	P - value
Total BMD	-0.004	± 0.004	-0.005	± 0.006	-0.005	± 0.006	0.058
Left arm	0.008	± 0.011	-0.009	± 0.009	-0.003	± 0.009	0.170
Right arm	0.018	± 0.010	-0.013	± 0.011	0.009	± 0.006	0.506
Left Leg	-0.008	± 0.010	-0.007	± 0.011	-0.027	± 0.008	0.590
Right Leg*	-0.042 ^b	± 0.010	-0.014 ^a	± 0.011	-0.019 ^b	± 0.009	0.003
Left Ribs	0.005	± 0.013	-0.004	± 0.009	0.046	± 0.016	0.512
Right Rib	-0.005	± 0.010	-0.001	± 0.009	0.017	± 0.010	0.813
T-Spine	0.022	± 0.010	-0.004	± 0.015	0.013	± 0.012	0.332
L-spine	0.019	± 0.013	0.023	± 0.015	-0.015	± 0.025	0.278
Pelvis*	0.013 ^a	± 0.011	0.001 ^a	± 0.013	-0.022 ^b	± 0.020	0.032

*Different letters in rows are significantly different ($P < 0.05$).

The changes in BMD observed in the present study after the consumption of LGS for 3 months can be considered biologically insignificant. The decreases for all treatments were on average less than 0.005 g/cm². The fact that LGS and S contain similar isoflavones content but different protein profiles, agree with the theory that the beneficial effects of soy products in bone health may be due to the synergistic action of proteins profile and isoflavone content (Arjmandi *et al.*, 1998; Bawa, 2010; Potter *et al.*, 1998). In this study, the high level of β -conglycinin and

low level of glycinin in LGS did not produce a significant effect on bone change. Other studies support the protective effect of the isoflavone content independent from the soy protein profile in rats after 91 days (Picherit *et al.*, 2001), in mice (Droke *et al.*, 2007) and in post and premenopausal Western women (Ricci *et al.*, 2010). In concordance, Alekel *et al.* (2000) compared the bone effect of isoflavone-rich soy, isoflavone-poor soy and whey protein during 24 weeks in perimenopausal women concluding that only isoflavone-rich soy ameliorated bone loss from lumbar spine. Other studies simply conclude that the consumption of whole soy products containing isoflavones reduce the risk of osteoporosis (Scheiber *et al.*, 2001). On the other hand, Nagata *et al.*, (2002) did not find any association of soy or soy-isoflavones intake with bone mineral density. Moreover, Wangen *et al.* (2000) did not observe any clinically important bone turnover after diets supplemented with soy-isoflavone for three months in pre and postmenopausal women. The lack of biologically relevant changes in BMD in our subjects may be attenuated by their overweight condition because there is some evidence that obesity has a protective effect against bone loss (Cifuentes *et al.*, 2003; Wearing *et al.*, 2006; El Maghraoui *et al.*, 2010).

Other factors like age, initial BMI, kilocalorie intake and physical activity did not have an influence in BMD in most parts of the body; however, age had an effect in pelvis ($P=0.021$) and left rib ($P=0.006$). These BMD changes showed slightly higher reductions in subjects older than 35 years.

5. Diet Records

Compliance was good for each of the three groups (>94% for all treatments) and all subjects effectively provided information about their diet intake. Dietary intake of specific food products increases calcium absorption and bone mineral density (Roberfroid *et al.*, 2010) and

factors such, age, BMI (El Maghraoui *et al.*, 2010), physical activity, dietary vitamin D (Cooper *et al.*, 2009), dietary calcium, phosphorus (Varley *et al.*, 2010), among others are influencing bone health. Despite the conclusion of Noakes *et al.*, (2005) affirming that high protein diets reduce bone calcium absorption and increase urinary calcium excretion, Heaney and Layman (2008) concluded that higher protein diets have benefit on bone mass with proper calcium intake. As shown in Tables 10, 11 and 12, the intake of dietary protein with consumption of LGS, S and M exceeded the dietary reference intake (DRI) for adult men of 56 g/day, however, none of the groups met the recommended calcium intake of 1000 mg/day or the vitamin D intake (200 IU/day). The intake of these three nutrients were not different among treatments which allowed us to discard any influence of total protein (Figure 23), calcium (Figure 24) and vitamin D (Figure 25) intakes on changes in bone mineral density during the short term of consumption. Doubts about a low calcium bioavailability in the soy protein beverages (Heaney *et al.*, 2000) is not a concern in the present study due to considerable evidence supporting that calcium fortification of soy protein beverages with calcium carbonate has an equivalent effect as bovine milk (Zhao *et al.*, 2005; Tang *et al.*, 2010). The slight reduction in BMD ($<0.005 \text{ g/cm}^2$) may be due to the lack of adequate dietary calcium and vitamin D, but it was not related to any of the treatments. The reduction in sodium, vitamin C and magnesium intake in the LGS group was the major difference in nutrient intake. We hypothesize that this change may be due the replacement in the consumption of certain food products like orange juice or soups by the protein beverage. The high level of sodium intake among all participants is within the regular average intake of American adult population (4000-5000 mg/day). However this exceeds the American Heart Association and the Institute of Medicine recommendations of sodium consumption no greater than 1500 mg/day (USDHHS:NIH, 2006).

Table 10. Macronutrient intake during three months of consumption of LGS, S and M.

Time	Baseline				Month 1				Month 2				Month 3				TRT/ time effect P- value
	LGS	S	M	P- value*	LGS	S	M	P- value*	LGS	S	M	P- value*	LGS	S	M	P- value*	
Energy (Kcal)	2904 ±329	2690 ±435	1930 ±176	0.20	2615± 267	2695 ±389	2023 ±235	0.29	2244 ±295	2135 ±124	2248 ±197	0.96	2395 ±244	2542 ±193	1892 ±212	0.10	0.13
Protein (g)	108 ±9	89 ±7	76 ±8	0.04	95 ±11	91 ±5	77 ±9	0.39	89 ±10	79 ±4	79 ±9	0.49	97 ±12	94 ±6	69 ±7	0.02	0.17
CHOS (g)	377 ±51	268 ±19	243 ±24	0.03	320 ±36	330 ±48	237 ±26	0.21	267 ±38	250 ±18	266 ±25	0.92	307 ±36	299 ±27	234 ±28	0.24	0.05
Fat (g)	109 ±14	94 ±10	69 ±8	0.07	99 ±11	110 ±21	81 ±12	0.43	86 ±15	88 ±6	91 ±11	0.99	85 ±9	102 ±9	69 ±8	0.02	0.09
Dietary fiber (g)	30 ±3	15 ±1	14 ±1	0.09	21 ±3	22 ±4	17 ±2	0.40	20 ±3	16 ±1	15 ±2	0.20	23 ±2	18 ±2	14 ±2	0.17	0.62

*P-value of differences among treatments. Data excludes treatment consumption (See treatment composition in Table 4)

Table 11. Minerals intake during three months of consumption of LGS, S and M.

Time Intake / day	Baseline				Month 1				Month 2				Month 3				Treatment/time effect P-value
	LGS	S	M	P-value*	LGS	S	M	P-value*	LGS	S	M	P-value*	LGS	S	M	P-value*	
Ca (mg)	825 ±217	692 ±102	639 ±100	0.75	783 ±128	641 ±60	559 ±83	0.53	700 ±118	650 ±53	607 ±64	0.78	744 ±90	758 ±77	553 ±58	0.93	0.73
Fe (mg)	26 ±7	13 ±1	13 ±1.8	0.05	18 ±3	13 ±1	11 ±1	0.17	19 ±5	12 ±1	11.7 ±0.9	0.08	24 ±7	14 ±1	11 ±1	0.03	0.19
Mg (mg)	200 ±41	132 ±16	91 ±12	0.02	178 ±29	107 ±12	90 ±11	0.01	181 ±37	123 ±12	97 ±11	0.02	170 ±33	168 ±31	82 ±10	0.04	0.004
P (mg)	908 ±175	681.3 ±93	521 ±74	0.15	859 ±153	564 ±47	576 ±77	0.13	866 ±181	641 ±54	624 ±58	0.16	813 ±161	719 ±72	490 ±72	0.09	0.65
K (mg)	1887 ±517	1531 ±362	1414 ±402	0.16	1498 ±461	1060 ±642	959 ±434	0.20	1477 ±434	1145 ±286	1030 ±351	0.57	1489 ±507	2006 ±445	921 ±393	0.16	0.06
Na (mg)	5310 ±517	4335 ±362	4017 ±402	0.16	5143 ±461	5107 ±642	3820 ±432	0.20	3731 ±434	4269 ±286	4097 ±351	0.57	4428 ±507	4665 ±445	3614 ±393	0.16	0.02
Caffeine (mg)	167 ±40	103 ±30	1093 ±803	0.28	173 ±36	114 ±33	102 ±27	0.29	173 ±46	139 ±38	100 ±29	0.31	198 ±55	549 ±388	114 ±29	0.44	0.52

*P-value of differences among treatments. Data excludes treatment consumption (See treatment composition in Table 4)

Table 12. Vitamins intake during three months of consumption of LGS, S and M.

Time	Baseline				Month 1				Month 2				Month 3				TRT/ time effect P- value
	LGS	S	M	P- value*	LGS	S	M	P- value*	LGS	S	M	P- value*	LGS	S	M	P- value*	
Vit D (IU)	61 ±22	61 ±21	33 ±15	0.65	22 ±67	23 ±7	24 ±6	0.81	12 ±4	22 ±7	32 ±6	0.12	18 ±5	21 ±9	17 ±5	0.87	0.49
Vit C (mg)	76 ±24	68 ±11	57 ±11	0.59	32 ±5	37 ±7	19 ±2	0.05	60 ±21	44 ±10	28 ±5	0.21	49 ±10	37 ±6	27 ±6	0.13	0.0002
Vit B12 (µg)	3.9 ±1	3 ±1	2 ±0.3	0.19	2 ±1	3 ±1	3 ±1	0.50	2 ±1	2 ±0	2 ±0	0.99	3 ±1	2 ±0	2 ±0	0.38	0.52
Vit K (IU)	38 ±18	18 ±4	12 ±3	0.24	17 ±4	13 ±2	11 ±1	0.33	41 ±12	22 ±5	21 ±5	0.10	13 ±3	21 ±4	13 ±4	0.42	0.17
Vit E (IU)	5 ±2	3 ±1	1.2 ±0.2	0.06	3 ±1	3 ±1	3 ±1	0.51	4 ±1	3 ±1	3 ±0	0.45	3 ±1	3 ±0	2 ±1	0.32	0.41
Vit A (IU)	5681 ±1669	2885 ±694	2629 ±267	0.11	4548 ±1158	4058 ±627	2627 ±530	0.22	3861 ±1110	3291 ±593	3123 ±534	0.88	4467 ±938	4102 ±999	2501 ±303	0.12	0.64

*P-value of differences among treatments. Data excludes treatment consumption (See treatment composition in Table 4)

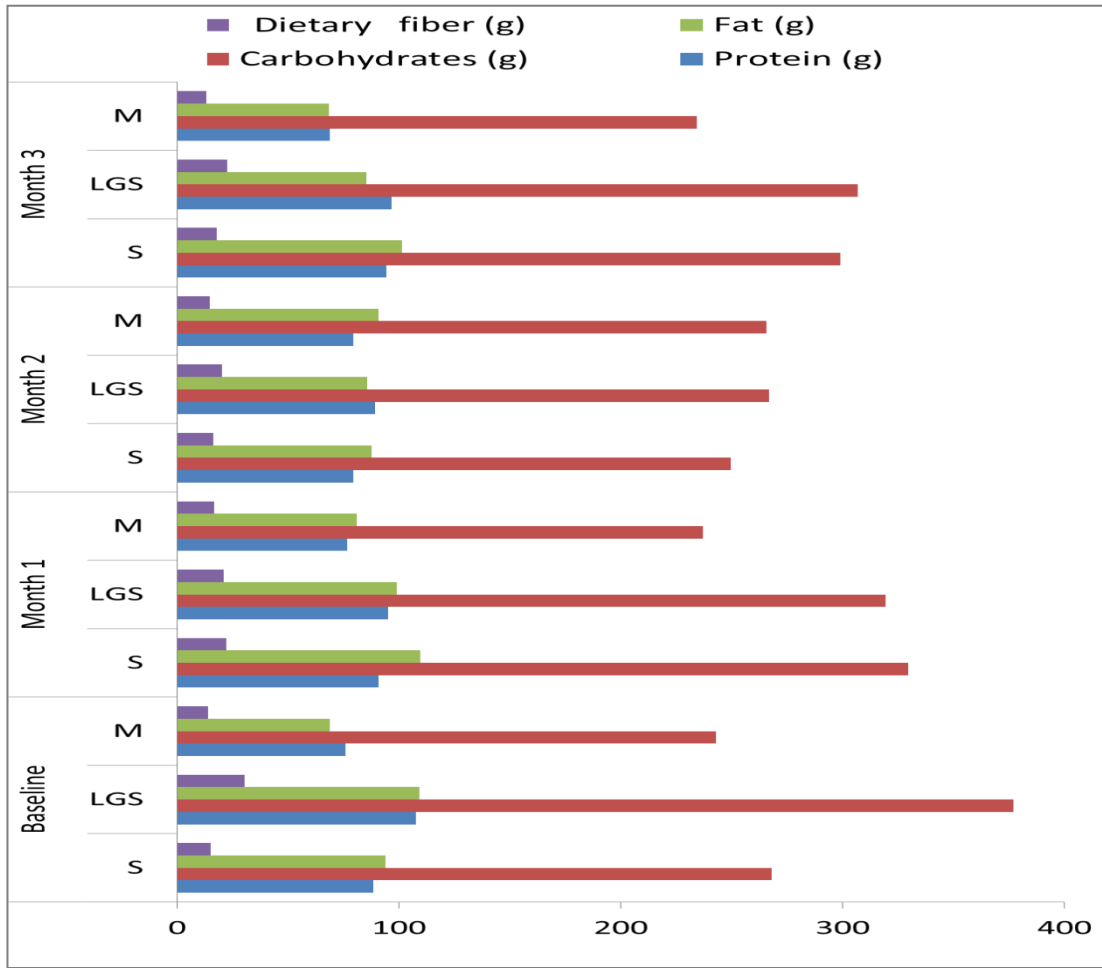


Figure 23. Main nutrient intake throughout the study ($P > 0.05$)

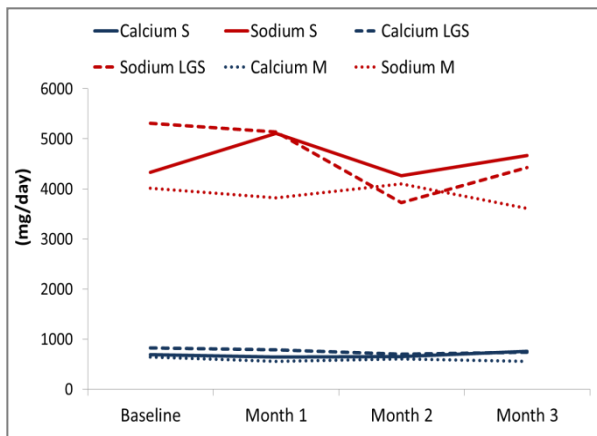


Figure 24. Calcium and sodium intake

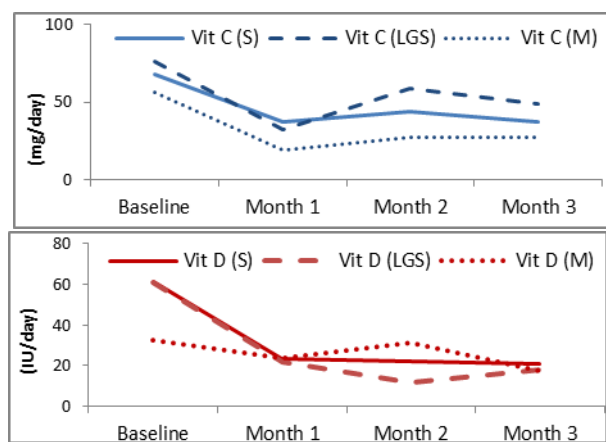


Figure 25. Vitamin D and C intake

6. Physical activity

Most participants had a sedentary life style and are part of the 32% of the American population physically inactive. There were not significant changes in physical activity during the study, eliminating any influence from this factor in the results observed. Changes from baseline in strenuous ($P = 0.489$) and moderate ($P = 0.209$) physical activity did not change more than 1h/week (Figure 26).

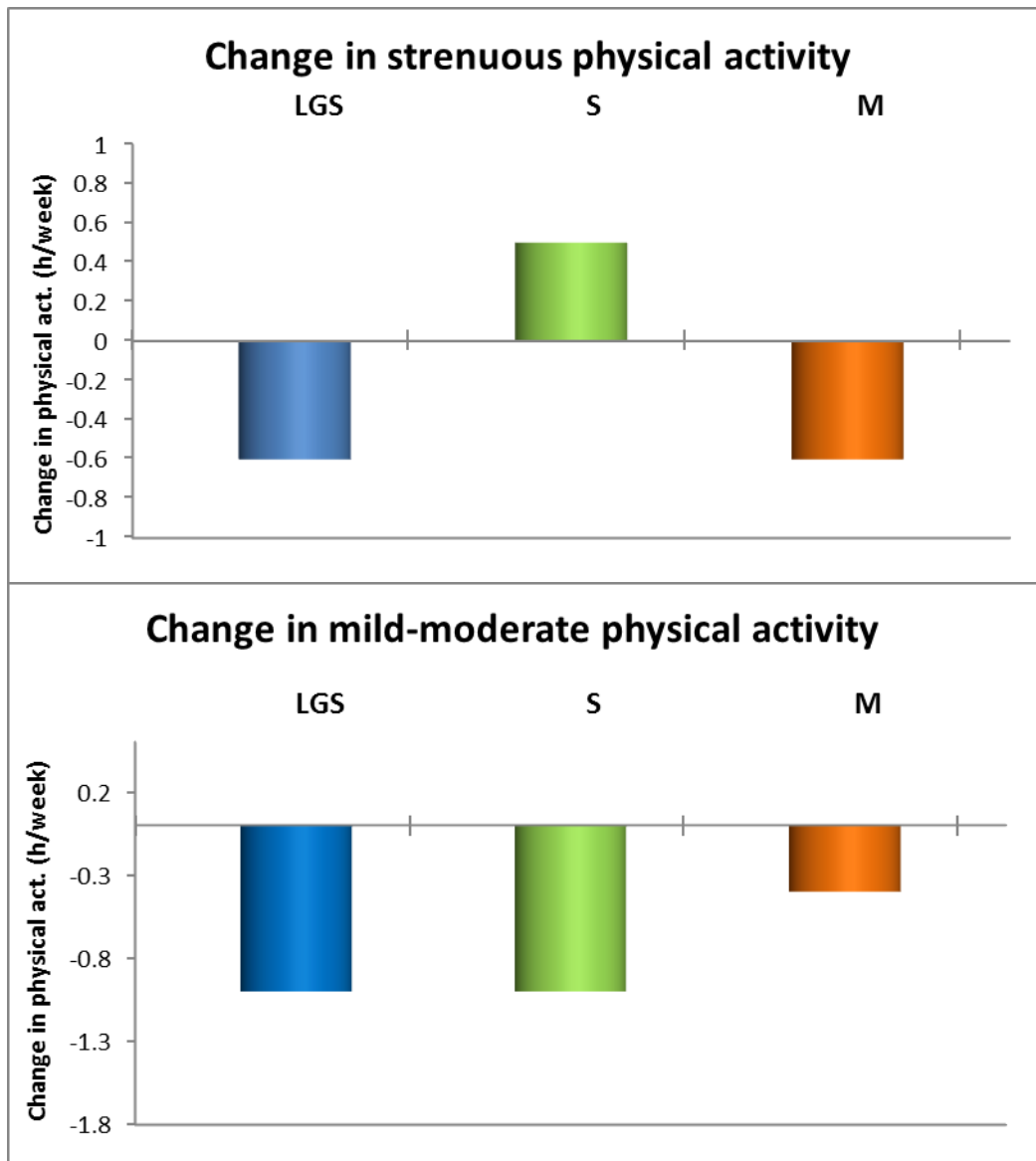


Figure 26. Moderate and strenuous physical activity changes

7. Lipid profile and adipokines

7.1. Total serum cholesterol, serum triglycerides and fatty acid synthase (FAS)

In agreement with Gardner *et al.* (2007), consumption of LGS and S had no impact on levels of serum total cholesterol and triglycerides. Total cholesterol after consumption of LGS, S and M was $16.6 \pm 7.6\%$, $-2.9 \pm 5.3\%$ and $10.8 \pm 6.7\%$ respectively ($P = 0.32$). A slight decrease in serum triglycerides was observed with LGS, S and M ($-8.8 \pm 10.7\%$, $-10.0 \pm 13.1\%$ and $-5.8 \pm 8.1\%$ respectively, however the changes among treatments were not significant ($P = 0.95$) (Table 13). This may be explained by differences in the types of heat treatments used in commercial soymilk production which may alter the ability of soy protein to lower cholesterol. For example, ultra-heat treatment, AS Ultra High Temperature (UHT) used in the present study, can destroy the cholesterol-lowering capacity of soy protein (Hoie *et al.*, 2006). In contrast, other soy protein studies have observed an important improvement in serum lipids after soy consumption (Borodin *et al.*, 2009). Fatty acid synthase was also analyzed and changes among treatments were not detected ($P = 0.976$). LGS, S and M changes were $-18.6 \pm 16.8\%$, -31.9 ± 11.5 and $40.0 \pm 21.6\%$ (table 11). Our results differed from a previous study developed by Martinez-Villalueva (2010) where purified peptides from β -conglycinin EITPEKPNQLR and RKQEEDEDEEQRE inhibited FAS *in vitro*.

7.2. Plasma adiponectin and leptin

Weight loss has been associated with the reduction of plasma leptin (Yamashina *et al.*, 1998). Dietary interventions performed in this study did not lead to weight loss in overweight men which may explain the maintenance of leptin levels (Table 13). LGS ($14.9 \pm 4.5\%$) and S ($2.0 \pm 5.2\%$) increased plasma adiponectin while M (-7.8 ± 4.4) decreased it (Figure 27). The elevation in plasma adiponectin associated with LGS consumption could partially explain the

differences in body fat composition due to the hormonal regulatory property of this hormone on fatty acid catabolism. Adiponectin is inversely correlated to the content of body fat in adults (Diez and Iglesias, 2003). Marecki *et al.* (2010) suggested age-related differences in the role of adiponectin in pathological responses associated with obesity. Higher levels of plasma adiponectin in overweight men after 3 months of LGS consumption may also contribute to decreasing oxidative stress and inflammatory state. Iwabu *et al.* (2010) determined that adiponectin reduced oxidative stress through the induction of Ca^{2+} influx in skeletal muscle via AdipoR1 (Iwabu *et al.*, 2010). Our results are also consistent with Martinez-Villaluenga *et al.* (2008), who determined *in vitro* that β -conglycinin induced more adiponectin expression in 3T3 L1 adipocytes than glycinin. A recent study confirmed that β -conglycinin increases adiponectin levels in rats more than soy protein isolate (SPI) and casein (Tachibana *et al.*, 2010). The increment in plasma adiponectin levels and the stability of plasma leptin after the consumption of LGS may have positively influenced energy expenditure without increasing satiety.

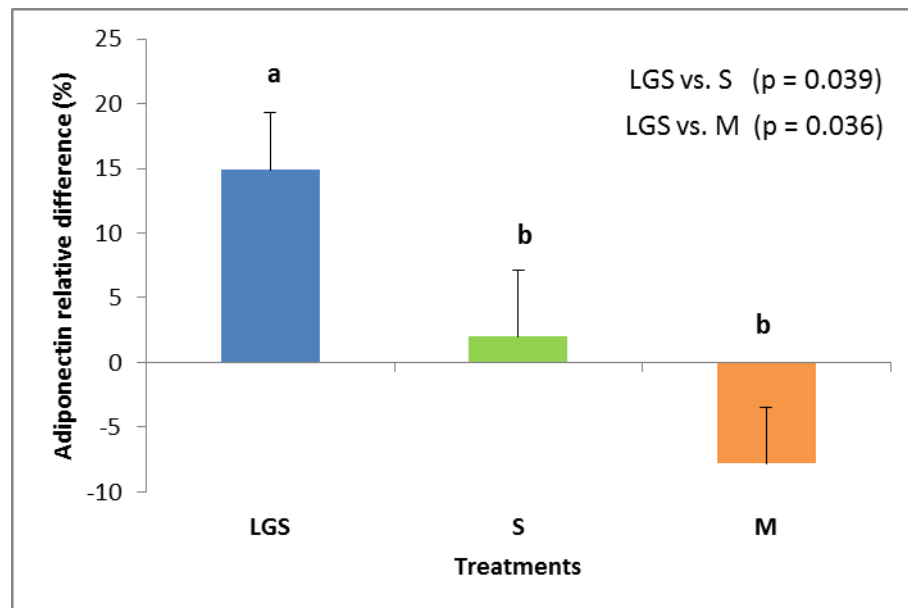


Figure 27. Relative differences in plasma adiponectin after three months

Table 13. Serum lipids, plasma leptin, C-reactive protein, tumor necrosis factor alpha and fatty acid synthase (FAS) at baseline and three months after consumption of the different types of LGS, S and M

Biomarker	LGS ¹		S ¹		M ¹		Relative differences from baseline (%) ^{2,3}			
	Baseline	Month 3	Baseline	Month 3	Baseline	Month 3	LGS	S	M	P-value ⁴
Total Cholesterol (mg/dL)	299.9 ± 24.5	334.5 ± 21.9*	396.3 ± 33.6	360.9 ± 23.3*	313.3 ± 22.9	337.6 ± 24.9*	16.6 ± 7.6 ^a	-2.9 ± 5.3 ^a	10.8 ± 6.7 ^a	0.32
Total Triglycerides (mg/dL)	143.6 ± 26.8	128.9 ± 36.6*	151.5 ± 26.9	135.5 ± 23.9*	105.4 ± 19.1	90.1 ± 12.5*	-8.8 ± 10.7 ^a	-10.0 ± 13.1 ^a	-5.8 ± 8.1 ^a	0.95
Leptin (ng/mL)	13.6 ± 1.9	14.4 ± 1.4*	14.9 ± 2.1	16.8 ± 1.9*	16.2 ± 3.9	18.5 ± 4.2*	6.6 ± 17.8 ^a	11.8 ± 5.9 ^a	20.4 ± 7.4 ^a	0.66
C-Reactive protein (ng/mL)	2022.9 ± 297	1903.7 ± 367*	1200.1 ± 258	978.4 ± 166*	1718.8 ± 316	1878.1 ± 374*	-6.6 ± 10.5 ^a	-19.7 ± 6.0 ^a	1.0 ± 6.8 ^a	0.96
Fatty acid synthase (mg/mL)	1.89 ± 0.90*	1.84 ± 1.11*	5.83 ± 0.97	4.58 ± 0.96*	2.77 ± 0.71	4.11 ± 1.12*	-18.6 ± 16.8 ^a	-31.9 ± 11.5 ^a	40.0 ± 21.6 ^a	0.98
Tumor Necrosis Factor α (pg/mL)	1.2 ± 0.3	1.2 ± 0.6	0.8 ± 0.1	0.7 ± 0.1*	1.4 ± 0.3	1.4 ± 0.4	-18.3 ± 17.8 ^a	-12.8 ± 14.2 ^a	-8.6 ± 8.7 ^a	0.61

¹ Values are means per treatment per time. ² Values are Means ± SEM of relative differences from baseline, n = 64. ³ Different letters in rows are statistically different ($P < 0.05$). ⁴ Variation probability among treatments. * Significantly different from baseline, $P < 0.0001$.

8. Oxidative stress

8.1. Oxidized LDL

Consumption of LGS for three months significantly reduced the levels of ox-LDL (Table 14). Consumption of S and M also reduced oxi-LDL; however, this reduction is smaller than the impact of LGS consumption (Figure 28). This reduction is similar to the therapeutic effect of statins when used in patients with hypercholesterolemia, in which the statin drug reduces up to 43% of ox-LDL (Nakhjavani *et al.*, 2010). A possible reason for this reduction in ox-LDL is that peptides from β -conglycinin have cell protective properties against oxidation by increasing the expression of thioredoxin 1 and cyclophilin B (Castiglioni, 2003; Van Ee, 2009), and preventing retinal ganglion cell death (Caprioli *et al.*, 2009). It has been also found that soy peptides, not the amino acid content, reduced the oxidative stress in rats (Takenaka *et al.*, 2003).

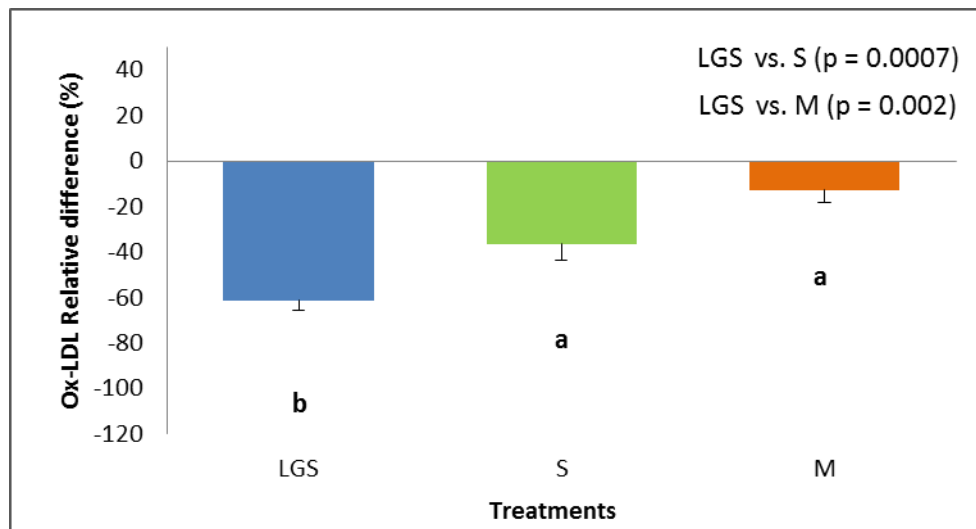


Figure 28. Relative difference in oxi-LDL after three months

The strong reduction in ox-LDL, as in the present study, has been considered independent of BMI in men (Weinbrenner *et al.*, 2006). However, our data show that subjects with lower

BMI presented higher reduction in oxi-LDL than those with higher BMI levels. Also younger subjects reduced more Oxi-LDL than the older participants (Table 15). Flavonoids present in soymilk can also exert an additional protective effect against LDL oxidation by binding directly to LDL molecules or acting as radical scavengers (Wu *et al.*, 2009). Based on our results, one can speculate that β -conglycinin may protect against plaque development by inhibiting oxi-LDL, and that by reducing systemic oxidative stress may also decrease risk factors for diabetes and obesity (Njajou *et al.*, 2009).

8.2. Antioxidant capacity

Oxidative stress is strongly associated with type 2 diabetes and cardiovascular mortality and serum antioxidant capacity may play an important role in metabolic syndrome prevention (Beydoun *et al.*, 2011). Both soymilk types improved serum antioxidant status compared to bovine milk (Table 14). However, the higher β -conglycinin content in soymilk did not provide additional improvement (Figure 29). In agreement with our results, a previous study comparing soybean and casein proteins determined that mice fed soybean protein lowered oxidative stress and increased antioxidant enzymes more than casein (Gu *et al.*, 2008). Similarly, soy consumption reduced plasma malondialdehyde (MDA) and increased plasma total antioxidant capacity (TAC) levels in postmenopausal women with metabolic syndrome (Azadbakht *et al.*, 2007). Antioxidant properties of soymilk have been attributed to soybean peptides (Suetsuna *et al.*, 1999) or specifically histidine-containing soybean peptides released after gastrointestinal digestion which act as hydroxyl radical scavengers (Chen *et al.*, 1998). However, the parental protein from which these peptides derived has not been reported. Soymilk also contains other nonpeptidic antioxidants such as tocopherols, isoflavones and phenolic acids which may contribute to its antioxidant capacity (Takahashi *et al.*, 2005).

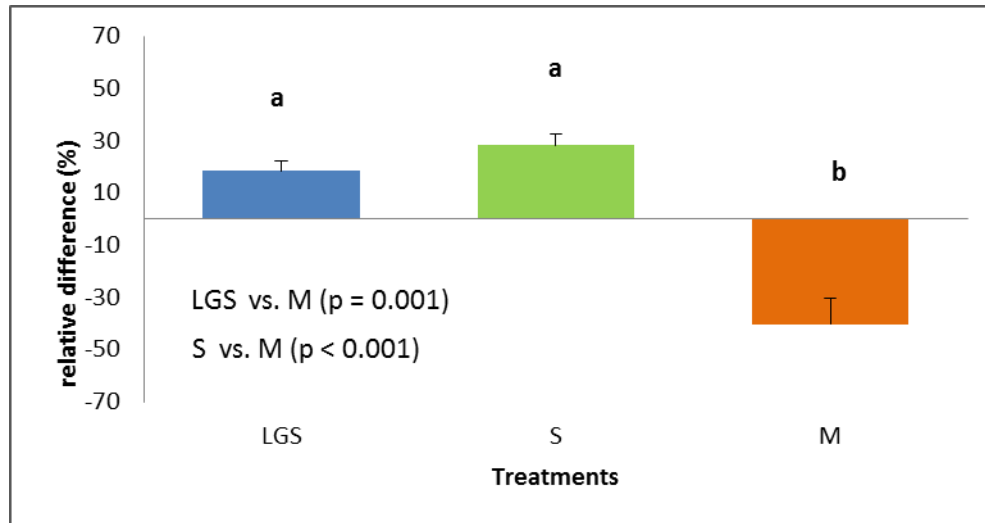


Figure 29. Relative difference in antioxidant capacity after three months.

Table 14. Oxi-LDL and antioxidant capacity at baseline and at the end of the study.

Biomarker	n	Treatments	Baseline U/mL	Month 3 U/mL	Relative difference (%)	P-value ¹	Contrasts
Serum Oxidized LDL	15	LGS	0.074	0.019	-61.3	0.0019	S vs. LGS = 0.0007
		SEM	0.012	0.004	3.8		
	20	S	0.099	0.051	-36.4		LGS vs. M < 0.0001
		SEM	0.039	0.014	7.1		
	16	M	0.144	0.136	-12.8		S vs. M = 0.8568
		SEM	0.062	0.061	5.2		
Biomarker	n	Treatments	Baseline $\mu\text{mol Trolox/ml serum}$	Month 3 $\mu\text{mol Trolox/ml serum}$	Relative difference (%)	P-value	Contrasts
Serum Antioxidant Capacity	18	LGS	16.28	19.22	18.5	< 0.0001	S vs. LGS = 0.9051
		SEM	0.59	0.79	3.5		
	22	S	15.59	19.67	28.1		LGS vs. M = 0.001
		SEM	0.67	0.84	4.6		
	21	M	17.02	9.46	-40.4		S vs. M < 0.0001
		SEM	0.58	1.37	9.7		

Data are means and SEM. ¹ Statistical effect of treatment in changes in Oxi-LDL and antioxidant capacity.

Table 15. Oxi-LDL relative differences by BMI and age

BMI	Ox-LDL (RD)	STD	n	P-value
25-27	-46.9 ^b	28.8	18	0.004
28-35	-36.3 ^b	39.0	27	
36-41	-9.1 ^a	46.0	6	
AGE	Ox-LDL (RD)	STD	n	P-Value
23-25	-52.8 ^b	51.5	7	0.046
26-30	-33.6 ^a	33.9	15	
31-35	-41.3 ^a	35.3	11	
36-40	-41.9 ^a	37.8	7	
41-45	-23.2 ^a	36.3	11	

*P-value of the comparison among groups

9. Inflammation

9.1 Plasma interleukin-6

Body fat accumulation leads to a proinflammatory state accompanied by an increased expression of pro-inflammatory cytokines and chemokines in circulating monocytes which control fat accumulation and leptin expression in human adipocytes (Christie *et al.*, 2010). The plasma concentration of IL-6, due to the consumption of LGS ($-22\% \pm 9$, $P = 0.025$) or S ($-26\% \pm 5$, $P = 0.014$) were also reduced in comparison to M ($10\% \pm 12$).

Our previous *in vitro* studies demonstrated that alcalase hydrolysates from β -conglycinin enriched soybean genotypes exhibited an anti-inflammatory effect through inhibition of NO/iNOS and PGE₂/COX-2 pathways in LPS-stimulated RAW 264.4 macrophages. However, the effect of β -conglycinin on pro-inflammatory mediators in overweight people has not been reported. In the present study we observed that consumption of soymilk was associated with reduced plasma IL-6 levels unlike in bovine milk consumption (Figure 30). Our results are in agreement with Christie *et al.* (2010) which determined that three months of soy supplementation in obese postmenopausal women reduced circulatory IL-6.

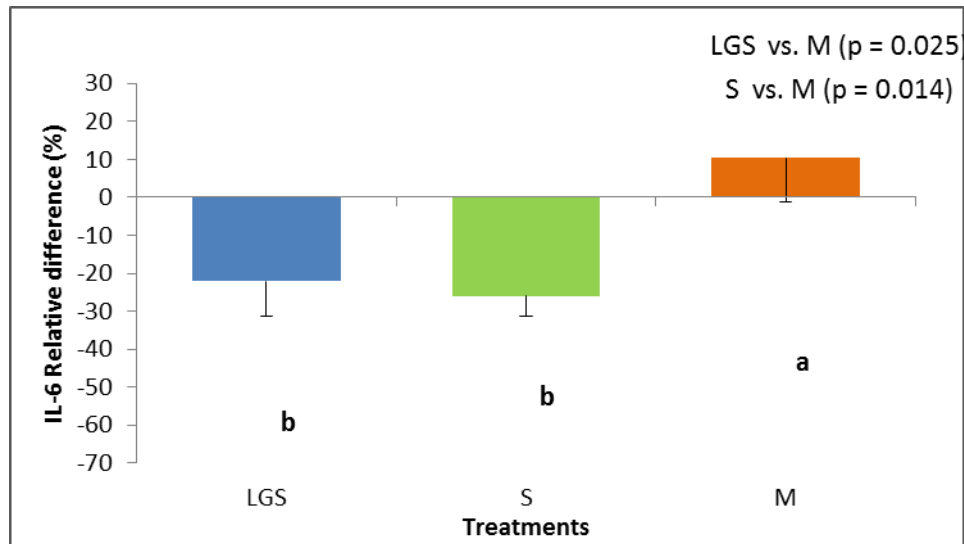


Figure 30. Relative difference in plasma interleukin-6 after three months

9.2. Plasma C-reactive protein and tumor necrosis factor alpha

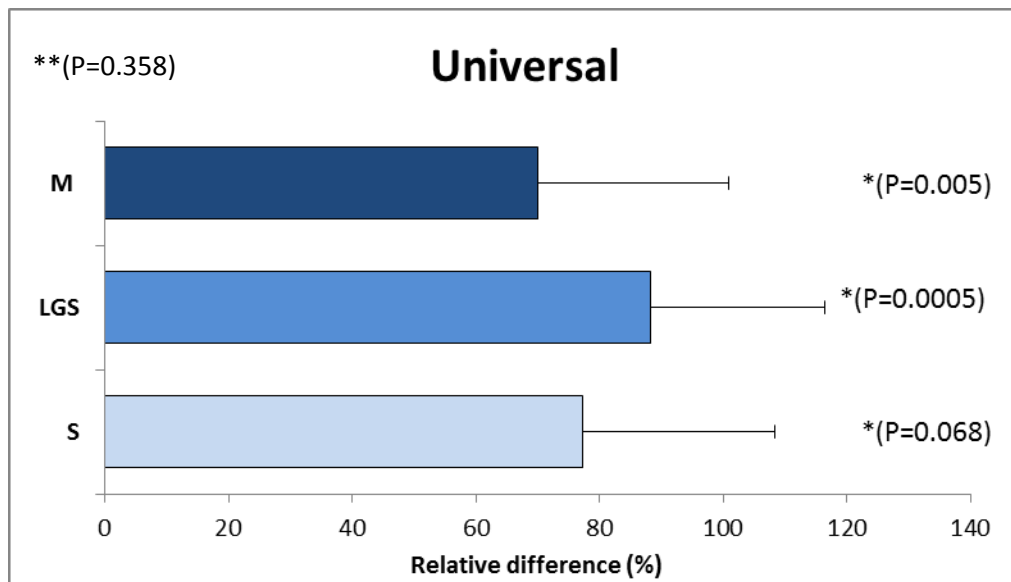
Contrary to plasma IL-6, relative differences of CRP and leptin levels in plasma were not affected by treatment in agreement with Maskarinec *et al.* (2009) who demonstrated that a high-soy diet for 3 months did not modify the levels of inflammatory markers in overweight individuals. Plasma CRP tended to decrease with LGS, S and M after three months ($-6.6 \pm 11\%$, $-19.7 \pm 6\%$ and $1.0 \pm 7\%$ respectively). However, changes were not significant among the treatments ($P = 0.96$). TNF α presented similar behavior ($P = 0.61$) (Table 13).

10. Gut microbiota composition

10.1. General gut microbiota

Evidence that gut microbiota composition can differ between obese and lean individuals led the hypothesis that gut microbiota manipulation may be one alternative mechanism to improve this epidemic. According to recent studies microbiota may play a strategic role in susceptible populations like the obese (Ley, 2010) and type 2 diabetic populations (Larsen *et al.*, 2010). Few researchers have investigated the impact of soy products or soy proteins in gastrointestinal

microbiota. Based on the primers used in this study, microbiota density increased after the consumption of LGS ($88.4 \pm 28\%$), S ($77.2 \pm 31\%$) and M ($70.1 \pm 31\%$). However, all treatments provided the same behavior ($P = 0.358$) (Figure 31). The prevalence of bacteria in the GI tract dependent on several factors, such as pH, peristalsis, redox potential, bacterial adhesion, bacterial co-operation and antagonism, mucin secretion, diet and nutrient availability among others. More density is not necessarily a positive effect; bacterial overgrowth has a negative impact on the function and morphological structure of the small bowel (Bures *et al.*, 2010), however, our results may carry positive effects because microbiota density increment was determined in feces.



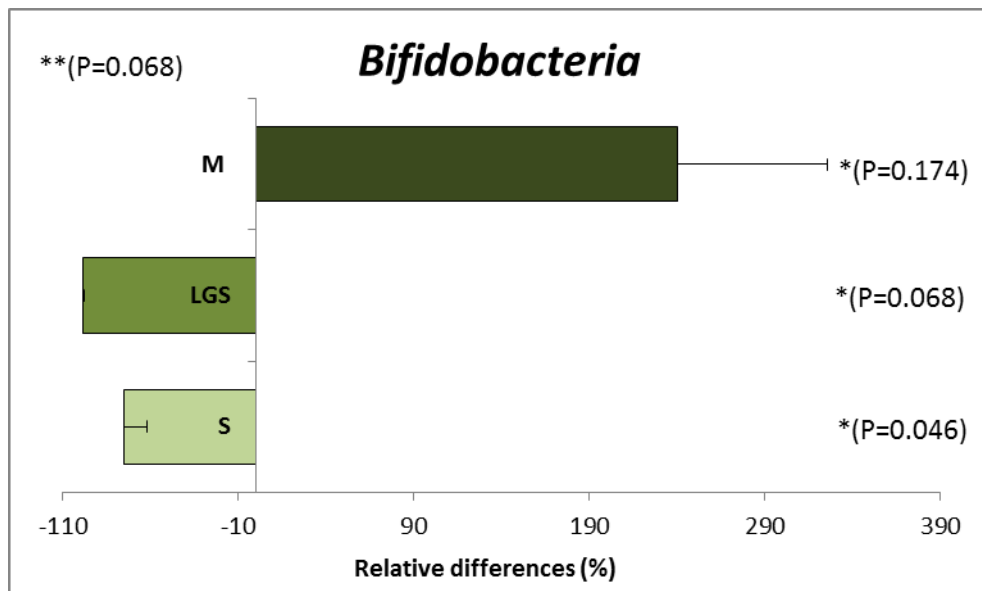
*P-value within treatments. **P-value among treatments.

Figure 31. Relative changes in universal microbiota after three months

The *Bifidobacteria* changes in LGS ($-98 \pm 1\%$) and S ($-76 \pm 13\%$) tended to decrease. However, under the condition of this study this change was not significantly different from

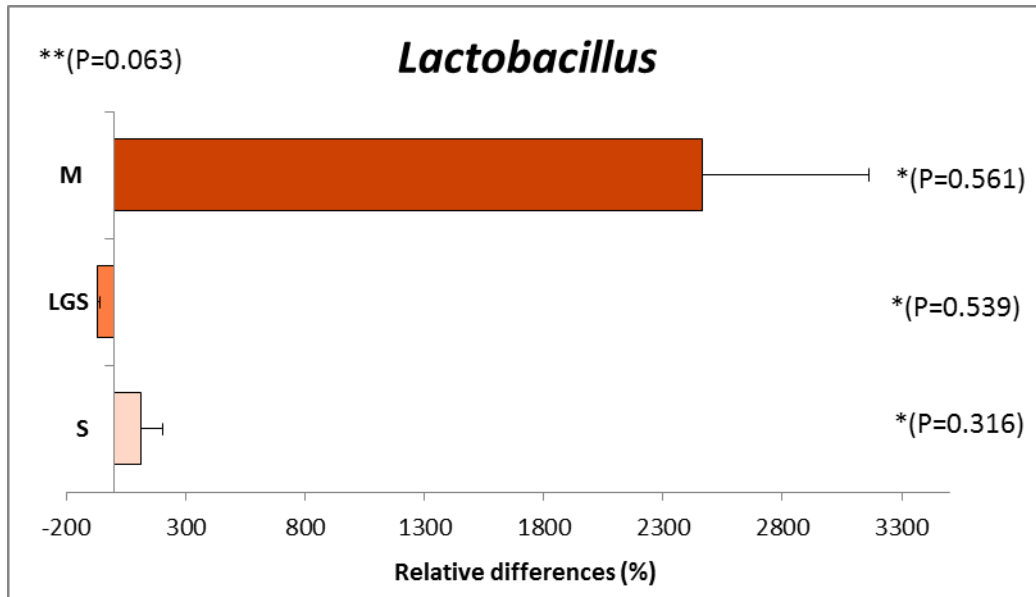
bovine milk ($240 \pm 85\%$) (Figure 32). Confusing tendencies were observed in *Lactobacillus* changes (Figure 33) where LGS reduced while S and M increased the *Lactobacillus* population but these changes were not different within or among groups. This phenomenon may be interpreted in the right direction considering the existing evidence that *Lactobacillus* in obese populations is higher than in lean populations (Armougon, 2009).

According to Santacruz *et al.* (2009) physical activity has an impact on gut microbiota. In our study, physical activity did not have an effect on gut microbiota changes which was expected due the physical inactivity of the subjects during the study. Santacruz *et al.* (2009) also found that overweight adolescent with higher fecal total bacteria and lower *Lactobacillus* and *Bifidobacteria* experienced higher weight loss which is relatively comparable to what happened after the consumption of soymilk in our study.



*P-value within treatments. **P-value among treatments.

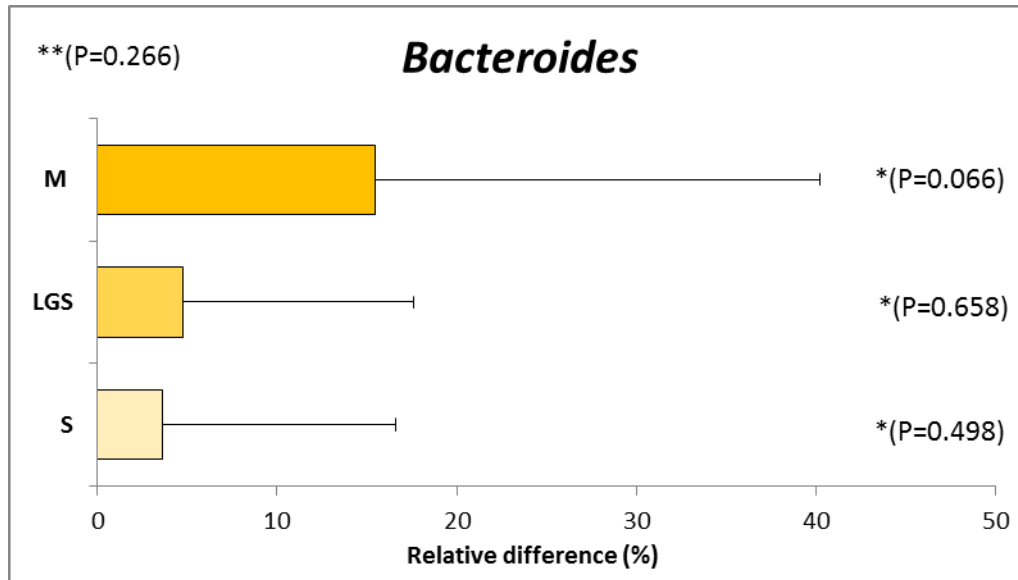
Figure 32. Relative changes in *Bifidobacteria* after three months



*P-value within treatments. **P-value among treatments.

Figure 33. Relative changes in *Lactobacillus* after three months

Bacteroides have a positive association with plasma biomarkers of lipid metabolism; higher gut *Bacteroides* composition is associated to higher HDL-cholesterol and lower triglycerides levels (Santacruz *et al.*, 2010). Nadal *et al.* (2009) confirmed a relationship between *Bacteroides* and *Clostridium* with weight loss. Subjects with higher weight loss had more *Bacteroides* and *Clostridium* in their microbiota profile than those who loss less weight. In our study, subjects did not lose weight with any treatment and changes in *Bacteroides* were not significant (Figure 34) which agrees with the studies previously mentioned. Alterations in *Bacteroides* number and its specific effect in body weight may be an interesting target for future human studies.



*P-value within treatments. **P-value among treatments.

Figure 34. Relative changes in *Bacteroides* after three months

Our results suggest that the consumption of LGS, S and M have similar impact on total microbiota, *Bacteroides*, *Bifidobacteria* and *Lactobacillus* after three months. These findings agree with Piacentini *et al.* (2010) who compared the effect of bovine and soy-based formula in infants for one month. They concluded that soymilk and bovine milk has a comparable impact in species biodiversity in infants and also concluded that soymilk does not alter the general intestinal flora of infants but it decreased *Bifidobacteria*.

10.2. Genera diversity analysis

High-throughput sequencing of the hypervariable V1-V3 region of the 16S rRNA was performed. Samples (n=384) were sequenced obtaining 1.88 million total sequences and 1.2 million sequences after trimming for selection of the high quality sequences. Sequences presented an average length of 491bp. Between 4600-4700 sequences were used and the taxonomic tree at the phyla level generated is shown in Figure 35. The diversity indices ACE and Chao1 showed very high sample richness estimating the potential OTU's that would be seen if

the sequence is completed. OUT 3% analysis showed a significant reduction in diversity within groups ($P < 0.05$) (Table 16). Approximate 50% of total diversity is represented by the sequences in this study and all treatments showed a slight reduction in the species diversity. These findings agree with Turnbaugh *et al.* (2009) who determined that obesity reduces bacterial diversity. Figure 36 shows the high quality of sequencing coverage (curves close to asymptote) indicating that overall diversity among people becomes more similar after three months of consumption.

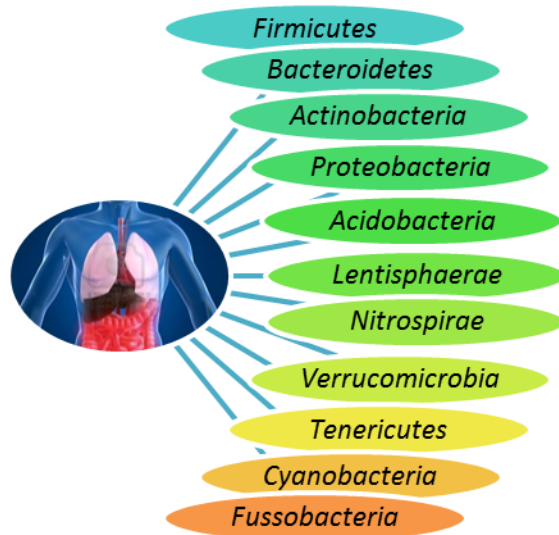


Figure 35. Taxonomic tree at the phyla level

Table 16. Microbiota diversity

TRT	Seq used		OUT 3%			ACE 3%		Chao1 3%	
	B	T3	B	T3	P-value*	B	T3	B	T3
LGS	4687	4730	1064	938	0.004	2191	1606	1749	1396
	(259)	(224)	(207)	(147)		(783)	(337)	(473)	(271)
S	4689	4671	1019	915	0.005	2065	1653	1663	1386
	(336)	(175)	(173)	(80)		(586)	(268)	(373)	(148)
M	4604	4625	1043	923	0.001	2150	1539	1722	1365
	(490)	(341)	(207)	(142)		(685)	(337)	(414)	(244)

Data are Means (STD). Operational taxonomic Units (OTU). OTU at 3% can show an identity at the species level of phylogeny. * P-value represents significant changes from baseline. B means baseline and T3 means after three months of consumption. Differences in OUT 3% among treatments were not significant ($P = 0.227$).

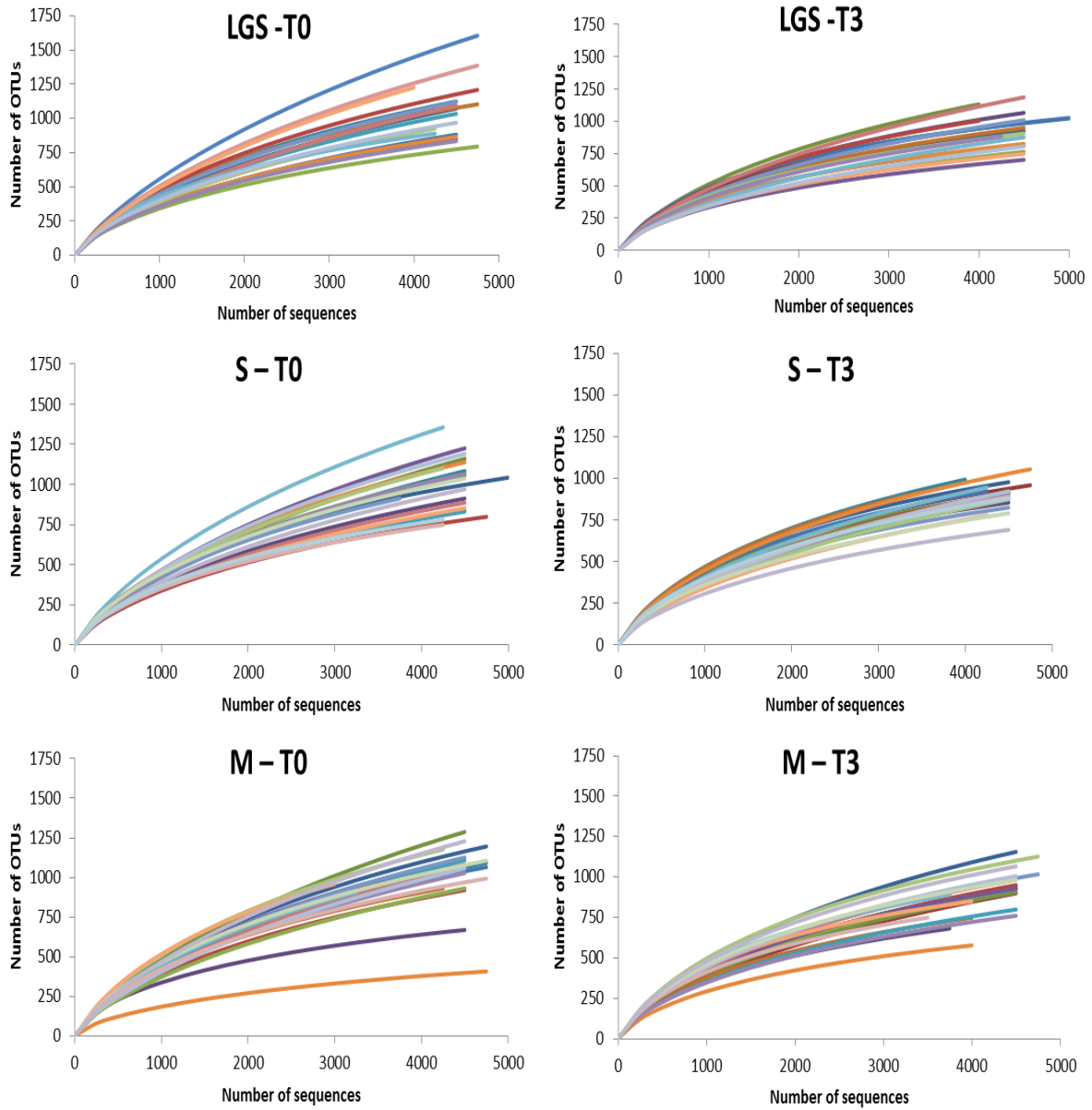
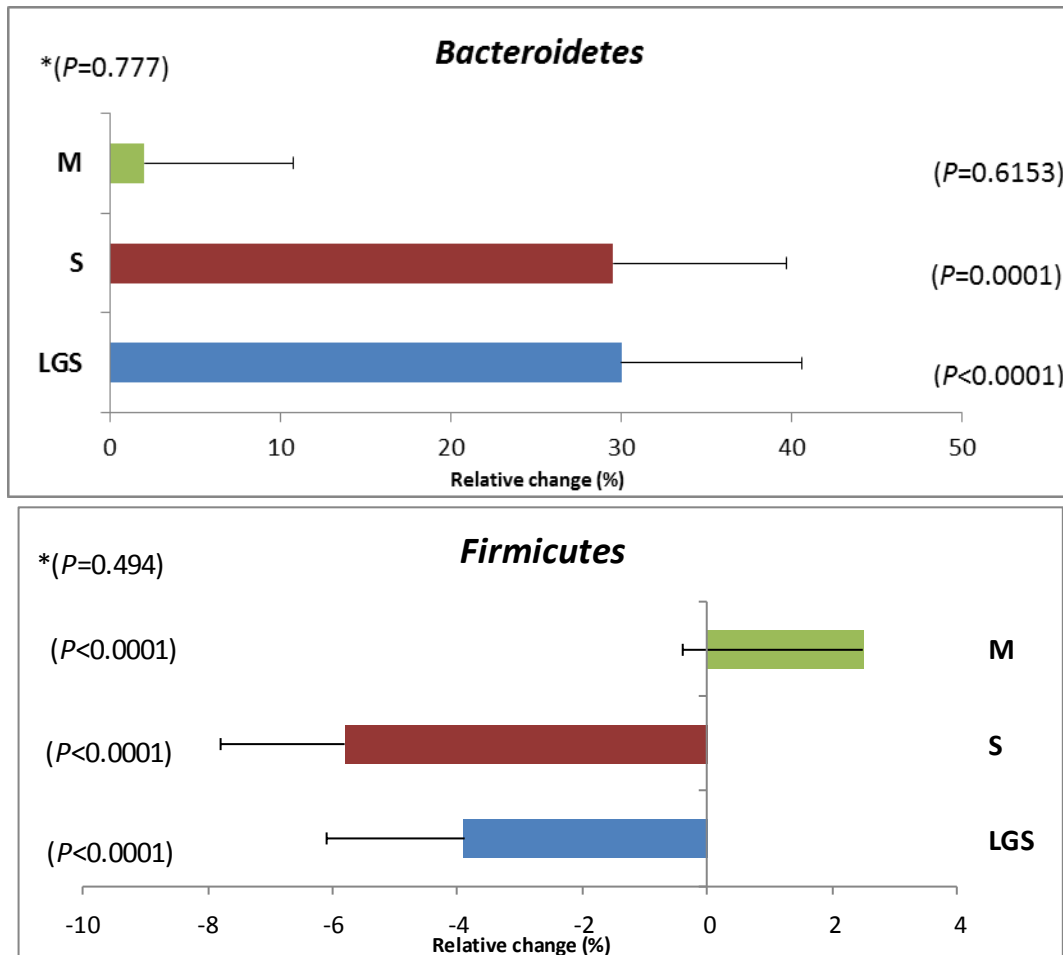


Figure 36. Rarefaction curves at baseline and after three months for LGS, S and M.

The majority of gut bacteria in mammals belong to the phyla *Bacteroidetes* and *Firmicutes* (Ley *et al.*, 2008) and there is a significant amount of evidence saying that elevated *Firmicutes* content and lower *Bacteroidetes* content characterizes the obese gut microbiota (Ley *et al.*, 2005; Turnbaugh *et al.*, 2009; Armougom *et al.*, 2009). Weight loss has been also directly related with a decrease in the *Firmicutes/Bacteroidetes* ratio (Ley *et al.*, 2006). After the pyrosequencing analyses performed in this study, phylum changes after three months in *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria* were not different among the treatments ($P > 0.05$). However, within treatments we were able to detect *Firmicutes* significant decreases with consumption of LGS ($-3.9 \pm 2.2\%$) and S ($-5.8 \pm 2\%$) and an increase with M ($2.5 \pm 2.9\%$). On the other hand, *Bacteroidetes* abundance increased with the consumption of LGS and S ($30.0 \pm 10.6\%$ and $29.5 \pm 10.2\%$, respectively) (Figure 37).

Additionally, our analyses detected an influence of age in the changes in *Bacteroidetes* ($P = 0.029$) and *Firmicutes* ($P = 0.041$). These findings agree with Mariat *et al.* (2009) who affirmed that *Firmicutes/Bacteroidetes* ratio increases with age, elevating the probabilities of gain weight. Initial BMI, changes plasma adiponectin, plasma ox-LDL, physical activity, plasma IL-6 and antioxidant capacity throughout the study were not a direct influence in phylum changes. Following the same trend, the *Firmicute/Bacteroidetes* ratio after three months of consumption tended to decrease with both soymilk treatments (LGS -1.9 ± 0.6 ; S -1.2 ± 0.5 and M 0.1 ± 0.6 ratio difference) (Table 17). These decreases in *Firmicute/Bacteroidetes* ratio may represent a potential weight lost in the medium or long term of consumption (Ley *et al.*, 2006) and may have effects in obesity related conditions.



*P-value of the among treatment comparison

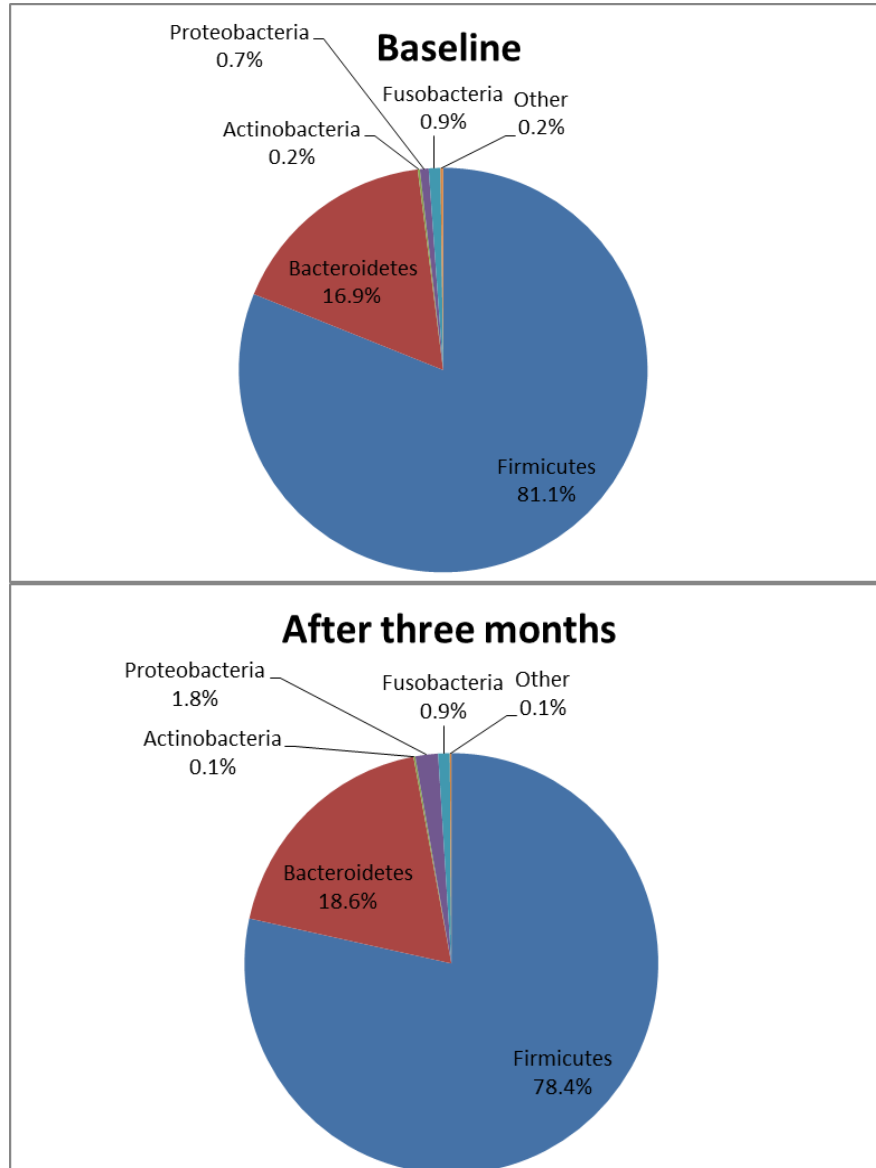
Figure 37. Relative change in *Firmicutes* and *Bacteroidetes* abundance after three months

Table 17. *Firmicutes/Bacteroidetes* ratio with LGS, S and M consumption

Firm/Bac	Baseline	T3	Diff	SEM	Within treatment P-value
LGS	6.8	4.9	-1.9	0.6	0.006
S	6.8	5.6	-1.2	0.5	0.018
M	6.4	6.5	0.1	0.6	0.847

*Significant changes P -value <0.05 . Changes among treatments were not significant ($P=0.757$).

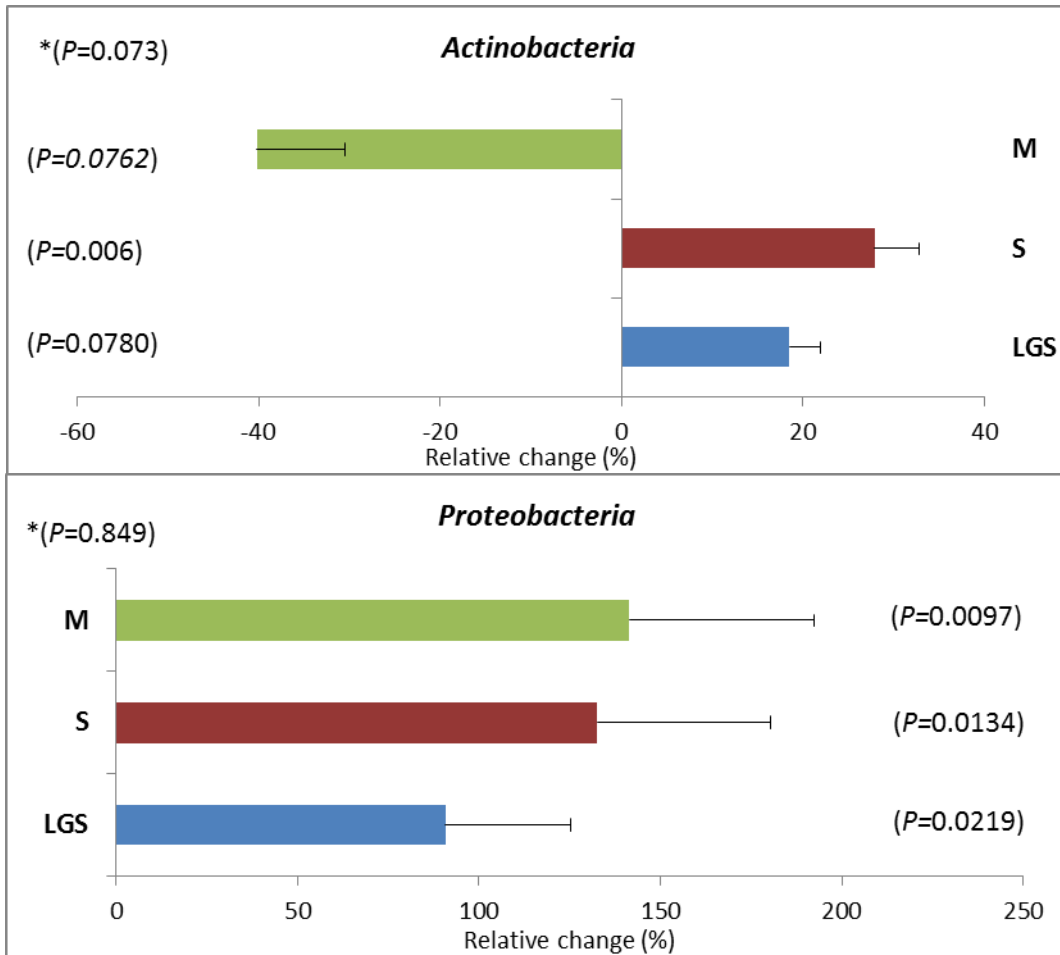
Our results showed that overweight and obese men phyla abundance at baseline and after three months is dominated by *Firmicutes* ($81.1 \pm 10.4\%$ and $78.4 \pm 10.6\%$ respectively). The second dominant phyla was *Bacteroidetes* ($16.9 \pm 13.3\%$ and $18.6 \pm 13.2\%$) at baseline and month 3 respectively. *Actinobacteria*, *Proteobacteria* and *Fusobacteria* represent less than 1% of the gut microbiota profile (Figure 38).



*Only major taxonomic groups are shown; these cover 90-100% of all reads assigned to phylum level.

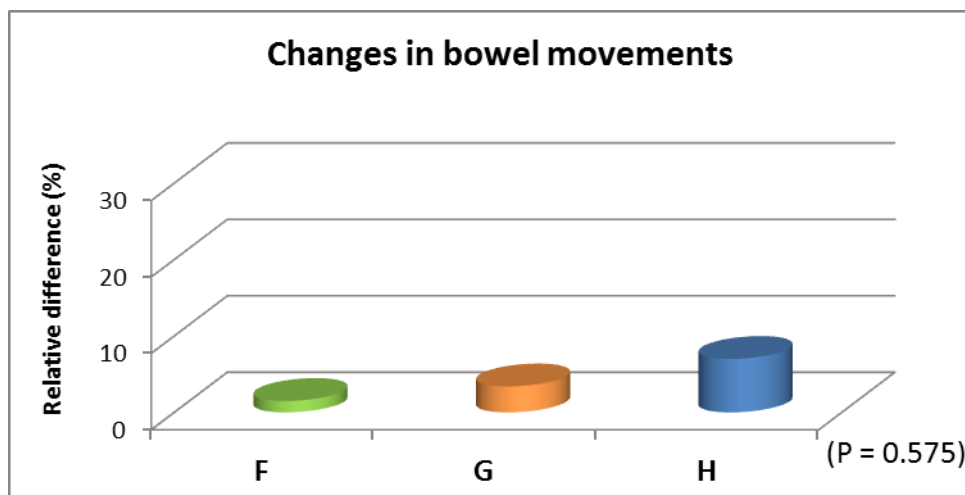
Figure 38. Dominant phyla aggregation intestinal microbiota in overweight and obese men

Actinobacteria abundance in young adults varies between 0.4-11.4% and *Proteobacteria* between 11-23% (Claesson *et al.*, 2010). Our *Actinobacteria* results are within the literature limits for young individuals; however the *Proteobacteria* abundance of overweight and obese men is more comparable to gut microbiota of elderly individuals (~2%). The phylum *Actinobacteria* contains *Bifidobacterium spp.*, which has been considered beneficial for human gastrointestinal health. *Proteobacteria* phylum is a gram-negative bacteria group containing a rich-lipopolisaccharides (LPS) outer membrane and includes important pathogens like *Salmonella*, *Vibrio*, *Escherichia*, among others. LPS is considered pro-inflammatory indicator generated by gram-negative bacteria and high circulating levels have been related with obesity (Amar *et al.*, 2008). Although *Actinobacteria* ($P = 0.073$) and *Proteobacteria* ($P = 0.849$) changes were not different among our treatments, a significant increment was observed in *Actinobacteria* with consumption of LGS ($18.5 \pm 3.5\%$; $P = 0.006$). Our previous results showed an apparent reduction in *Bifidobacteria* with the soymilk consumption which let us conclude that the increment in *Actinobacteria* is not due to *Bifidobacteria* abundance. On the other hand, *Proteobacteria* gut colonization significantly increased with the consumption of LGS ($90.7 \pm 34\%$), S ($132 \pm 47.6\%$) or bovine milk ($141.1 \pm 50.9\%$) (Figure 39). The decrease in *Actinobacteria -Bifidobacteria* and increase in *Proteobacteria* observed must be of special consideration due their relationship with type 2 diabetes (Wu *et al.*, 2010; Larsen *et al.*, 2010). Through a brief question about the bowel movement changes during the monthly interviews we determined no significant changes in defecation frequency ($P=0.575$) during the study (Figure 40). Phylogenic abundance profile in Table 18 provides more specific description of the gut microbiota genera and species changes in this study.



*P-value of the among treatment comparison

Figure 39. Relative change in *Firmicutes* and *Bacteroidetes* abundance after three months



Changes in bowel movement were less than 1 time/day for all treatments

Figure 40. Relative changes in bowel movements after three months

Table 18. Phylogenic abundance profile related with LGS, S and M consumption

ABUNDANCE (%)			LGS			S			M			
Phylum	Genera	Species	B	T3	P-value*	B	T3	P-value*	B	T3	P-Value*	P-value**
<i>Bacteroidetes</i>			15.54	17.97	<00001	16.32	20.37	0.0001	18.78	17.54	0.6153	0.777
	<i>Bacteroides</i>		11.11	13.78	0.658	9.03	9.90	0.498	10.83	11.29	0.066	0.261
		<i>Bacteroides vulgatus</i>	3.51	4.55	0.027	3.00	2.97	0.003	3.79	3.69	0.233	0.309
<i>Firmicutes</i>			83.43	79.59	<0.0001	82.93	78.08	<0.0001	77.02	77.64	<0.0001	0.494
	<i>Eubacterium</i>		16.28	16.73	0.049	14.65	16.68	<0.0001	14.06	16.67	0.130	0.356
		<i>Eubacterium rectale</i>	9.36	9.72	0.166	9.19	11.05	0.016	9.28	10.98	0.644	0.518
		<i>Eubacterium eligens</i>	2.23	2.48	0.233	2.52	2.18	0.038	1.49	1.73	0.537	0.589
	<i>Faecalibacterium</i>		35.26	33.53	<0.0001	39.89	31.65	<0.001	30.02	24.97	0.0007	0.501
		<i>Faecalibacterium prausnitzii</i>	35.26	33.53	<0.0001	39.89	31.65	<0.001	30.02	24.97	0.0007	0.501
	<i>Roseburia</i>		6.27	6.38	<0.0001	7.35	5.34	<0.0001	5.22	6.71	0.0007	0.851
		<i>Roseburia intestinalis</i>	4.12	4.28	0.463	5.11	3.72	0.651	3.87	4.88	0.592	0.759
		<i>Roseburia cecicola</i>	2.15	2.10	0.002	2.25	1.62	0.000	1.35	1.83	0.038	0.417
	<i>Ruminococcus</i>		3.94	3.71	0.502	3.12	3.68	0.005	2.60	3.78	0.006	0.867
	<i>Coprococcus</i>		0.54	0.40	0.003	1.27	1.15	0.000	0.99	0.89	0.006	0.754
	<i>Lachnospira</i>		0.79	0.95	0.776	0.49	0.76	0.025	0.67	0.62	0.648	0.713
	<i>Pseudobutyrvibrio</i>		0.42	0.25 ^a	0.0009	0.37	0.24 ^b	0.001	0.37	0.20 ^a	0.011	0.001
	<i>Clostridium</i>		15.85	13.22	0.002	11.76	13.73	<0.0001	18.20	17.91	<0.0001	0.497
		<i>Clostridium orbiscindens</i>	2.77	2.30	0.800	2.17	2.70	0.115	2.68	3.10	<0.0001	0.521
		<i>Clostridium bolteae</i>	1.98	1.60	0.081	1.57	1.84	<0.0001	2.92	2.72	0.016	0.427
		<i>Clostridium methylpentosum</i>	1.28	1.08	0.327	1.06	1.02	0.092	1.65	1.66	0.004	0.621
		<i>Clostridium aminophilum</i>	2.03	1.62 ^b	0.071	0.90	1.33 ^a	0.000	1.15	0.84 ^b	<0.0001	0.024
	<i>Clostridium symbiosum</i>	1.33	1.24	0.390	1.01	1.04	0.025	1.52	1.51	0.422	0.703	
	<i>Clostridium saccharolyticum</i>	0.83	0.59	0.775	0.77	0.82	0.813	1.54	1.48	0.523	0.260	
<i>Actinobacteria</i>			0.10	0.13	0.078	0.13	0.09	0.006	0.29	0.19	0.076	0.073
<i>Proteobacteria</i>			0.65	2.24	0.022	0.55	1.22	0.013	0.87	1.87	0.009	0.849

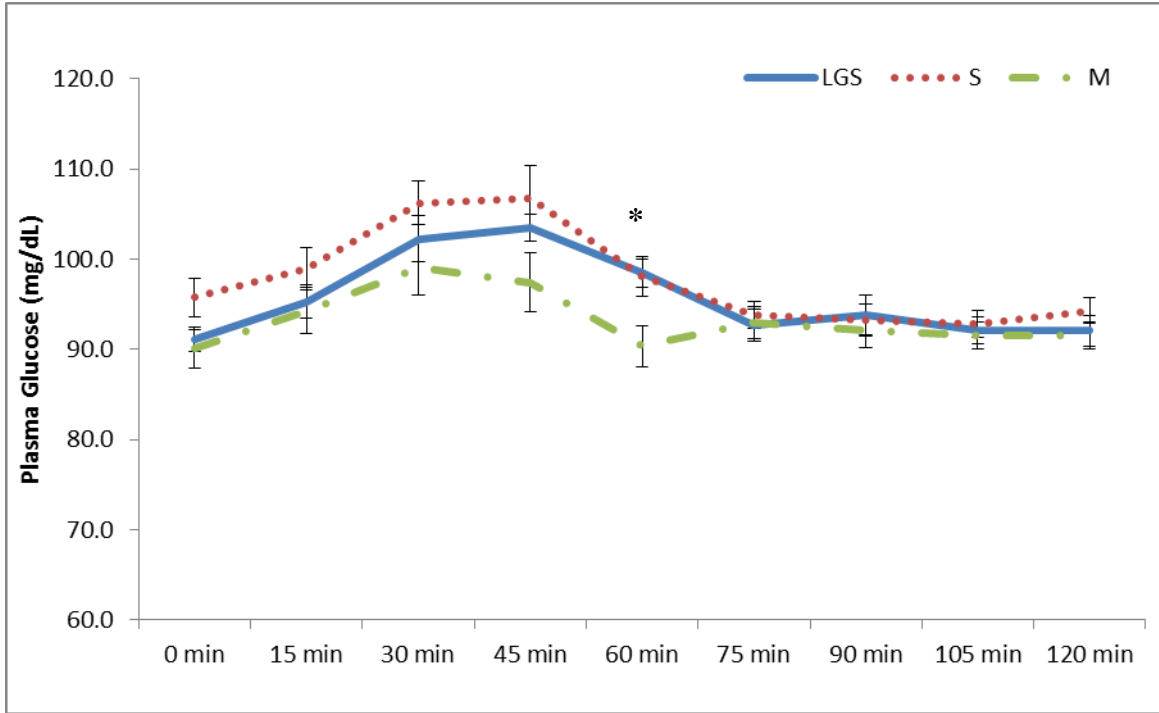
* P-value of changes within treatment. ** P-value of changes among treatment. B mean Baseline and T3 means after three months of consumption. Different letter in rows represent significant changes from baseline among treatments.

11. Glycemic response

LSG, S and M were processed and stored under the same conditions to eliminate bias due to treatment preparation. Analysis of low glycinin soymilk (49.9% β -conglycinin) and conventional soymilk (26.5% β -conglycinin) resulted in GI of 41.0 ± 7.2 and 40.4 ± 9.6 , respectively. The effect of both soymilk treatments was compared against bovine with GI of 29.2 ± 6.1 in overweight men. All beverages are low glycemic index products according to the American Diabetes Association (ADA, 2011) (GI of 55 or less). The international tables of glycemic index also classify our treatments as low GI food product.

Glucose maintenance (Barclay *et al.* 2010; Stevenson *et al.*, 2005), better glycemic responses (Jenkins *et al.*, 2002) and increase in satiety have been associated with low GI products. In contradiction, other authors did not find differences in glucose response in short term low GI food consumption (Jenkins *et al.*, 2011; Kurotobi *et al.*, 2010; Isken *et al.*, 2010; Cocate *et al.*, 2010; Nuttall *et al.*, 2011).

Animal studies developed by Tachibana *et al.*, (2010) and Moriyama *et al.* (2003) affirmed that β -conglycinin improves glucose levels more than casein. Our results contradict such findings, with no differences in postprandial blood glucose response among the high, regular and none β -conglycinin treatments throughout the 120 min of the analysis ($P = 0.338$) (Figure 41). The maximum glucose peak was observed at 45 min after consumption for all treatments. We also failed to prove that consumption of food products with similar carbohydrate content but different GI's, as shown in our soymilk and bovine beverage, promote different postprandial blood glucose response as shown by Parillo *et al.*, (2010).



Data are: Means \pm SEM. *($P < 0.05$). Time X treatment effect ($P = 0.331$)

Figure 41. Glycemic response of LGS, S and M.

The only difference observed in the postprandial glucose response throughout the time among treatments was a slight lower blood glucose concentration at minute 60 with bovine milk ($P = 0.021$). However, after 75 minutes all treatments tended to stabilize the glucose concentration between 90-100 mg/dL. LGS and S showed less monophasic than biphasic glucose curves. However, the glucose curve type did not influence the glycemic response (Table 19). According to Tschirritter *et al.* (2003) biphasic glucose curves are more associated with normal glucose tolerance than monophasic curves, and most of the curves observed with soymilk consumption were biphasic. The influences of BMI ($P = 0.177$), age ($P = 0.712$) and serum triglycerides ($P = 0.617$) were explored and none of them had an effect in postprandial glucose response under the condition of this study.

Nutrient intake analysis generated through a one-day diet record, for assessing of energy, carbohydrates, fat and protein intake were analyzed and showed no effect on postprandial glucose, except for protein intake ($P = 0.018$). However the difference of 6 g/day of protein intake was not enough to produce significant changes in glycemic response among treatments (Table 19). LGS, S and M presented fasting blood glucose of 91.1 ± 1.3 mg/dL, 94.1 ± 2.0 mg/dL and 91 ± 2.0 mg/dL respectively. These small differences were influencing the postprandial blood glucose response ($P = 0.0006$). However, the association with glycemic response is not clear. The power analysis for the postprandial glucose response was 96% increasing our confidence about our results and confirming the proper amount of subjects used for this study. After an analysis of the glycemic response using low glycinin soymilk, conventional soymilk and bovine milk we can conclude that β -conglycinin does not have an effect in blood postprandial glucose response in overweight and obese mean.

Table 19. Glycemic response of LGS, S and M.

TRT		LGS	M	S	<i>P</i> -value*
n		16	16	14	
Glycemic index		41.0 ± 7.2	40.4 ± 9.6	29.2 ± 6.1	
F a c t o r s o f I n f l u e n c e					
Fasting glucose (mg/dL)		91.1 ± 1.3	94.1 ± 2.0	91.2 ± 2.0	0.0006
Curve type	Monophasic	5	6	5	0.072
	Biphasic	9	7	8	
	Triphasic	1	1	3	
BMI		29 ± 1	29 ± 1	30 ± 1	0.177
Age	years	30.4 ± 1.4	30.1 ± 1.6	30.1 ± 1.5	0.712
Serum Triglycerides	(mg/dL)	78.9 ± 11.3	78.1 ± 12.1	80.2 ± 11.4	0.617
Dietary energy	Kcal	2472 ± 251	2699 ± 192.3	2611 ± 282	0.219
Dietary CHOs	g	366.7 ± 41.3	348.5 ± 34.2	340.6 ± 39.2	0.350
Dietary protein	g	103.9 ± 9.6	102.4 ± 7.5	96.4 ± 10.3	0.018
Dietary fat	g	121.9 ± 16.8	98.7 ± 9.5	97.3 ± 14.7	0.795

* *P*-values of the influence of variables in the glycemic index response (MANOVA). Data are Mean ± SEM. The treatment effect in glycemic index was not significant (*P* = 0.338)

VII. SUMMARY AND INTEGRATION

Obesity has many correlated negative effects in human health and affect a wide range of the U.S. population, approximately $\frac{3}{4}$ of the adult men population is obese or overweight. The use of dietary bioactive compounds in soybean has been studied as new strategic solutions to this epidemic problem; the present study focused its effort to evaluate the effect of β -conglycinin in body composition, inflammation, oxidative stress, gut microbiota and glycemic response.

Low glycinin soymilk (LGS) consumption caused less body fat accumulation than conventional soymilk (S) and bovine milk (M) ($P = 0.017$) after three months of consumption however no biological relevant changes among treatments were observed in total lean composition ($P = 0.201$), bone mass density ($P = 0.060$), BMD-t-scores ($P = 0.094$), waist-hip ratio ($P = 0.454$), BMI ($P = 0.721$) or weight ($P = 0.836$).

Relative differences from baseline in serum oxidized LDL decreased after LGS consumption ($-61 \pm 7.1\%$) in comparison to S ($-36 \pm 4.3\%$) and M ($-12 \pm 5.4\%$), ($p = 0.001$). Serum antioxidant capacity increased in LGS ($18\% \pm 3.5$) and S ($28\% \pm 4.6$) in comparison to M ($-40 \pm 9.9\%$), ($p < 0.0001$). Plasma adiponectin increased after three months of consumption of LGS ($14.9 \pm 5.3\%$) compared with S ($3.8 \pm 4.5\%$) and M ($-7.8 \pm 4.4\%$), ($p = 0.034$). The results suggest that LGS consumption can significantly improve the oxidative stress status. Considering that oxidized LDL is a biomarker of diabetes and other metabolic syndrome related conditions, serum antioxidant capacity and high level of adiponectin have been prove to decrease oxidative stress, under the condition of this study we can conclude that the consumption of soymilk with high content of β -conglycinin reduce oxidative stress in overweight men.

Plasma interleukin-6 after the consumption of LGS ($-22 \pm 9\%$) and S ($-26 \pm 5\%$) were significantly reduced in comparison to M (10.29 ± 12), ($p = 0.044$). Plasma CRP and TNF α did

not present differences after three months among treatments. The clear reduction in plasma interleukin-6 observed, confirms the hypothesis that soymilk has potential to protect against inflammation. Other biomarkers like plasma leptin ($P = 0.655$), plasma CRP ($P = 0.959$), plasma fatty acid synthase FAS ($P = 0.976$), plasma tumor necrosis alpha TNF α ($P = 0.610$), serum total cholesterol ($P = 0.320$) and serum triglycerides ($P = 0.947$) did not have an effect under the conditions of this study with the treatments.

Moderate physical activity ($P=0.210$) and strenuous physical activity ($P=0.490$) did not change or influence other data. Changes in dietary intake of energy, total protein, fat, carbohydrates, dietary fiber, Ca, Fe, P, K, caffeine, vitamins D, B-12, K, and E were not influenced by treatments during the study ($P >0.05$). However, sodium ($P = 0.019$) and vitamin C ($P = 0.0002$) intakes were reduced throughout time with the consumption of LGS.

Based on q-PCR, gut microbiota density increased with consumption of all treatments ($P < 0.05$). Changes in universal microbiota ($P = 0.266$), *Bacteroides* ($P = 0.266$), *Bifidobacteria* ($P = 0.068$) and *Lactobacillus* ($P = 0.063$) were not different among treatments; however, the effect of the treatments were nearly significant in *Bifidobacteria* abundance showing a decrease with the soymilk treatments. After a high throughput analysis of fecal samples, our results shown an increase in *Bacteroidetes* relative abundance with consumption of LGS ($30 \pm 11\%$, $p < 0.0001$) and S groups ($29 \pm 10\%$, $p = 0.0001$). The relative abundance of *Firmicutes* decreased for both the LGS ($-4 \pm 2\%$, $p < 0.0001$) and S groups ($-6 \pm 2\%$, $p < 0.0001$) yet increased for the M group ($3 \pm 3\%$, $p < 0.0001$). Based in the clear evidence of *Firmicutes* – *Bacteroidetes* composition in obese gut microbiota, we considere a positive that consumption of LGS and S may have a positive impact on *Bacteroidetes* and *Firmicutes* relative abundance and may help to reverse metabolic disorders in overweight men.

All treatments used in the present human study are considered low glycemic index. Postprandial glycemic responses were not affected with the consumption of LGS, S or M ($p = 0.338$) allowing us to conclude that beverages with different protein profiles with same glycemic index does not have different glucose response and specifically β -conglycinin does not affect blood glucose concentration in overweight men. A graphic summary of the results generated in this study is presented in Figure 42.

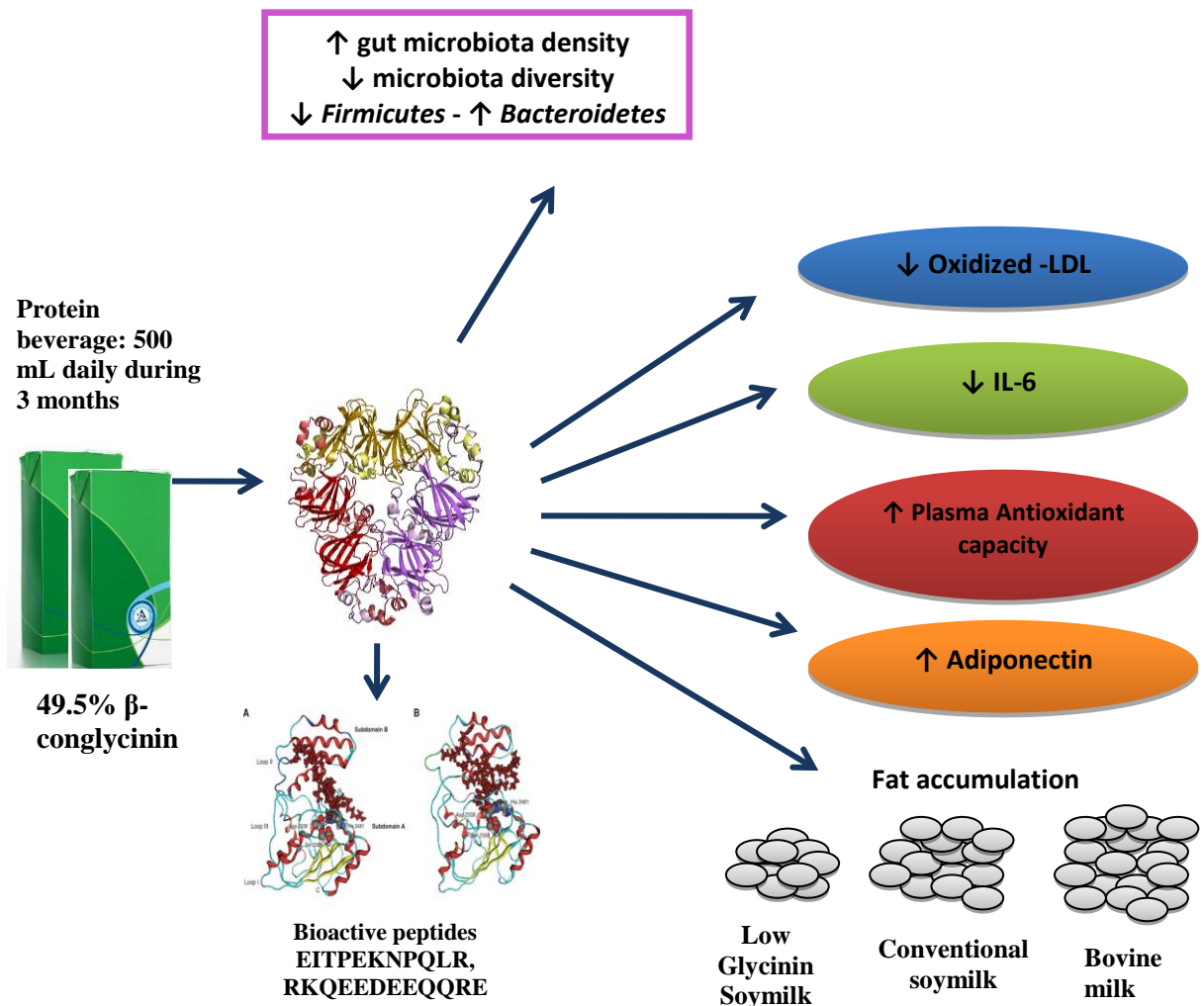


Figure 42. Summary of effect of LGS consumption in overweight men.

VIII. CONCLUSIONS

- ❖ Low glycinin soymilk ameliorates a modest but significant amount of body fat accumulation in comparison to conventional soymilk and bovine milk in overweight men.
- ❖ Low glycinin soymilk decreases serum oxidized LDL more than conventional soymilk and bovine milk in overweight men.
- ❖ Low glycinin soymilk reduces plasma interleukin-6 and improves serum antioxidant status compared to bovine milk in overweight men.
- ❖ The consumption of low glycinin soymilk may be useful to prevent body fat accumulation and reverse oxidative stress.
- ❖ Three months of low glycinin soymilk consumption increased plasma adiponectin in overweight men.
- ❖ The consumption of low glycinin soymilk for three months may not have any physiologically important effect on lean composition or bone mineral density in overweight men.
- ❖ Nutrients intake was maintained with low glycinin soymilk consumption, however reduction of vitamin C and sodium intake was observed.

- ❖ Under the conditions of this study microbial density increased in the same proportion with soymilk and bovine milk consumption.
- ❖ Gut *Bifidobacteria* abundance decreased with soymilk consumption following the normal tendency in overweight and obese individuals; however the total *Actinobacteria* phylum abundance increased.
- ❖ Low glycinin soymilk and conventional soymilk had a positive decrease in *Firmicutes* abundance and increase in *Bacteroidetes*.
- ❖ Our results suggested that the high content of β -conglycinin in low glycinin soymilk did not have an effect in postprandial blood glucose response in overweight men.
- ❖ The high content of β -conglycinin in low glycinin soymilk contain bioactive peptides that may be a useful therapeutic strategy to include in meals designed to reduce inflammation, oxidative stress and to alters gut microbiota composition with potential anti-obesity effect in overweight men.

IX. FUTURE STUDIES

- i. Based on the body composition results, and other studies in the literature, a longer period of treatment may have allowed more changes in body composition. DXA scans may be performed at baseline, 3 months and 6 months of consumption.
- ii. An increment in sample size (63/treatment), to compare among treatments, will increase the power analysis for parameters such as plasma C-reactive protein, serum leptin, plasma tumor necrosis factor α , serum total cholesterol and plasma fatty acid synthase. Based on power analysis, more than 1000 subjects per treatment may be required to see changes in serum triglycerides among overweight men. For future studies, the subject eligibility criteria should be narrowed to avoid influences of age and BMI as observed in this study with the serum oxidized-LDL changes. Subjects with a BMI between 25 - 29 and age of 18 - 35 years may reduce the variability.
- iii. To detect differences among treatments in *Bifidobacteria*, *Firmicutes*, *Lactobacillus* and *Proteobacteria*, the sample size should increase to 56 subjects per treatment to have a power analysis of 80%. For *Bacteroides* analysis more than 4000 subject per treatment will be required to have a sample power of 80%.
- iv. More detailed restrictions on carbohydrate intake one day before blood glucose assessment will standardize the conditions of the subjects in future glycemic response studies.

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APPENDIX A


\$500 - Soymilk protein and weight loss

SECOND ROUND OF PARTICIPANTS

Who can participate:

- Male between 18 – 45 years old
- Must not have any chronic disease, especially gastrointestinal disease.
- Must have a Body Mass Index (BMI) of 25 or more (overweight or obese) but less than 300 pounds. Check your BMI in www.freebmiccalculator.net/ or use the formula [WEIGHT (pounds) / (HEIGHT (in)²) x 703
- Nonsmokers, non vegetarian, can be recreationally active, but not athletes. Must not be taking antibiotics.
- Must be present on campus during the fall 2009.
- Individuals must be willing to consume two glasses of soymilk/cow milk per day, during a three month period at home and attend to four sampling sessions (or laboratory visits).
- You will be compensated up to **\$500** for participation.

Contact
Dina Fernández
dinafer@illinois.edu
217-244-3198



Aug -17 - 2009

Flier used in the recruitment process

APPENDIX B

Health questionnaire

"Soymilk protein profile modulates human adipogenesis through changes in gut microbiota."

Complete name: _____ Age: _____

Are you: American or International → Country: _____

Email: _____ Phone: _____

Local Address: _____

INSTRUCION: Mark or give color to the word **YES** or **NO**. Change the name of the file with your name and send this document to dinafer@illinois.edu

1. Are you allergic to soy products? Yes No
2. Are you lactose intolerant? Yes No
3. Do you weigh more than 300 pounds? Yes No
4. Are you an athlete? Yes No
5. Do you suffer from any physical or mental disease, or have you ever had a major surgery? Yes No
6. Do you take antibiotics or dietary supplements? Yes No
7. Do you smoke? Yes No
8. Are you a vegetarian? Yes No
9. Do you suffer from any chronic disease? Yes No
(i.e., chronic constipation, diarrhea, ulcerative colitis, irritable bowel syndrome, diverticulosis, stomach or duodenal ulcers, hepatitis, HIV, cancer, etc.)
10. Are you overweight (BMI 25-30). Yes No
Your weight is _____(Pounds) and your height is _____(feet)
11. Are you willing to avoid the consumption of any soy food products during the week before the study? Yes No
12. Are you willing to consume two (250 ml) Tetra-Pak containers of soymilk or cow milk every day during three months? Yes No
13. Are you willing to avoid the intake of additional soymilk or cow milk during the study? Yes No
14. Are you willing to give blood and feces specimens? Yes No
15. Are you willing to let us scan you body with DXA machine and to measure your weight, height and circumferences every month? Yes No

16. Are you willing to avoid any food product with pre-probiotics? Yes No

17. Are you willing to don't change your normal physical activity during the next three months?

Yes No

18. Are you student?

Yes No

If yes:

Department: _____

Are you graduate or undergrad student? _____

19. **If you are international student**; do you have a 50% of appointment?

Yes No

20. **If you are International**; are you willing to provide copies of personal documents like; (Visa, passport ID, I-20 or DS2019, entry stamps, I-94, social security, I-card) this documents are required to receive a payment of \$500.

Yes No

21. Are you employee of the university of Illinois

Yes No

Department: _____

22. Are you available to attend meetings the following dates?

First meeting for samples collection: September 21, 22 or 23th Yes No

Second meeting for samples collection: October 19, 20 or 21th Yes No

Third meeting for samples collection: November 16, 17 or 18th Yes No

Last meeting for samples collection: December 14, 15 or 16th Yes No

Comments about the dates:

Thanks for your participation in this study

APPENDIX C

DOCUMENTS USED FOR DIETARY INSTRUCTIONS

List of prebiotic and probiotic products to avoid during the study

Probiotic and prebiotic supplements

All food products with level highlighting phrases like active bacteria, live cultures, active culture, prebiotic and prebiotic.

Yogurts with pro and pebiotics

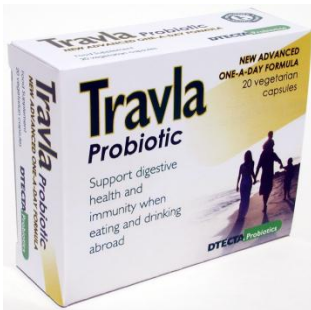
- Dannon
- Yoplait plus
- Stonyfield Farm
- Whole Soy & Co.
- Turtle Mountain

Milk, cereals and breakfast bars with probiotic yogurt powder or active cultures

Smoothies

- **Healthy Dairy**
- **Stonyfield Farm**
- **Satiety Smoothies**
- **Dannon**

Probiotic Frozen Yogurt



Foods to Avoid during one - week before the study

- Any food containing the word soy, for example, soy milk, soy flour, soy yogurt... etc
- Meat alternatives
- Prebiotic and probiotic yogurt, kefir, fermented products, pre and probiotics supplements
- Veggie/Garden Burgers
- Soy Fiber (Okara, Soy Bran, Soy Isolate Fiber)
- Kashi products
- Yuba (Yuba is made by lifting and drying the thin layer formed on the surface of cooling hot soymilk. It has high protein content and is commonly sold fresh, half-dried and dried)
- Dry blended beverages
- Food bars
- Protein bars
- Dijon Style Mayonnaise or mustard
- Chai
- Keto Mix (mix for pancakes and muffins)
- Lean Shakes
- Canned chicken broth
- Fast Food (example, McDonald's, Wendy's, Burger King)
- Vegetable broth, gum, and starch
- Bouillon cubes (beef, chicken, vegetable, etc.)
- Smoothies
- Textured vegetable protein: Textured soy protein (TSP) usually refers to products made from textured soy flour, although the term can also be applied to textured soy protein concentrates and spun soy fiber.
- Hydrolyzed vegetarian protein (HVP): HVP is a flavor enhancer that can be used in soups, broths, sauces, gravies, flavoring and spice blends, canned and frozen vegetables, meats and poultry
- Lecithin: Lecithin is used in food manufacturing as an emulsifier in products high in fats and oils. It also promotes stabilization, antioxidation, crystallization and spattering control.
- Miso: Miso is a rich, salty condiment that characterizes the essence of Japanese cooking. The Japanese make miso soup and use it to flavor a variety of foods. Miso is made from soybeans and a grain such as rice, plus salt and a mold culture, and then aged in cedar vats for one to three years
- Natto: Natto is made of fermented, cooked whole soybeans.
- Soy Sauce (Tamari, Shoyu, Teriyaki)
- Tempeh: Is a traditional Indonesian food, is a chunky, tender soybean cake. Whole soybeans, sometimes mixed with another grain such as rice or millet, are fermented into a rich cake of soybeans with a smoky or nutty flavor. Tempeh can be marinated and grilled and added to soups, casseroles, or chili.
- Tofu: A type of cheese made out of soybeans.

Some other things to keep in mind Some salad dressings, mayonnaise, sauces, or gravies may contain soy products

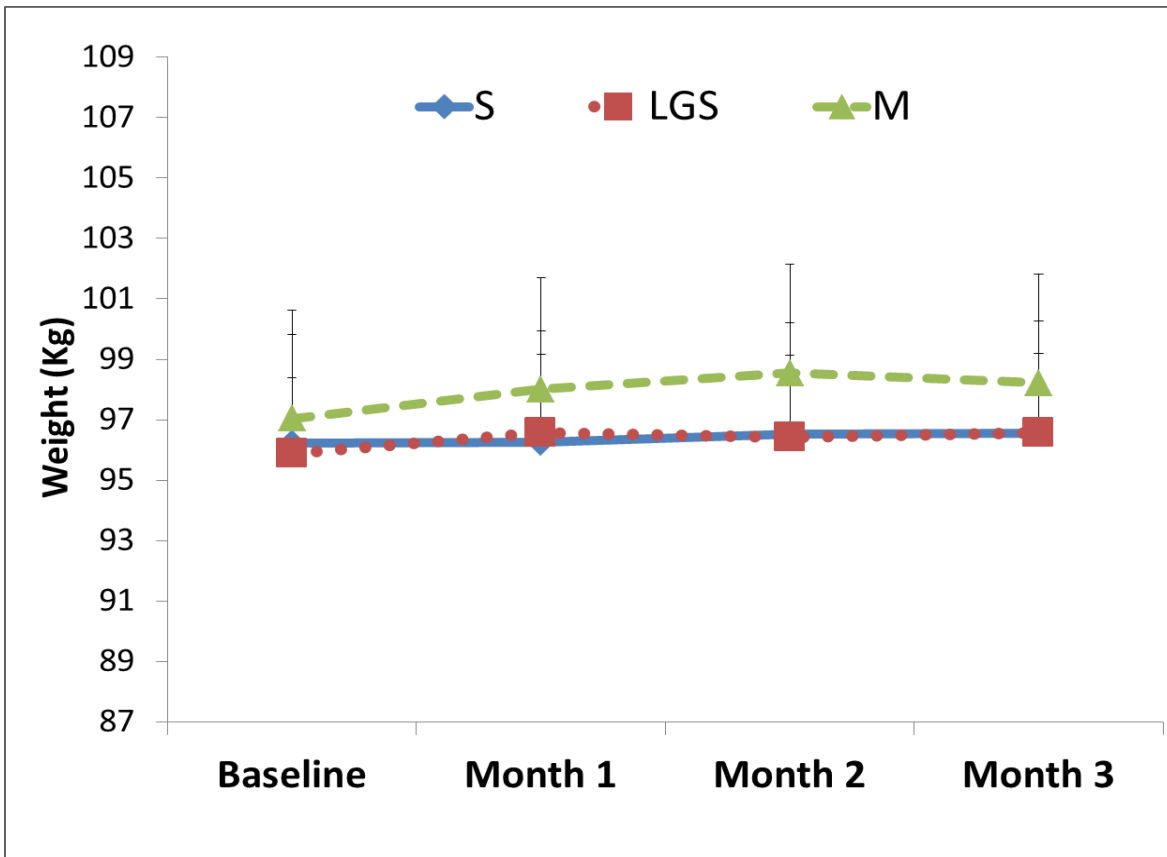
- Margarine and butter substitutes, may contain soy products
- Soy products may be used in some commercial ice creams and other frozen desserts
- Baked goods, such as cakes or cookies which contain soy flour
- Soy is used in many canned soups, commercial entrees, and combination foods
- Some commercially prepared meats use soy as a meat extender
- Pork link sausage, deli/luncheon meats may be made with soy

APPENDIX D

Panelists	1ST MONTH	2ND MONTH	3RD MONTH
	Returned Codes	Returned Codes	Returned Codes
Conventional soymilk	52	52	55
%	92	93	98
Low glycinin soymilk	50	53	52
%	90	94	92
Bovine milk	54	54	55
%	96	96	98

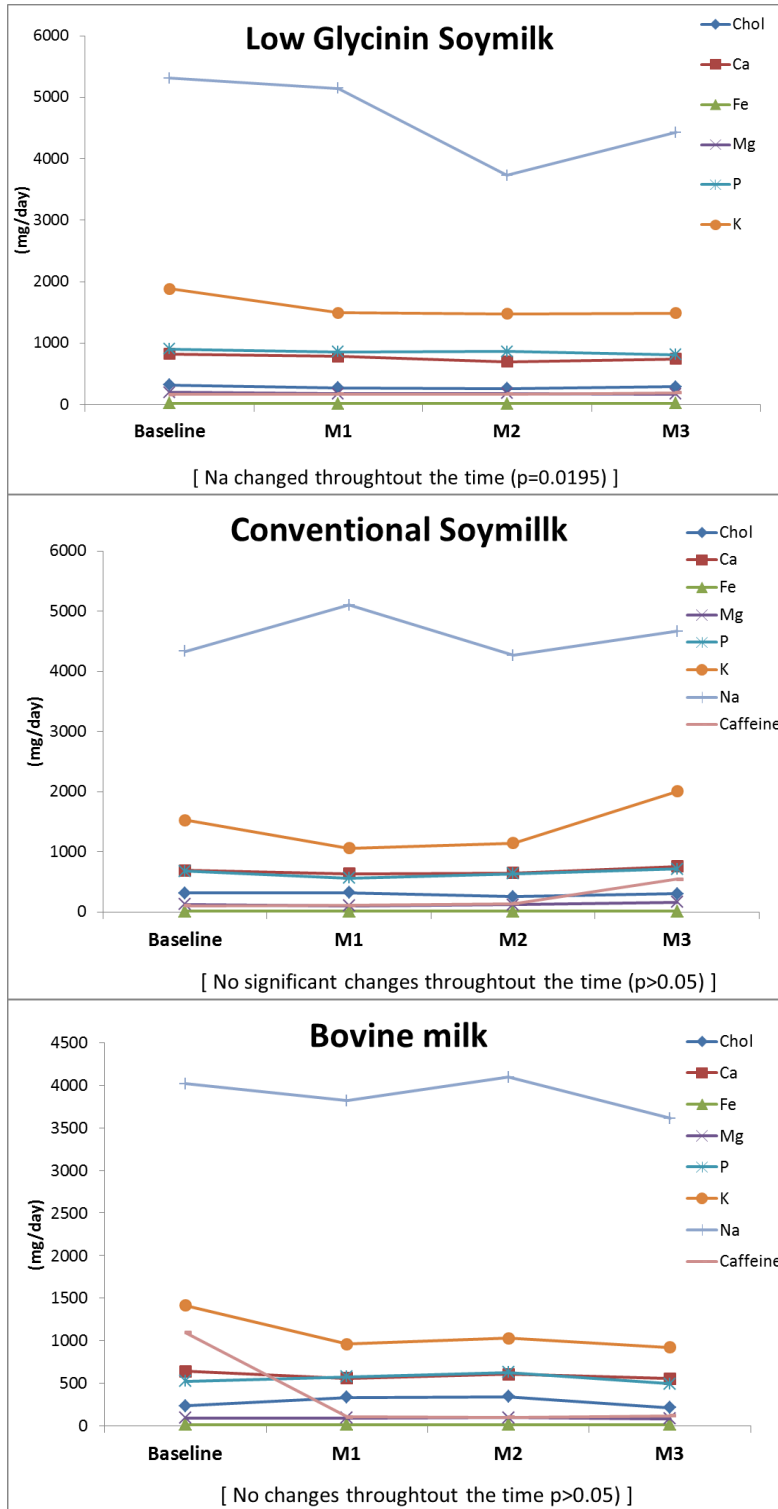
Compliance assessment (returned codes)

APPENDIX E

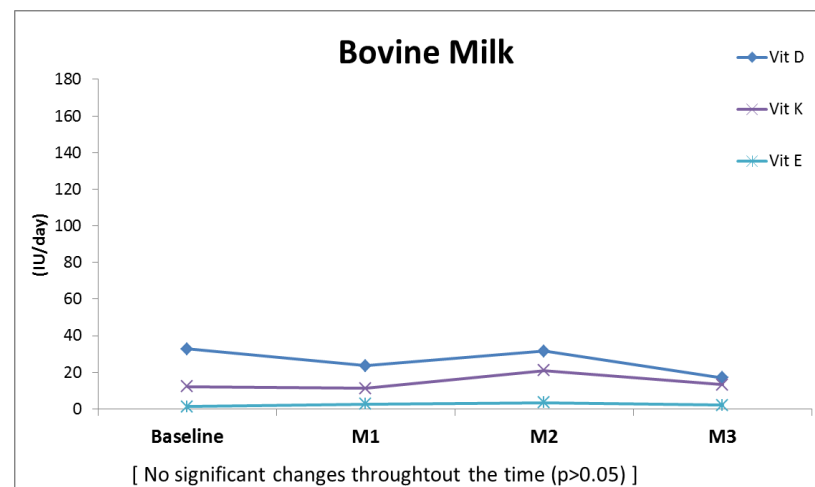
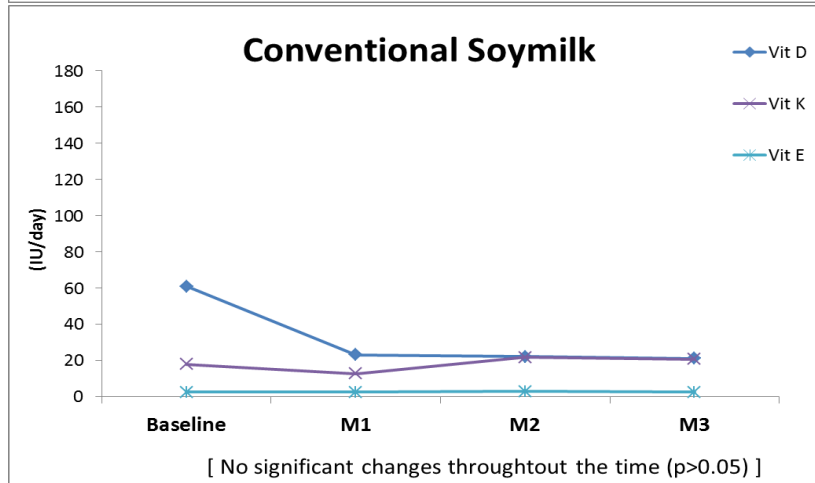
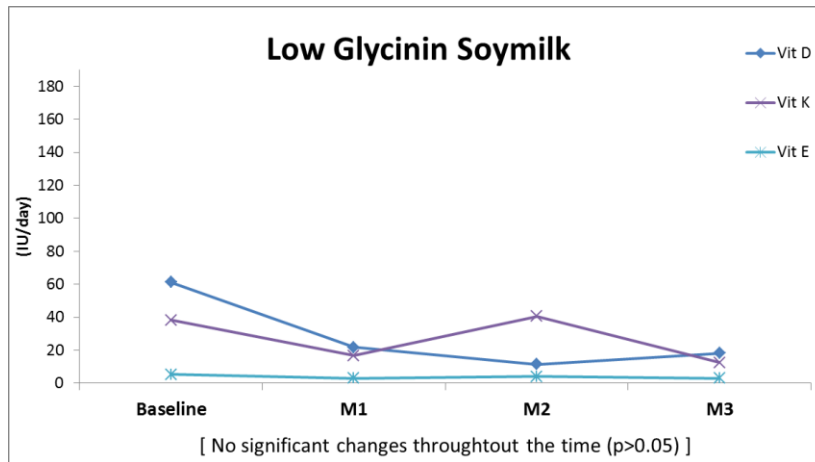


Body weight changes over time, among treatments, was observed ($p = 0.932$), means \pm SEM.

APPENDIX F



Minerals intake during the three months of treatment consumption



Vitamins intake during the three months of treatment consumption

APPENDIX G

Sample	Daidzin	Daidzein	Genistin	Genistein	Glycintin	Glycitein	Total If	Total If (Aglycone Equiv)
S	97.5 ± 0.7	2.0 ± 0	132.5 ± 0.7	2.0 ± 1.4	8.0 ± 0	<1 ± 0	242.0 ± 2.8	151.5 ± 2.1
LGS	95.5 ± 0.7	3.0 ± 0	98.0 ± 1.4	2.5 ± 0.7	5.5 ± 0.7	<1 ± 0	205.0 ± 0	129.5 ± 0.7

* Data are means ± SD (p > 0.05). Total isoflavones concentration in bovine's milk was < 1 ppm

Isoflavones (If) concentration (ppm) in S and LGS.*

	Asp	Ser	Glu	Pro	Gly	Val	Lys	His	Arg
S	117.6 ± 8.1	50.5 ± 4.1	180.2 ± 11.4	50.8 ± 2.8	43.3 ± 3.1	47.8 ± 0.3	61.2 ± 5.0	24.2 ± 2.3	83.1 ± 3.8
LGS	113.8 ± 8.4	49.3 ± 5.2	168.7 ± 12.6	47.7 ± 2.2	40.2 ± 2.7	45.9 ± 0.0	67.1 ± 6.9	25.5 ± 1.6	92.3 ± 4.4

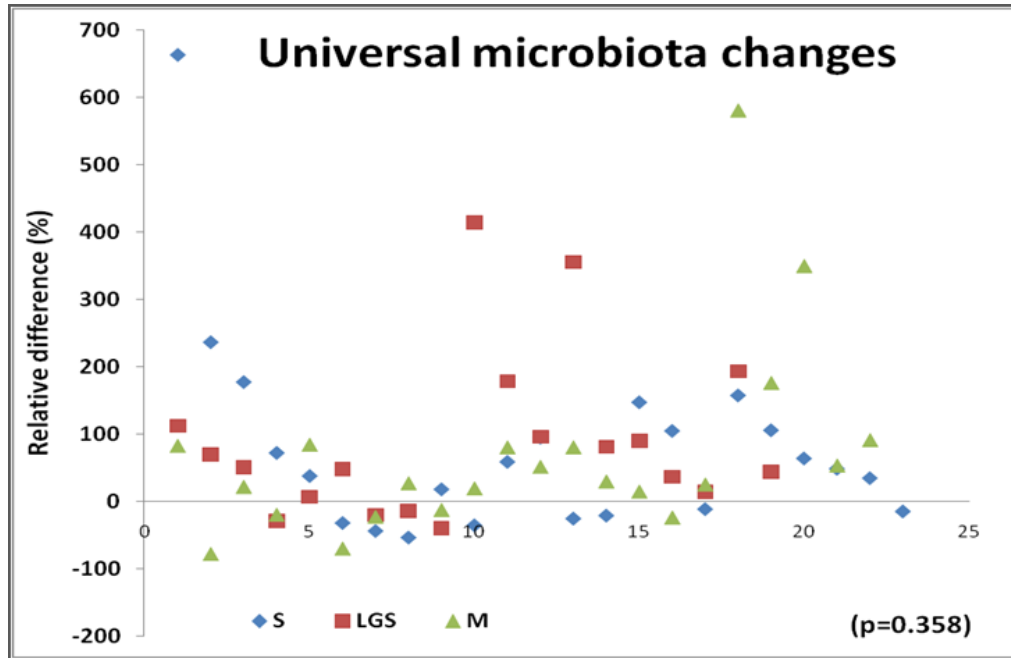
*Differences in Thr, Ala, Gly, Ile, Leu, Tyr, Phe, Cys and Met between S and GLS were < 1 mg/g. Means ± SD.

Amino acids concentration (mg/g) in S and LGS.* SDS-PAGE of soymilk powders

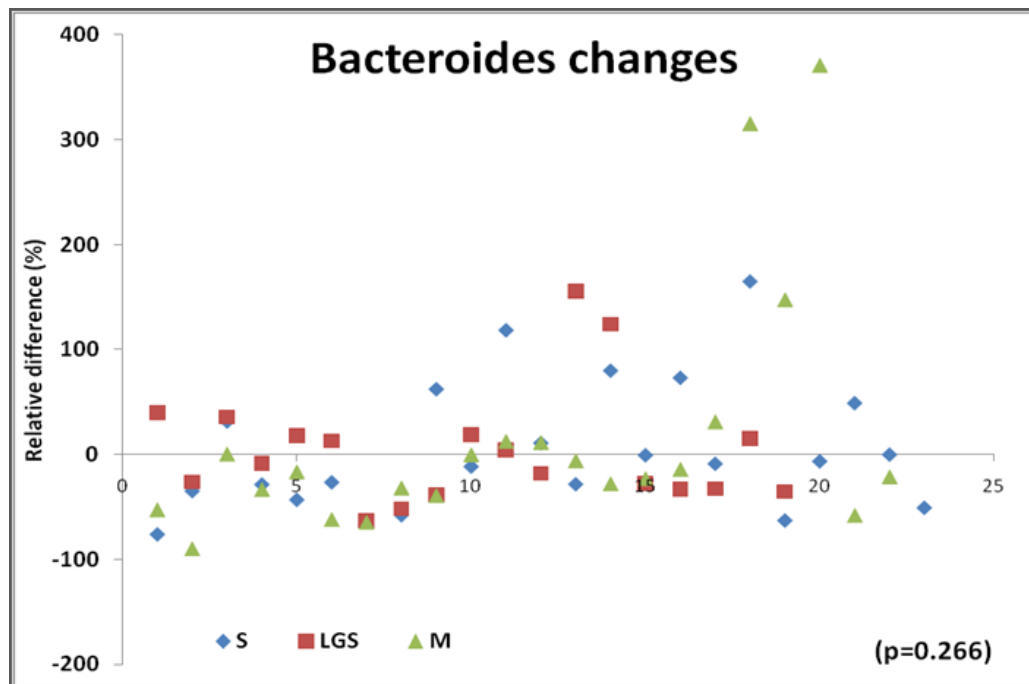
Type	SDS-PAGE Gel Lane	LOX 2&3 %	LOX 1 %	Alph a' BC %	Alpha BC %	alpha / alpha'	Beta BC %	Total BC %	A3 gly %	A1,2,4 Gly %	Basic Gly %	Total Glycini n %	KTI %
Control	E-9204 123-05	3.8	1.6	8.7	12.1	1.4	5.4	26.2	3.4	16.5	18.8	38.6	3.6
HBC	E-9204 123-03	4.1	1.7	18.5	22.3	1.2	7.1	48.0	0.0	2.4	3.5	5.9	4.8
HBC	E-9204 123-04	4.1	1.6	17.7	22.7	1.3	7.5	47.9	0.0	2.7	3.6	6.3	4.5
HBC	E-9204 123-02	4.3	1.7	19.0	23.5	1.2	7.2	49.6	0.0	2.4	3.5	5.8	4.5
HBC	E-9204 123-07	4.0	1.7	17.9	22.5	1.3	8.2	48.6	0.0	2.8	3.5	6.3	4.4
HBC	E-9204 123-06	4.1	1.7	17.8	22.8	1.3	7.9	48.5	0.0	2.8	3.6	6.4	4.5
HBC	E-9204 123-01	4.4	1.7	18.2	23.6	1.3	7.1	49.0	0.0	2.4	3.6	6.0	4.8
Control	E-9204 123-14	4.0	1.6	9.0	12.2	1.4	5.6	26.8	3.5	16.5	18.8	38.8	3.2
HBC	E-9204 123-12	4.0	1.5	18.3	23.8	1.3	7.6	49.7	0.0	2.4	3.3	5.7	4.3
HBC	E-9204 123-13	4.2	1.6	18.6	24.3	1.3	7.7	50.5	0.0	2.7	3.1	5.8	4.1
HBC	E-9204 123-11	4.0	1.6	18.3	24.2	1.3	7.8	50.4	0.0	2.5	3.2	5.6	3.5
HBC	E-9204 123-16	4.3	1.7	18.6	23.9	1.3	7.9	50.3	0.0	2.7	3.1	5.8	3.4
HBC	E-9204 123-15	4.3	1.7	18.6	24.2	1.3	7.8	50.5	0.0	2.9	3.1	6.0	3.4
HBC	E-9204 123-10	4.1	1.6	18.7	23.9	1.3	7.7	50.3	0.0	2.6	3.3	5.9	3.9

Control = Conventional soymilk, HBC = Low glycinin soymilk

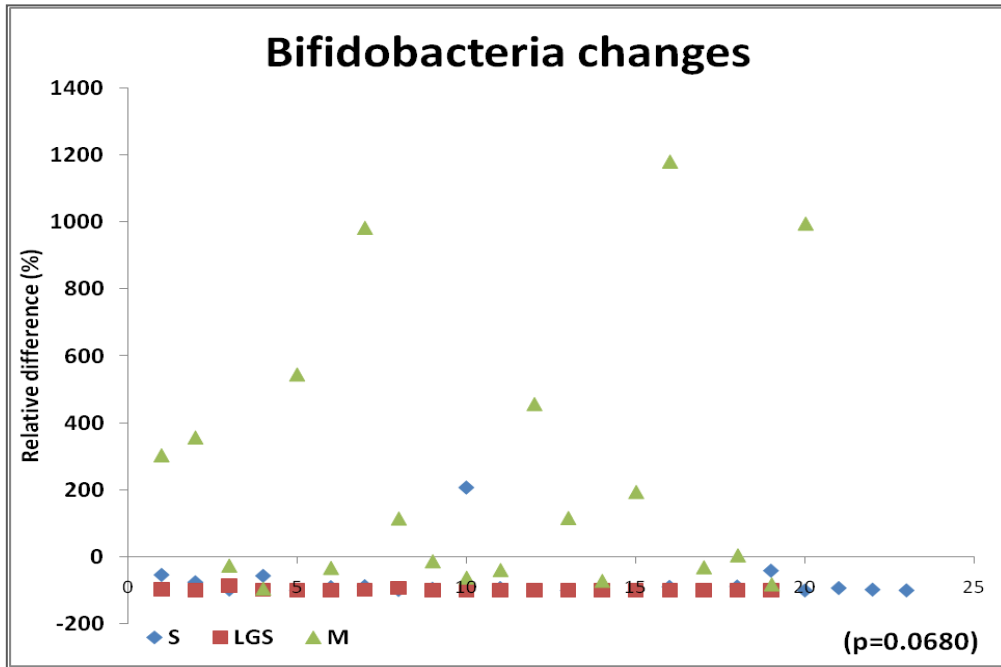
APPENDIX H



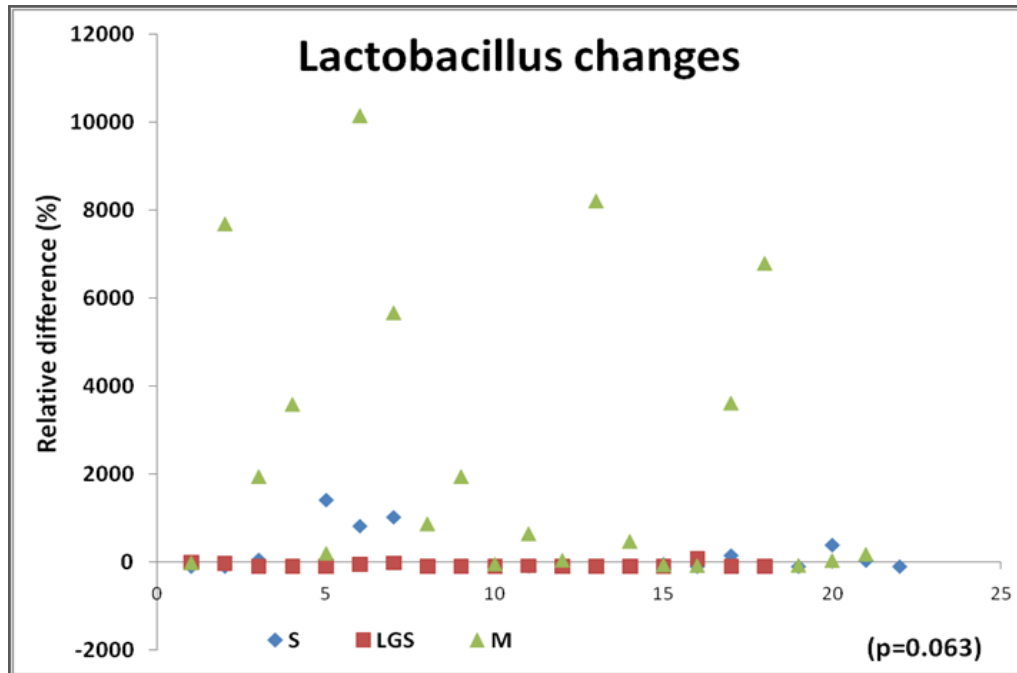
Individual subject analysis of changes in universal microbiota per treatment



Individual subject analysis of changes in *Bacteroides* per treatment

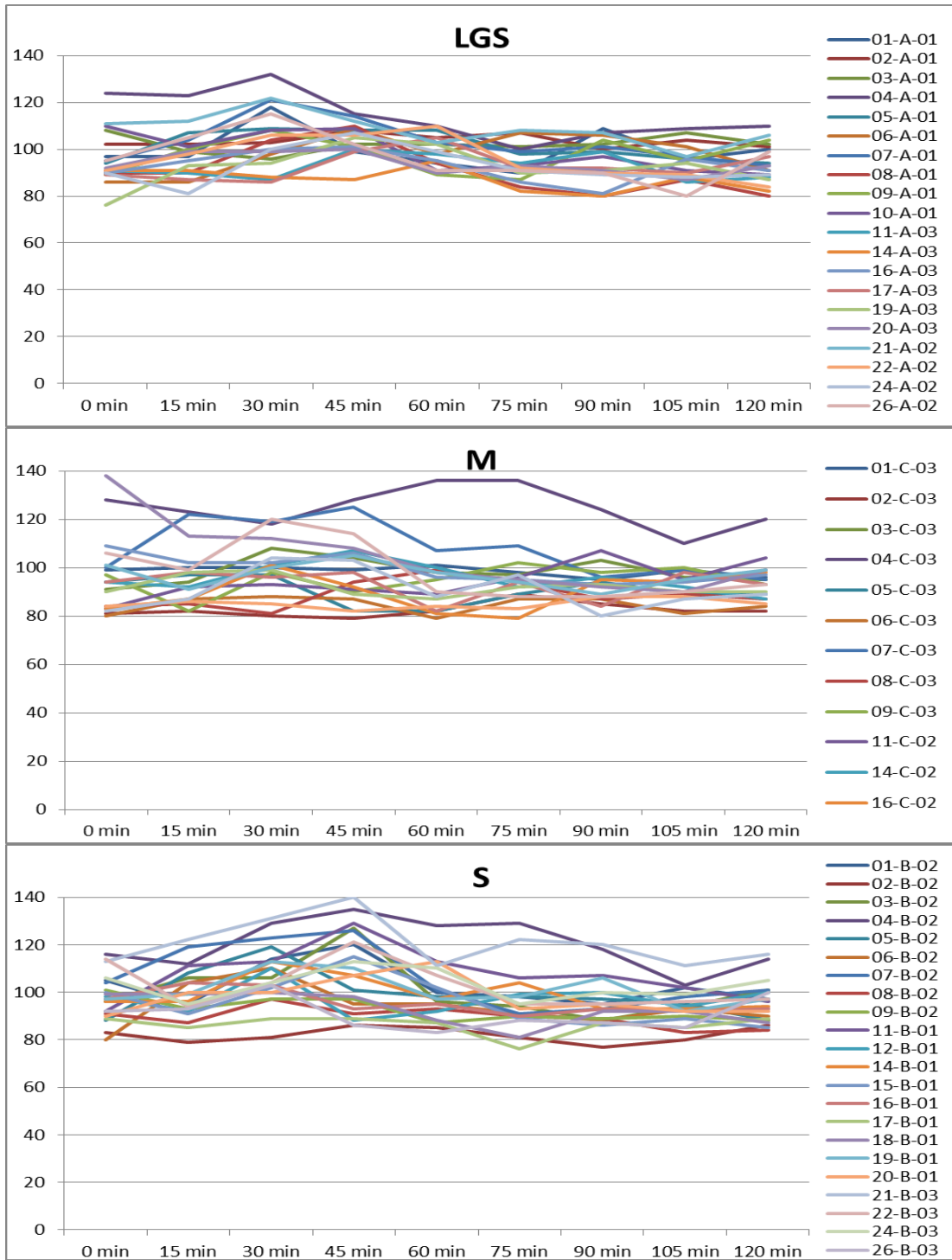


Individual subject analysis of changes in *Bifidobacteria* per treatment



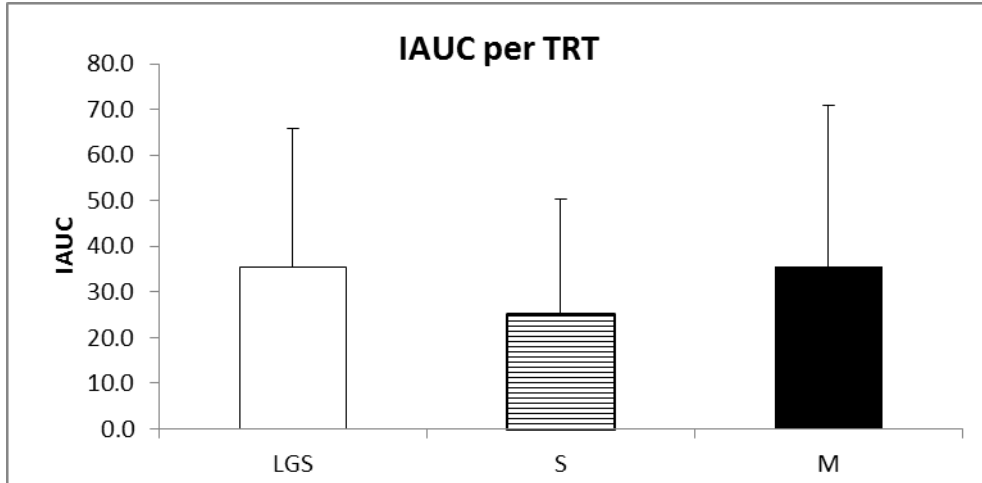
Individual subject analysis of changes in *Lactobacillus* per treatment

APPENDIX I

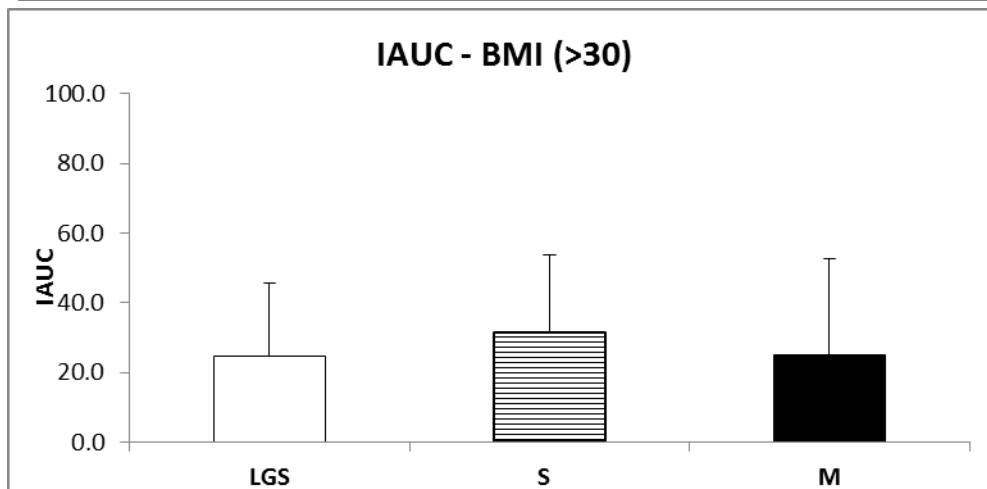
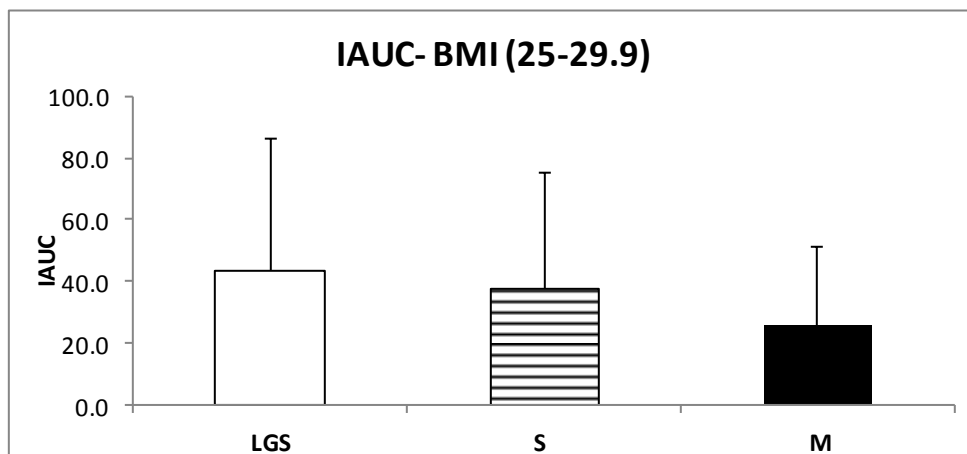


Postprandial blood glucose response after LGS, S and M consumption

APPENDIX J



Incremental area under the curve of the postprandial glycaemic response per treatment



Exploratory analysis of incremental areas under the curve postprandial glycaemic response per treatment per BMI

APPENDIX K

Low glycinin soybeans were enriched in β -conglycinins, with similar amino acids, isoflavones and fatty acids profiles as conventional soybean. β -conglycinin and other soybean components are potential protective agents against body fat accumulation and inflammation. The objective was to compare the effect of low glycinin soy milk (LGS), conventional soy milk (S) and cow's milk (M) on body fat accumulation, serum lipids, biomarkers of inflammation and oxidative stress in overweight men. In a randomized, double-blind, placebo controlled IRB approved study, 64 overweight men (BMI > 25) were divided into three groups and fed daily for 3 months 500 ml of LGS, S or M. No changes were observed in their caloric intake during the study ($p > 0.05$). LGS consumption caused less total body fat accumulation compared to S ($p = 0.015$) and M ($p = 0.011$). Oxidized LDL was dramatically decreased after LGS consumption (-61%) in comparison to S (-36%, $p = 0.0007$) and M (-12%, $p = 0.002$). Serum antioxidant capacity increased significantly in LGS (18%, $p = 0.001$) and S (28%, $p < 0.001$) compared with M (-40%). The decrease in plasma interleukin-6 after 3 months of LGS (-22%, $p = 0.025$) and S (-26%, $p = 0.014$) consumption was significantly different to M. LGS ameliorated body fat accumulation and improved oxidative stress and antioxidant status compared to M in overweight men. LGS consumption may be useful to prevent body fat accumulation and reverse oxidative stress.



Low glycinin soymilk ameliorates body fat accumulation and improves serum antioxidant status in overweight men

Dina Fernández,^a Cristina Martínez-Villaluenga,^a Neal A. Bringe^b and Elvira Gonzalez de Mejia^a

^aDepartment of Food Science and Human Nutrition, University of Illinois at Urbana Champaign, 228 ERLM, 1201 W. Gregory Dr., Urbana, IL 61801. ^bThe Monsanto Company, St Louis MO.

INTRODUCTION

- Soy protein consumption may reduce body fat, increase satiety, lower blood lipids and produce a positive effect in energy balance (1).
- β -conglycinin is an effective food ingredient that can be used to reduce obesity (2).
- β -conglycinin embeds more active peptides than glycinin subunits that inhibit lipid accumulation and induce adiponectin in 3T3-L1 adipocytes (3).

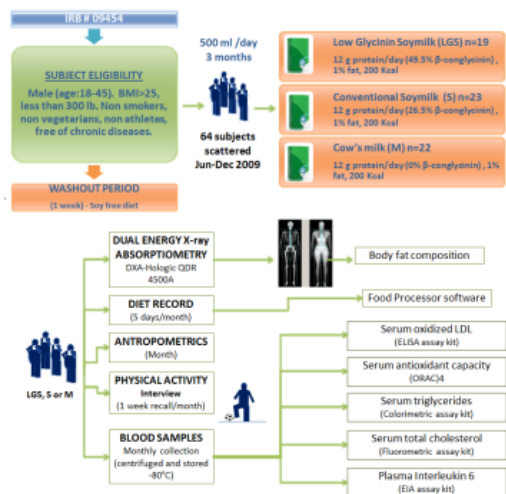
HYPOTHESIS

Low glycinin soymilk (LGS), suppresses body fat, serum lipids, biomarkers of inflammation and oxidative stress.

OBJECTIVE

Compare the effect of low glycinin soymilk (LGS), conventional soymilk (S) and cow's milk (M) on body fat accumulation, serum lipids, biomarkers of inflammation and oxidative stress in overweight men.

MATERIALS AND METHODS



Statistical analysis: SAS, version 9.1 (SAS Institute). Univariate analyses with data transformations, General linear model (GLM), repeated measures analysis and mixed model with random effect were used ($p < 0.05$). Influence of age, initial BMI, diet intake and physical activity were considered in the mixed model.

RESULTS

Table 1. Baseline characteristics of subjects per treatment.*

Characteristic	Low glycinin soymilk (LGS)	Conventional soymilk (S)	Cow's milk (M)
Age (years)	35 ± 6	32 ± 8	32 ± 6
Weight (kg)	95.8 ± 11.1	96.2 ± 17.3	97.0 ± 17.2
BMI	29 ± 5	29 ± 4	30 ± 4
Total Fat (%)	28 ± 3.5	27 ± 6.0	28 ± 5.6
Waist-Hip ratio	0.95 ± 0.05	0.94 ± 0.07	0.94 ± 0.06

*Data are means ± SD ($p > 0.05$)

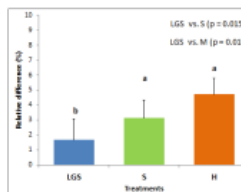


Figure 1. LGS ameliorates total body fat accumulation after 3 months of consumption, means ± SEM.

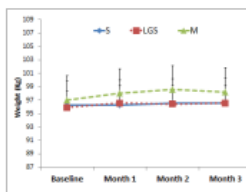


Figure 2. No significant change of body weight over time, among treatments, was observed ($p = 0.932$), means ± SEM.

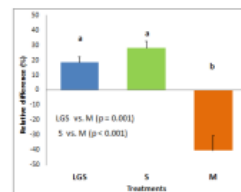


Figure 3. LGS and S increased serum antioxidant capacity after 3 months of consumption, means ± SEM.

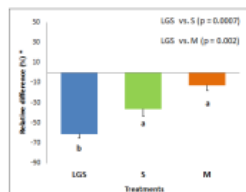


Figure 4. LGS consumption decreased serum oxidized LDL after 3 months, means ± SEM. *Change affected by age ($p = 0.03$) and initial BMI ($p = 0.02$)

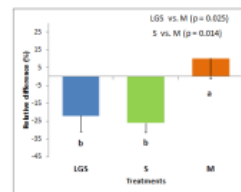


Figure 5. Plasma interleukin-6 decreased after 3 months of LGS and S consumption, means ± SEM.

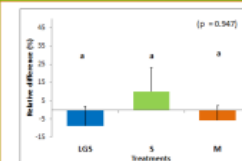


Figure 6. Serum triglycerides did not present significant changes, after 3 months of consumption, means ± SEM.

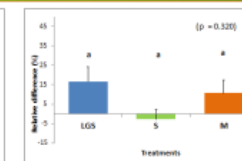


Figure 7. Serum total cholesterol did not present significant changes after 3 months of consumption, means ± SEM.

Dietary intake ($p = 0.399$), moderate physical activity ($p = 0.209$) and strenuous physical activity ($p = 0.489$) did not change significantly in the three months of consumption.

Table 2. Isoflavones (If) concentration (ppm) in S and LGS.*

Sample	Daidzin	Daidzein	Genistin	Genistein	Glycitin	Glycitein	Total If	Total If (Alycyone Equiv)
S	97.5 ± 0.7	2.0 ± 0	132.5 ± 0.7	2.0 ± 1.4	8.0 ± 0	<1 ± 0	242.0 ± 2.8	151.5 ± 2.1
LGS	95.5 ± 0.7	3.0 ± 0	98.0 ± 1.4	2.5 ± 0.7	5.5 ± 0.7	<1 ± 0	205.0 ± 0	129.5 ± 0.7

*Data are means ± SD ($p > 0.05$). Total isoflavones concentration in Cow's milk was < 1 ppm

Table 3. Amino acids concentration (mg/g) in S and LGS.*

	Asp	Ser	Glu	Pro	Gly	Val	Lys	His	Arg
S	117.6 ± 8.1	50.5 ± 4.1	180.2 ± 11.4	50.8 ± 2.8	43.3 ± 3.1	47.8 ± 0.3	61.2 ± 5.0	24.2 ± 2.3	83.1 ± 3.8
LGS	113.8 ± 8.4	49.3 ± 5.2	168.7 ± 12.6	47.7 ± 2.2	40.2 ± 2.7	45.9 ± 0.0	67.1 ± 6.9	25.5 ± 1.6	92.3 ± 4.4

*Differences in Thr, Ala, Gly, Ile, Leu, Tyr, Phe, Cys and Met between S and LGS were < 1 mg/g. Data are means ± SD, ($p > 0.05$).

CONCLUSIONS

- > Low glycinin soymilk ameliorates body fat accumulation in comparison to conventional soymilk and cow's milk in overweight men.
- > Low glycinin soymilk decreases serum oxidized LDL more than conventional soymilk and cow's milk in overweight men.
- > Low glycinin soymilk reduces plasma interleukin-6 and improves serum antioxidant status compared to cow's milk in overweight men.
- > The consumption of low glycinin soymilk may be useful to prevent body fat accumulation and reverse oxidative stress.

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[1] Velasquez and Bhattacha. 2007. Int. J. Med. Sci. 4, 72-82. [2] Kohno, M., et al. 2006. J. Athl. Therabiosci. 13, 247-255. [3] Martínez-Villaluenga, et al. 2008. J. Agric. Food Chem. 56, 10533-10543. [4] Prior, et al. 2003. J. Agric. Food Chem. 51:3273-3279.

ACKNOWLEDGEMENTS

Archer Daniels Midland, Decatur IL, Illinois Soybean Association, IL, Jenny Lotton, Elizabeth Pletsch, Sherline Sherline, Vermont Dia, Amber Yudel, Dr. Luis Mejia and all participants in this study.

Several studies suggest that consumption of soy protein has positive effects on preventing obesity. Preliminary *in vitro* studies with β -conglycinin showed significant reduction in lipid accumulation compared with glycinin. The objective was to compare the effect of low glycinin soy milk (LGS) (49.5% β -conglycinin) with conventional soy milk (S) (26.5% β -conglycinin) and cow's milk (M) (0% β -conglycinin) on body fat accumulation, serum lipids and adipokines in overweight men. A randomized, double-blind, placebo, controlled study was conducted with 64 overweight men (BMI > 25; 18-45 years old). Subjects consumed 500 ml of each beverage daily for 3 months. Fasting blood samples, dual X ray absorptiometry scans, anthropometrics, lipid profile, plasma leptin, adiponectin, fatty acid synthase and C-reactive protein analyses were executed at baseline and after 3 months. Total energy, total fat and total protein content were standardized among treatments. LGS and S contained similar levels of total isoflavones and amino acids. No changes were observed in dietary intake ($p = 0.399$) and physical activity ($p = 0.489$) throughout the study. LGS consumption caused less body fat accumulation after 3 months compared to S and M ($p = 0.017$). No effects were detected in BMI ($p = 0.721$), WHR ($p = 0.454$), weight ($p = 0.836$), triglycerides ($p = 0.947$) and total cholesterol ($p = 0.320$), leptin ($p = 0.655$), FAS ($p = 0.976$) and CRP ($p = 0.959$) among treatments. However, LGS and S dramatically decreased α -LDL (75% and 56%, respectively) compared with M (-38%). LGS consumption significantly increased plasma adiponectin (44%) compared with S (4%, $p = 0.039$) M (-8%, $p = 0.036$). In conclusion, LGS consumption ameliorated body fat accumulation, reduces α -LDL and increased plasma adiponectin in overweight men after 3 months. Therefore, LGS may be a useful product to include in meals designed to improve the health of overweight individuals.



Effect of low glycinin soy milk on body fat accumulation, lipid profile and adipokines in overweight men

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INTRODUCTION

It is believed that soy protein consumption reduces body fat, increases satiety, lowers blood lipids and produces a positive effect in energy balance [1]. β -conglycinin embeds more active peptides than glycinin that inhibit lipid accumulation and induces adiponectin in 3T3-L1 adipocytes [2,3]. It also increases adiponectin levels in rats more than soy protein isolate (SPI) and casein [4].

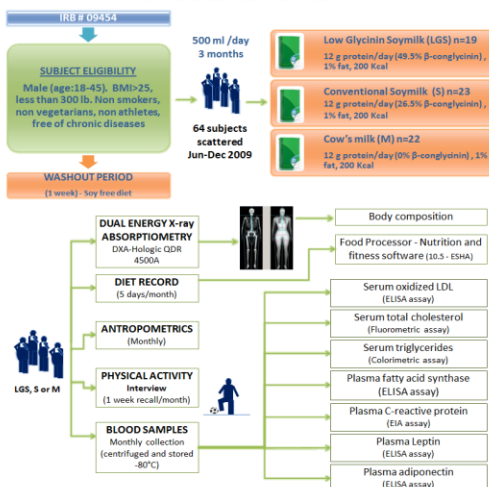
HYPOTHESIS

Low glycinin soy milk (LGS) suppresses body fat accumulation, serum lipids and biomarkers of oxidative stress in overweight men.

OBJECTIVE

Compare the effect of low glycinin soy milk (LGS), conventional soy milk (S) and cow's milk (M) on body fat accumulation, serum lipids and biomarkers of oxidative stress in overweight men.

MATERIALS AND METHODS



Statistical analysis: SAS, version 9.1 (SAS Institute). Univariate analyses with data transformations, general linear model (GLM), repeated measures analysis and mixed model with random effect were used ($p < 0.05$). Influence of age, initial BMI, diet intake and physical activity were considered in the mixed model.

RESULTS

Figure 1. Low glycinin soy milk (LGS) and conventional soy milk (S) did not change in isoflavones and amino acid concentrations.

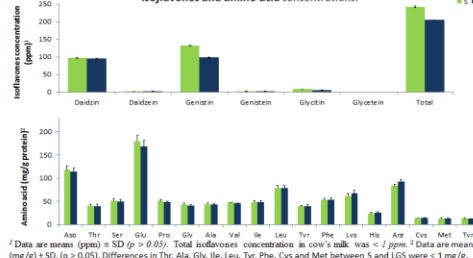


Table 1. Baseline characteristics of subjects per treatment.*

Characteristic	Low glycinin soy milk (LGS)	Conventional soy milk (S)	Cow's milk (M)
Age (years)	33 ± 6	32 ± 8	32 ± 6
Weight (Kg)	95.8 ± 11.1	96.2 ± 17.3	97.0 ± 17.2
BMI	29 ± 3	29 ± 4	30 ± 4
Total Fat (%)	28 ± 3.5	27 ± 6.0	28 ± 5.6
Waist-Hip ratio	0.95 ± 0.05	0.94 ± 0.07	0.94 ± 0.06

*Data are means ± SD ($p > 0.05$)

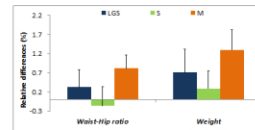


Figure 2. Anthropometric parameters did not change from baseline after three months of consumption, means ± SEM.

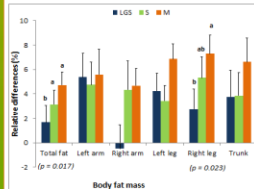


Figure 3. LGS consumption accumulated less total body fat than S and M and less right leg fat than M, means ± SEM.

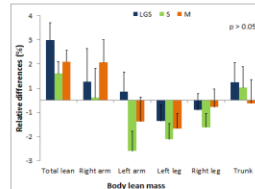


Figure 4. Body lean composition changes were not significant after 3 months of consumption, means ± SEM.

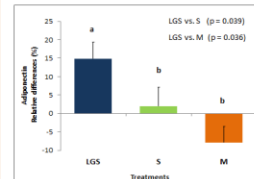


Figure 5. LGS consumption increased plasma adiponectin after 3 months, means ± SEM.

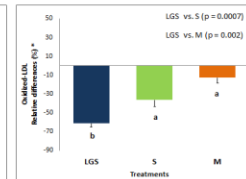


Figure 6. LGS consumption decreased serum oxidized LDL after 3 months, means ± SEM. *Change affected by age ($p = 0.03$) and initial BMI ($p = 0.02$)

Table 3. Serum lipids, plasma leptin, C-reactive protein and fatty acid synthase did not change significantly after three months of consumption of the different types of milk.

Biomarker	Relative differences from baseline (%) ¹			p-value ²
	LGS	S	M	
Total cholesterol (mg/dl)	16.59 ± 7.62	-2.85 ± 5.25	10.77 ± 6.66	0.320
Total triglycerides (mg/dl)	-8.83 ± 10.66	9.97 ± 13.14	-5.75 ± 8.13	0.947
Leptin (ng/ml)	6.64 ± 17.76	11.78 ± 5.88	20.37 ± 7.43	0.655
C-Reactive protein (ng/ml)	-6.57 ± 10.49	-19.73 ± 5.99	1.00 ± 6.81	0.959
Fatty acid synthase (mg/ml)	-26.14 ± 16.67	-31.92 ± 11.50	40.00 ± 21.60	0.976
Interleukin-6 *	-22.09 ± 9.07 ^a	-26.03 ± 5.29 ^b	10.29 ± 11.65 ^a	0.044

¹ Values are Means ± SEM of relative differences from baseline, n = 64. ² Variation probability among treatments.

*Different letters in a row indicates statistically significant differences ($p < 0.05$).

Dietary intake ($p = 0.399$), moderate physical activity ($p = 0.209$) and strenuous physical activity ($p = 0.489$) did not change significantly in the three months of consumption.

CONCLUSIONS

- Three months of low glycinin soy milk consumption increased plasma adiponectin and decreased serum oxidized-LDL levels in overweight men.
- Low glycinin soy milk consumption ameliorated a modest but significant accumulation of body fat.
- Low glycinin soy milk may be a useful product to include in meals designed to reduce oxidative stress and to improve the health of overweight individuals.

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Protein profile in low glycinin soymilk does not alter bone density or intestinal microbiota in overweight men

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The objective was to compare the effect of consuming low glycinin soymilk (LGS, 49.5% β-conglycinin/6% glycinin), conventional soymilk (S, 26.5% β-conglycinin/38.7% glycinin) or bovine milk (M, 0% β-conglycinin) on bone mineral density (BMD) and composition of intestinal microbiota in overweight individuals. In a randomized double-blind study 64 men BMI>25, 18-45 years old consumed 500 ml of either soymilk or bovine milk daily for 3 months. Each product contained fat (1%), energy (200 kcal) and protein (12 g). BMD was determined by DXA and fecal material composition with real-time quantitative PCR, both at baseline and after 3 months; diet and physical activity were recorded every month. Energy intake (p=0.40), moderate physical activity (p=0.21) and strenuous physical activity (p=0.49) did not change during the study period. LGS did not alter BMD and T-scores (p>0.05); changes in total microbiota (p=0.36), *Bacteroides* (p=0.27), *Bifidobacteria* (p=0.07) and *Lactobacillus* (p=0.06) were not significant among treatments. Dietary intakes of protein, fat, carbohydrates, dietary fiber, Ca, Fe, P, K, caffeine, vitamins D, B-12, K, and E were not influenced by LGS, S or M (p>0.05). However, sodium (p=0.02) and vitamin C (p=0.0002) intakes were reduced with LGS consumption. In conclusion, consumption of LGS for three months did not have an effect on bone mineral density and increased microbial density however a high throughput analysis is needed.

INTRODUCTION

The contributions of soy proteins on bone density and intestinal microbiota are still inconclusive. Soy protein (1) and isoflavones (2) have been related with benefits in human bone health; however, other authors contradict such findings concluding that soy products have small or no effect on bone mineral density (3, 4). The intestinal microbiota composition has an important role in the energy balance and weight dynamic (5).

HYPOTHESIS

Low glycinin soymilk (LGS) does not affect bone mass density and benefits gut microbiota composition in overweight men.

OBJECTIVE

To compare the effect of consuming low glycinin soymilk, conventional soymilk and bovine milk on bone mineral density (BMD) and composition of intestinal microbiota in overweight men.

MATERIALS AND METHODS

SUBJECTS:

64 healthy male subjects, 20-45 year old, BMI >25, non-vegetarian, non-athletes, non-smokers

TREATMENTS:

(LGS) Low glycinin soymilk (49.5% β-conglycinin)
(S) Conventional soymilk (26.5% β-conglycinin)
(M) Bovine milk (0% β-conglycinin)

One week washout period + 3 months of consumption (12 g protein/day)
Measurements at baseline and after 3 months

Bone mineral density

Dual Energy X Ray Absorptiometry (DXA) (Illinois: Holistic 4500A, software version 11.1.3)



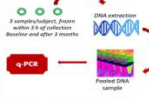
Nutrient Intake and physical activity:

Five-days Diet Record

Food Processor SQL, Nutrition Analysis and Fitness Software (version 10.5.0, ESHA Research)

Seven-day physical activity recall (SDPAR) (personal interviews)

Gut microbiota composition



Results are relative differences (%) from baseline to the number of 25S rRNA gene copies per acquisition (on average)

Target group Primer
Total bacteria 1H-3315, 1H-3771
Bacteroides A1B2-2365, A1B2-2328
Bifidobacterium B1F1-47, B1F6-24
Lactobacillus L4F5, L4F7

Statistical analysis: SAS, version 9.1 (SAS Institute). Univariate analyses with data transformations, GLM, repeated measures and mixed with random effect procedures (p<0.05). BMI, body fat changes, adiponectin, oxidized LDL, physical activity, interleukin-6 and antioxidant capacity did not influence microbiota analysis.

Table 1. Baseline characteristics of subjects *

Characteristic	Low glycinin soymilk (LGS)	Conventional soymilk (S)	Bovine milk (M)
Age (years)	33 ± 6	32 ± 8	32 ± 6
Weight (kg)	95.8 ± 11.1	96.2 ± 17.3	97.0 ± 17.2
BMI	29 ± 3	29 ± 4	30 ± 4
Total Fat (%)	28 ± 3.5	27 ± 6.0	28 ± 5.6
W-H ratio	0.95 ± 0.05	0.94 ± 0.07	0.94 ± 0.06

*Data are means ± SD (p>0.05)

RESULTS

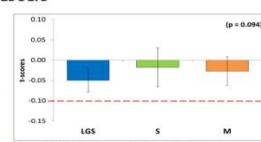


Figure 1. BMD t-scores did not change among treatments, Mean/SEM.

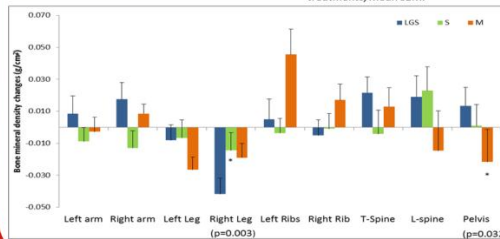


Figure 2. Changes in bone mass density were less than 0.05 g/cm² and did not differ among treatments after three months of consumption, Mean/SEM.

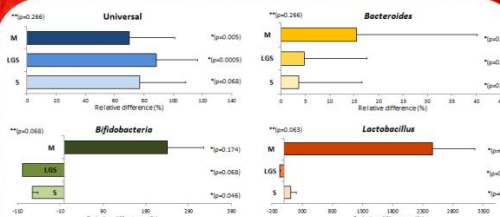


Figure 3. Based on the primers used, no significant changes among treatments were observed, however changes in *Lactobacillus* and *Bifidobacteria* were short of significance (p=0.06) **Differences among treatments. *Changes from baseline. Mean/SEM. Age influenced universal microbiota (P=0.046).

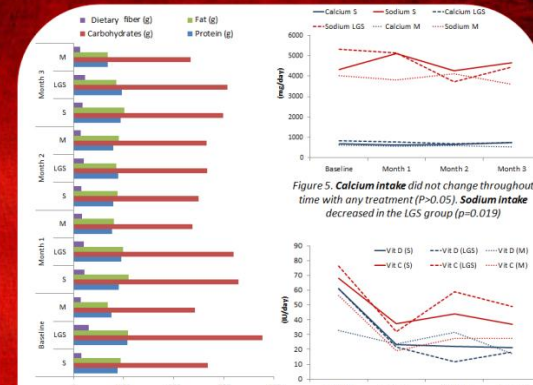


Figure 4. Protein, fat, carbohydrate and dietary fiber intake did not significantly change throughout the study (p>0.05). Intake of Fe, P, K, caffeine, vitamins D, B-12, K, E, energy intake, moderate and strenuous physical activity did not change significantly during the study among groups (p>0.05).
Figure 5. Calcium intake did not change throughout time with any treatment (p>0.05). Sodium intake decreased in the LGS group (p=0.019)
Figure 6. Vit D did not change significantly throughout time in any group (p>0.05). Vit C intake decreased in the LGS and M groups (p=0.0002)

CONCLUSIONS

- The consumption of low glycinin soymilk for three months may not have any physiologically important effect on bone mineral density in overweight men.
- Most nutrients intake were maintained except vitamin C and sodium which decreased with low glycinin soymilk consumption.
- Under the conditions of this study microbial density increased in the same proportion with soymilk and bovine milk consumption.
- Gut *Bifidobacteria* decreased with soymilk consumption; however a high throughput analysis of specific genera and species are needed to better understand the nature of the intestinal microbiota in overweight men. (Oral presentation - Abstract number 4368, Wednesday 10:45 AM).

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Research poster presentation – Experimental Biology, Washington DC 2011.

APPENDIX L

Parameter	Sample Power	# of subjects per treatment for 80% power	# of subjects per treatment for 90% power	# of subjects per treatment for 99% power
Plasma C-reactive protein	32%	47	61	113
Plasma leptin	29%	63	83	155
Plasma TNF α	45%	39	51	95
Serum cholesterol	45%	39	51	95
Serum Triglycerides	9%	348	456	857
Plasma FAS	30%	62	82	147
Universal microbiota	43%	45	59	109
Bacteroides	5%	4728	6209	11681
Bifidobacteria	45%	46	59	110
Firmucutes	37%	56	74	137
Lactobacillus	60%	33	42	78
Proteobacteria	39%	53	69	128
Actinobacteria	19%	121	159	297

*Based on Sample Power analysis performed in SPSS for non-significant parameters.

APPENDIX M

Sub	RMR	Baseline	Month 1	Month 2	Month 3	Energy intake	Difference	Adiponectin
		Kcal/Day	Kcal/Day	Kcal/Day	Kcal/Day	Average		RD %
F6-01	1981.1	2697.3	2683.0	1137.4	.	2172.6	191.5	31.1
F6-02	1714.9	1317.0	2288.4	1958.1	1703.5	1816.7	101.9	29.2
F6-03	1934.8	1949.6	1896.9	.	1918.8	1921.7	-13.1	-8.1
F6-04	2140.8	2032.1	2196.6	2052.3	2324.5	2151.4	10.5	16.2
F6-05	2198.9	3385.1	1986.3	2304.8	2400.6	2519.2	320.3	-11.6
F6-06	1986.3	3081.0	2913.5	2831.0	2675.6	2875.3	889.0	-7.4
F6-07	1916.5	3197.9	.	2350.7	3279.3	2942.6	1026.1	-5.6
F6-08	2166.9	2000.4	7203.5	2146.9	4043.9	3848.7	1681.7	38.9
F6-09	1761.0	1629.2	2111.1	2691.3	2277.6	2177.3	416.3	20.3
F6-10	1699.0	1607.2	2127.1	1627.0	1642.7	1751.0	52.0	-23.3
F6-11	1838.3	1639.1	904.9	2025.9	1953.6	1630.9	-207.5	10.6
F6-12	2131.4	1589.7	3781.0	2304.1	3728.1	2850.7	719.3	21.2
F6-13	2253.4	2772.9	2383.1	1566.6	2244.9	2241.9	-11.5	-11.2
F6-15	2029.0	8216.1	3077.4	2262.6	2670.8	4056.7	2027.7	10.6
F6-16	1892.2	3232.5	2172.2	2636.2	2728.3	2692.3	800.1	0.5
G6-01	2183.9	1623.6	4153.2	3116.1	1880.7	2693.4	509.5	-8.9
G6-02	1841.2	1557.1	1830.7	810.1	1202.6	1350.1	-491.1	14.9
G6-03	2009.8	4331.4	3151.0	2781.1	2619.2	3220.7	1210.8	3.4
G6-04	1941.9	4063.1	2426.2	3817.1	3219.4	3381.4	1439.6	17.4
G6-06	1927.9	1748.1	2309.5	1924.5	2725.2	2176.8	248.9	6.1
G6-07	1677.9	2224.8	1551.2	1170.1	1747.3	1673.3	-4.6	23.4
G6-10	1927.4	3664.9	2984.6	3097.6	2919.3	3166.6	1239.2	-15.9
G6-12	2033.8	3065.4	3333.7	2873.8	3601.0	3218.5	1184.7	5.1
G6-13	1875.6	2206.6	1305.4	1049.8	1565.4	1531.8	-343.8	43.0
G6-14	2134.3	4380.1	2218.8	2186.8	2472.5	2814.5	680.2	48.9
H6-01	1683.2	2280.2	2491.2	2495.3	2258.5	2381.3	698.1	17.6
H6-02	2276.5	904.0	718.5	1006.4	870.1	874.7	-1401.8	16.2
H6-03	1939.4	2559.9	2302.3	2211.8	2103.8	2294.4	355.0	-5.3
H6-04	1795.6	1987.2	2376.3	1901.1	1192.1	1864.1	68.5	2.0
H6-05	2208.4	2167.7	2379.8	2858.3	2221.8	2406.9	198.5	3.2
H6-07	1679.2	1518.3	531.5	1393.0	917.9	1090.2	-589.0	5.2
H6-08	1914.4	2706.8	3160.9	3602.8	3450.0	3230.1	1315.7	12.5
H6-09	1910.6	1488.7	1742.4	1958.2	1432.8	1655.5	-255.1	9.4
H6-11	2108.7	2109.3	1667.6	2247.2	1995.3	2004.8	-103.9	1.5
H6-12	1913.7	2180.5	2814.0	2719.2	2401.5	2528.8	615.1	-33.9
H6-13	1867.7	845.3	1473.0	2026.6	1593.6	1484.6	-383.1	-11.8
H6-14	1724.3	2408.1	2620.2	2554.1	2264.8	2461.8	737.5	.

ESTIMATED ENERGY INTAKE PER SUBJECT DURING THE STUDY

* BMR (basal metabolic rate) was calculated with the Mifflin-St Jeor formula ($BMR = 9.99 \times \text{weight} + 6.25 \times \text{height} - 4.92 \times \text{age} + 5$). According to the energy consumption declared by participants, some of the participants did not meet the energy requirement, however, after an analysis of energy intake throughout the time, no significant differences were found among groups (LGS, S and M) every month and not significant changes were detected ($P = 0.130$) throughout the three months of consumption. Also a mixed model analysis, showed that energy intake did not influenced changes in plasma adiponectin ($P = 0.642$).

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Low glycinin soymilk ameliorates body fat accumulation and improves serum antioxidant status in overweight men

Dina Fernandez¹, Cristina Martinez-Villaluenga¹, Neal A Bringe² and Elvira Gonzalez de Mejia¹

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² The Monsanto Company, St. Louis, MO

ABSTRACT

Low glycinin soybeans were enriched in β -conglycinins, with similar amino acid, isoflavone, and fatty acid profiles as conventional soybean. β -conglycinins and other soybean components are potential protective agents against body fat accumulation and inflammation. The objective was to compare low glycinin soymilk (LGS) with conventional soymilk (S) and cow's milk (M) for effects on body fat accumulation, serum lipids, antioxidant capacity and biomarkers of oxidative stress and inflammation. In a randomized, double-blind, placebo controlled, IRB approved study, overweight men (BMI > 25) were divided into three groups and fed daily for 3 months 500 ml of LGS, S or M. No changes were observed in their calorie intake and physical activity ($p > 0.05$). LGS consumption caused less total body fat accumulation compared to S and M. Oxidized LDL was dramatically decreased after LGS consumption (-75%) in comparison to M ($p = 0.03$). The rate of increase of serum antioxidant capacity was faster after consumption of LGS (8%, 16%, and 21%) compared to S (-3%, 12%, and 18%) at 1, 2 and 3 months, respectively, but no increase was observed for M. Interleukin-6 decreased after 3 months for LGS consumption but not M ($p < 0.03$). LGS ameliorated body fat accumulation and improved antioxidant status compared to

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Description

Summary


A unique blend of fundamentals and applied information, this book first provides readers with understanding of the chemistry and properties of food-derived proteins and peptides before looking at ways to maximize functional utilization in foods and dietary supplements. A valuable resource for nutritionists, nutraceutical experts, and pharmacologists involved with product development and dietary supplement applications, the book describes physicochemical properties and interactions involved in regulating biological function. It also covers practical considerations regarding properties such as solubility, emulsification, foaming, and gelling, as well as protein modifications, both chemical and enzymatic, to improve functionalities.

Gonzalez de Mejia E., Martínez-Villaluenga, C., **Fernandez, D.**, Urado, D., Sato K. Bioavailability and safety of food peptides. *In: Food Proteins and Peptides Chemistry, Functionality Interactions, and Commercialization*. Chapter 19. Taylor & Francis. Edited by **Navam S Hettiarachchy, Kenji Sato, Maurice R Marshall** ISBN: **978-1-4200-9341-4**, **CRC Press**, Publication Date: **12/01/2011**, Pages: **456**, Trim Size: **6-1/8 x 9-1/4**. In Press

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Presentation Abstract

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Presentation Number: 295-23

Presentation Title: Effect of low-glycinin soymilk on body fat accumulation, lipid profile and adipokines in overweight men

Division: Nutraceuticals and Functional Foods

Presentation Time: Tuesday, Jul 20, 2010, 1:30 PM - 4:00 PM

Location: Hall A

Author Information: Dina Fernandez-Raudales, Univ of Illinois at Urbana-Champaign, Urbana, IL; Neal A. Bringe, The Monsanto Company, St Louis, MO; Elvira Gonzalez de Mejia, Univ of Illinois at Urbana-Champaign, Urbana, IL

Presentation Description/Abstract: Several studies suggest that consumption of soy protein has positive effects on preventing obesity. Preliminary *in vitro* studies with β -conglycinin showed significant reduction in lipid accumulation compared with glycinin. The objective was to compare the effect of low glycinin soymilk (LGS) (48% β -conglycinin) with conventional soymilk (S) (28% β -conglycinin) and cow's milk (C) (0% β -conglycinin) on body fat accumulation, serum lipids and adipokines in overweight men. A randomized, double-blind, placebo, controlled study was conducted with 27 overweight men (BMI > 25; 18-45 years old). Subjects consumed 500 ml of each beverage daily for 3 months. Fasting blood samples, Dual X ray absorptiometry scans, anthropometrics, lipid profile, plasma leptin and adiponectin analyses were executed at baseline and after 3 months. Total energy, total fat and total protein content were standardized among treatments. LGS and S contain similar levels of total isoflavones. No changes were observed in dietary intake ($p = 0.399$) and physical activity ($p = 0.489$) throughout the study. LGS consumption caused less body fat accumulation at 3 months vs. baseline compared to S and C. No effects were detected in BMI ($p = 0.86$), triglycerides ($p = 0.86$) or total cholesterol ($p = 0.49$) among treatments, however LGS and S dramatically decreased Oxi LDL (-75% and -56%, respectively) compared with C (-38%). LGS ameliorated the secretion of plasma leptin (1% relative difference), S (7.4%) and C (17.7%) ($p = 0.54$). LGS consumption significantly enhanced plasma adiponectin (16%) compared with C (-19%) ($p = 0.02$). In conclusion, LGS consumption inhibited the accumulation of body fat and increased plasma adiponectin in overweight men after 3 months. Therefore, LGS may be a useful product to include in meals designed for maintaining healthy levels of body fat.

CURRICULUM VITAE

Dina G. Fernandez

EDUCATION

University of Illinois at Urbana-Champaign

Masters of Science in Food Science and Human Nutrition, Expected Graduation: May 2011

Zamorano University, Honduras

Bachelor of Science in Food Science and Technology, December 2002.

WORK EXPERIENCE

RESEARCH ASSISTANT, University of Illinois, FSHN Department (Aug 2009-Present)

- Conduct research to evaluate the effect of three functional protein beverages on body composition, bone mineral density, gut microbiota and biomarkers of oxidative stress and inflammation in overweight men.
- Teaching assistant for FSHN 416 Food Chemistry Laboratory, Fall 2010.
- Research mentor of two undergraduate students (Fall 2009, Spring 2010, Summer 2010 and Fall 2010).
- Mentor for the Research Apprentice Program (RAP), College of Agricultural, Consumer and Environmental Sciences, University of Illinois (Summer 2009 and 2010). High school student project won the oral presentation and research paper competition (Summer 2010).
- National Soybean Research Laboratory collaboration: “Evaluation of micronutrient supplements acceptability in infants” (Jan –Jul 2009).
- Led research and non-research laboratory activities.
- Conducted research to evaluate the glycemic index of protein beverages in overweight men.

VISITING SCHOLAR, University of Illinois, FSHN Department (Jan –Jul 2009)

- Developed and oversaw Institutional Review Board (IRB) processes for human trials.
- Oversaw and contributed to the manufacturing of soy-based beverages using UHT technology at Tetra-Pack® TX.
- Gained analytical experience in dietary record and physical activity using the Nutrition Analysis and Fitness Software (ESHA).
- Participated in the processing demonstrations in the “International workshop on soy applications for the food industry”, INTSOY-UIUC, IL -2009.

ASSISTANT PROFESSOR, Food Sci. and Tech. Department, Zamorano University, Honduras (2004 -Dec 2009)

- Taught courses at the undergrad level: Sensory Science; Food Service; and Food Processing and non-Food Product Processing.
- Establishment and validation of a sensory evaluation laboratory and its manual.
- Food safety instructor for food companies in Honduras and El Salvador.
- Instructed food chemistry and food analysis practical laboratories and edited the respective laboratories manuals.

- Developed and oversaw the International Professional Practices Program for Food Science students in Honduras.
- Undergraduate student advisor for several research projects in the areas of: product development, sensory evaluation, food safety, food service and food processing.

Publications

- **Fernandez-Raudales D.**, Martinez-Villaluenga C., Bringe NA., Gonzalez de Mejia E. Low glycinin soymilk reduced serum oxidized LDL and ameliorates body fat accumulation in overweight men. *British J of Nutr* (2011). Submitted.
- **Fernandez-Raudales D.**, Hoeflinger J., Bringe N.A., Dawn Sm, Miller M., González de Mejia E. Low glycinin soymilk decreases *firmicutes:bacteroidetes* ratio and increases intestinal microbiota density in overweight men. *Plos One* (2011). In preparation.
- **Fernandez-Raudales D.**, Bringe N.A., Lotton J., Diaz K., Chapman-Novakofski K., González de Mejia E. Effect of beverages with different protein profiles on postprandial blood glucose response in overweight and obese men. *Diabetes Care* (2011) In preparation.
- Gonzalez de Mejia E., Martínez-Villaluenga, C., **Fernandez, D.**, Urado, D., Sato K. Bioavailability and safety of food peptides. *In: Food Proteins and Peptides Chemistry, Functionality Interactions, and Commercialization*. Chapter 19. Taylor & Francis. Edited by **Navam S Hettiarachchy, Kenji Sato, Maurice R Marshall** ISBN: 978-1-4200-9341-4, CRC Press, Publication Date: 12/01/2011, Pages: 456, Trim Size: 6-1/8 x 9-1/4. In Press

Abstract & poster presentations

- **Fernandez D.**, Martinez-Villaluenga C., Bringe NA., Gonzalez de Mejia E. Low glycinin soymilk ameliorates body fat accumulation and improves serum antioxidant status in overweight men. 2009 FASEB journal, 721.3 Vol 24, No. 11
- **Fernandez D.**, Bringe NA., Gonzalez de Mejia E. Effect of low glycinin soymilk on body fat accumulation, lipid profile and adipokines in overweight men. Finalist in Nutraceuticals & Functional Foods Division: Graduate student research paper poster competition, COMP11-13. 2009 Institute of Food Technologist (IFT) annual meeting, Chicago IL.
- **Fernandez D.**, Bringe N.A., Lotton J., Hoeflinger J., Miller M. González de Mejia E. Protein profile in low glycinin soymilk does not alter bone mineral density or intestinal microbiota in overweight men. Poster # 4368. Experimental Biology annual meeting, Washington DC, 2011.
- Hoeflinger J., **Fernandez D.**, Bringe N., Dowd S., Miller M., De Mejia E. Effect of soymilk with different protein profiles on the relative abundance of the major bacterial phyla in feces of overweight men. Oral presentation # 4368. Experimental Biology annual meeting, Washington DC, 2011.

Oral presentations

- Soy β -conglycinin and adipogenesis. Research Laboratory Meeting, University of Illinois, October 2009.
- Low glycinin soymilk ameliorates body fat accumulation and improves serum antioxidant status in overweight men. Preliminary results. Research Laboratory Meeting, University of Illinois, March 2010.
- Effect of low glycinin soymilk on body fat accumulation, biomarkers of inflammation and oxidative stress and gut microbiota in overweight men. FSHN 595 seminar, October 2010.

- Protein profile in low-glycinin soymilk does not alter bone density or intestinal microbiota in overweight men. Research Laboratory Meeting, University of Illinois, November 2010.

Honors & Awards

- M.S. Candidate to the ACES-FUNK graduate student research award 2011.
- Research assistant 2009-2011.
- Senior student representative, International Division- IFT, 2010-2011.
- Junior student representative, International Division-IFT, 2009-2010.
- Alice and Charlotte Biester Merit Award, 2010.
- Beatriz and Francis Mar Graduate Student Endowment Fund– FSHN University of Illinois, 2009 and 2010.
- Award for excellent performance and consistency on quality work, Zamorano University, Honduras, 2008.
- Award for outstanding contribution to the training program for Exports promotion, USAID, El Salvador 2004-2008.
- Award for outstanding performance and contributions in the Food Sci. and Agro-industry. Zamorano University, 2005.
- Academic Scholarship NIPPON Foundation, Japan 2002.
- Academic Scholarship, Swiss Agency for Development and Cooperation (SDC), 1999-2001.
- Member of American Society of Nutrition (ASN).
- Member of Institute of Food Technologist (IFT).
- Member of American Oil Chemist' Society (AOCS).
- Member of the Food Science Honorary Society- PHI TAU SIGMA.

Technical expertise

Randomized, double blind human trials.

IRB application procedures.

Beverage processing (UHT technology at Tetra-Pack®).

Anthropometric measurements.

Dual Energy X-ray Absorptiometry (DXA) scans.

Biochemical analyses of human blood biomarkers of inflammation, oxidative stress, serum lipids such as ELISA, EIA, ORAC, among others.

Affinity chromatography and gel filtration.

Dietary recalls: Nutrition Analysis and Fitness Software (ESHA).

Physical activity assessments.

Statistical analysis (SAS System).

Practical experience in processing of dairy, meat and vegetable products.

LANGUAGES: Fluent in Spanish and English.

ACKNOWLEDGEMENT OF RESEARCH

The research was performed under the direction and supervision of Dr. Elvira Gonzalez de Mejia, associate professor in the Department of Food Science and Human Nutrition at the University of Illinois at Urbana-Champaign. Funding to the de Mejia's laboratory was provided by Illinois Soybean Association and The Monsanto Company