THE EFFICACY OF FUNCTIONAL FOODS FOR THE MITIGATION OF
OBESITY-RELATED PATHOLOGIES

A Thesis in
Food Science
by
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ABSTRACT

Obesity is a major health concern in the United States and the rest of the developed world. Currently, more than 35% of adults and 15% of children in the U.S. are obese. The treatment of obesity-related illnesses accounts for $200 billion or more than 20% of total healthcare expenditures in the U.S. every year. Several diseases have been linked to obesity including cardiovascular disease, type-2 diabetes mellitus and non-alcoholic fatty liver disease (NAFLD). Nearly a quarter of adults in the U.S. have NAFLD. NAFLD is characterized by the accumulation of excess lipids in the liver in the absence of excessive alcohol consumption. Steatosis can progress to non-alcoholic steatohepatitis in which hepatocytes become inflamed, and fibrosis develops. In a small number of cases, severe fibrosis can lead to cirrhosis, liver failure, and death. Dietary interventions offer a safer and more affordable means of treating obesity and its related pathologies compared to interventions such as medication or surgery. Previous research has shown that a large number of foods have physiological activities beyond their nutritional value. In this thesis, we investigated the efficacy of tart cherries, soy protein concentrate, and high-oleic soybean oil in treating obesity and its related pathologies. The objectives of this project were to: 1.) determine if substituting a fiber-rich soy protein concentrate into a high-fat fed mouse diet prevents the development of obesity and NAFLD 2.) determine if substituting high-oleic soybean oil into a high-fat fed mouse diet prevents the development of obesity and NAFLD 3.) determine if tart cherries reduce the severity of obesity and its related inflammation in vivo 4.) determine if a tart cherry derived extract is capable of mitigating inflammation in TNF-α-stimulated human colonic epithelial cells

Previous research has indicated that dietary supplementation with soy protein may mitigate the severity of NAFLD. Several studies have shown that replacing dietary
saturated fatty acids with monounsaturated fatty acid (MUFA) may also ameliorate
NAFLD. We examined the efficacy of dietary supplementation with soy protein
concentrate (SPC), high-oleic soybean oil (HOS), and the combination to reduce markers
of fatty liver disease and its associated inflammation in a high-fat (HF) induced NAFLD
mouse model. Male C57BL/6J mice were given experimental diets *ad libitum*. The HF
diets consisted of 60% kcal from lard; the HOS diets consisted of 20% kcal from lard and
40% kcal from high-oleic soybean oil, and diets supplemented with SPC replaced one-third
of the casein with soy protein. Mice fed SPC had reduced visceral adiposity, hepatic
steatosis, and markers of inflammation compared to casein-fed control mice. Mice fed
high-oleic soybean oil gained significantly less weight and visceral adipose but had
increased expression of several inflammatory markers.

Previous studies have shown that tart cherry (TC) products may mitigate symptoms
of obesity and obesity-related inflammation. Tart cherries contain large amounts of
anthocyanins and proanthocyanidins which have antioxidant and anti-inflammatory
properties. We examined the effects of supplementing 1% tart cherry powder into a diet-
induced obesity mouse model. Mice were provided *ad libitum* access to either a HF or a
HF+TC diet for 18-weeks. No differences in weight gain or visceral adiposity were
observed between the treatment groups. Mice fed the HF+TC diet had a slightly lower
hepatic expression of inflammatory markers monocyte chemoattractant protein-1,
interleukin (IL)-1ß, IL-6 and tumor necrosis factor (TNF)-α. We also examined the effects
of a tart cherry extract (TCE) on colonic inflammation *in vitro*. Inflammation was
stimulated in HT29 colonic epithelial cells with TNF-α and treated with TCE.
Inflammation was measured as the concentration of IL-8 secreted by the cells and
normalized to viability. The TCE had a small anti-inflammatory effect at higher concentrations when applied simultaneously with TNF-α.

In conclusion, we have found that both a fiber-rich soy protein concentrate and tart cherry powder may be effective in mitigating the effects of obesity-related inflammation and NAFLD in a diet-induced obesity mouse model. We also found that high-oleic soybean oil may be pro-inflammatory in the same model. Further research will evaluate the constituent compounds of these foods to identify the key active components.
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Chapter 1: Literature Review

1.1 Obesity

1.1.1 Overview

Obesity is defined as having a body mass index (BMI) greater than 30 kg/m$^2$. Rates of overweight (BMI $> 25$ kg/m$^2$) and obesity are on the rise in the United States and in the rest of the developed world. Around 35% of adults in America fall into the obese category while over two-thirds of adults are overweight or obese [1]. These numbers are substantially higher than they were a few decades ago. In 1960, while the percentage of overweight adults was similar to current day, only 12.8% of the population was obese [2]. Rates of childhood overweight and obesity have seen similar increases; rising from around 15% in 1972 to over 35% in 2012 [3]. Children who are overweight at younger ages are highly predisposed to developing obesity later in life [4]. These data indicate that a rapidly increasing portion of the population is at risk for obesity-related diseases.

While the major cause of obesity in the world is an excess consumption of a diet rich in energy-dense foods and a lack of exercise brought on by a cultural normalization of a sedentary lifestyle, genetics can play a role as well. Several twin studies have tracked the BMIs of genetically identical siblings and found that they tend to develop similarly during childhood indicating that obesity may be, in part, a heritable trait [5]. This effect appears to decrease with age and therefore, plays a smaller role when considering the etiology of adult obesity. Genetic predisposition to obesity may be augmented by epigenetic alterations from environmental factors discussed previously such as diet and activity level [6]. In essence, genes may predispose some individuals to obesity, but lifestyle choices are still
the key factors in the development of obesity. Obesity is a major risk factor for insulin resistance and type-2 diabetes mellitus (T2DM), hypertension, cardiovascular disease, and nonalcoholic fatty liver disease (NAFLD) [7]. Obesity places a heavy burden on the healthcare system. Current estimates state that the cost of obesity in the United States is almost $200 billion per year and accounts for approximately 20% of total healthcare expenses.

Several other factors are correlated with the incidence of obesity such as gender, race, and socioeconomic status. A greater percentage of men above the age of 20 are overweight, but recent statistics show a roughly equal percentage of men and women are obese [8, 9]. This indicates that more men fall into the overweight category and are therefore at risk of becoming obese. Gender differences are observed in adolescents as well, with roughly 33% of boys and only 30% of girls being overweight or obese [10]. Obesity tends to be more prevalent in certain ethnic groups. Around 66% of white Americans are overweight or obese. This value spikes to 76% in black Americans and 79% in Hispanic Americans [10]. Black Americans have the highest rates of obesity and extreme obesity in the country. These ethnic differences may be due to factors related to food security and access to “healthy” foods [11].

A number of risk factors have been identified that may predispose children to obesity later in life. Children who have at least one obese parent are more likely to become overweight or obese [12]. Other studies have found that children that are not breastfed are more likely to develop obesity [13, 14]. Activity level is also predictive of obesity development. Children who watched more than 8 hours of television per week were 58%
more likely to be obese [14]. Obese children are at risk for developing many of the same health issues as obese adults.

1.1.2 Adipose Signaling and Obesity-related Inflammation

Adipocytes store excess fat in the body and function as endocrine cells regulating lipid homeostasis in the body through the release of signaling proteins called adipokines. The function of these adipokines is altered during the development of obesity. Leptin, a signaling protein produced by white adipocytes, regulates lipid metabolism in adipose and other organs in the body [15]. Leptin secretion increases as adipocytes take on more triglycerides and leptin binds with receptors in the hypothalamus resulting in reduced appetite and upregulation in fatty acid oxidation [16]. In obesity, adipose tissue produces large amounts of leptin in an effort to reduce energy intake and increase energy expenditure, but over time the body develops a resistance to the effects of leptin [17]. The effects of leptin can be seen well in two common mouse models for obesity: the ob/ob and the db/db mouse models. The ob/ob model contains a knock-out mutation of the gene that encodes for leptin while the db/db model contains a knockout mutation of the gene that encodes for the leptin receptor [18]. Both models develop extreme obesity due to the absence of leptin signaling.

Adiponectin is another signaling protein secreted by adipocytes. Adiponectin functions as an anti-inflammatory protein through the inhibition of tumor necrosis factor-α (TNF-α). It has also been shown to reduce gluconeogenesis and activate fatty acid β-oxidation in the liver through the activation of 5’-AMP-activated protein kinase which may protect against the development of non-alcoholic fatty liver disease [19-21]. Mice lacking
adiponectin experience diet-induced insulin resistance indicating that adiponectin may regulate insulin response. Adiponectin concentrations decrease with increasing obesity, contributing to obesity-related inflammation and the onset of insulin resistance [22, 23].

In obesity, adipocytes become overloaded with triglycerides and become inflamed. They begin to release the inflammatory cytokine TNF-α [24]. TNF-α can stimulate an inflammatory response both in the adipose tissue and in other organs. In the adipose tissue, TNF-α stimulates the release of Monocyte Chemoattractant Protein-1 (MCP-1) by preadipocytes which leads to the recruitment of macrophages to the adipose tissue [25]. Both TNF-α and MCP-1 have been implicated in the development of insulin resistance [26, 27]. Obese mice in which TNF-α has been knocked out are protected from the development of insulin resistance [28]. Further research has revealed that TNF-α reduces the expression of the insulin receptor and inhibits its activation in vitro [26].

Recruited macrophages produce additional TNF-α along with other inflammatory cytokines including interleukin (IL)-1β, and IL-6. TNF-α and IL-6 can circulate to other organs such as the liver stimulating hepatocyte inflammation and apoptosis, contributing to the development of non-alcoholic fatty liver disease (NALFD) [25, 29, 30]. It is this increase in systemic inflammatory signaling that links the obesity to metabolic syndrome and NAFLD.
1.1.3 Metabolic Syndrome

Obesity is often grouped together along with several other related conditions including dyslipidemia, hyperglycemia, and hypertension into an overarching disease state referred to as metabolic syndrome [31]. Metabolic syndrome is currently defined and diagnosed as being obese along with at least two of the following conditions: elevated triglycerides, low high-density lipoprotein cholesterol (HDL), hypertension, or hyperglycemia (Defined in Table 1-1) [31, 32].

<table>
<thead>
<tr>
<th>Condition</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Obesity</td>
<td>BMI &gt; 30 kg/m²</td>
</tr>
<tr>
<td>Hyperglycemia</td>
<td>Fasting blood glucose ≥ 100 mg/dL</td>
</tr>
<tr>
<td>Dyslipidemia</td>
<td>TG ≥ 150 mg/dL or HDL cholesterol ≤ 40 mg/dL</td>
</tr>
<tr>
<td>Hypertension</td>
<td>Systolic &gt; 130 mmHg or Diastolic &gt; 85 mmHg</td>
</tr>
</tbody>
</table>

Metabolic syndrome is a valuable tool used to identify patients that are at increased risk of developing cardiovascular disease or T2DM, both of which are in the top ten leading causes of death in the United States (CDC) [33-35]. Metabolic syndrome is also predictive for the development of NAFLD, a major health concern for an increasing percentage of the adult population and a principle focus of this dissertation. All co-morbidities of obesity are tied together by obesity-related inflammation.
1.2 Non-alcoholic Fatty Liver Disease

1.2.1 Prevalence

NAFLD is a common comorbidity of obesity. NALFD encompasses a range of hepatic disorders ranging from simple hepatic steatosis all the way to liver cirrhosis in the absence of excessive alcohol consumption or another liver disease such as viral hepatitis [36]. NAFLD has become an increasingly common malady in the world, affecting between 9% and 36% percent of the world’s adult population (23% in the United States) and is well correlated with the incidence of obesity [36, 37]. Between 10-15% of healthy-weight adults have fatty liver disease [38]. Prevalence increases with increasing body weight to between 70 and 80% in obese individuals. Fatty liver disease has been on the rise in children as well [39].

1.2.2 Definitions and Progression

The first stage of NAFLD is simple steatosis in at least 5% of hepatocytes. This state is reversible and generally has no observable symptoms beyond the histopathological visibility of lipid droplets in hepatocytes. These lipid droplets are composed of triglycerides (TGs) and are similar to the lipid droplets usually found in adipose tissue [40]. The TGs are made up of fatty acids from peripheral adipose tissue, dietary fatty acids, and new fatty acids formed by de novo lipogenesis [41]. Hepatic lipid droplets are still metabolically active and the TGs can be used for energy in the liver by beta-oxidation or delivered to other tissues in very low-density lipoproteins (VLDL) [41-43]. Early stages of NAFLD are usually identified through the use of ultrasound, magnetic resonance imaging, or histology of a liver biopsy [44, 45]. While a rough estimation of the degree of steatosis can
be made through non-invasive imaging, a biopsy remains the definitive method for diagnosing NAFLD and identifying the degree of steatosis [45, 46].

In some patients, hepatic steatosis progresses to non-alcoholic steatohepatitis (NASH) in which the liver tissue becomes inflamed. It is difficult to differentiate simple steatosis from steatohepatitis [47]. The liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are often examined in a clinical setting, but they may be unchanged in simple steatosis and even in liver fibrosis [45, 47]. The gold standard for identifying NASH is, again, histological evaluation of a liver biopsy. Several histopathological markers help to identify and grade the severity of NAFLD/NASH including macrovesicular lipid droplets occurring in zone 3 of the lobule, hepatocyte ballooning, monocyte infiltration, and perisinusoidal fibrosis [48]. The degree of steatohepatitis, as well as fibrosis, can be given a grade based on defined histopathological criteria [49]. Steatohepatitis is also reversible, but the immune response may lead to fibrosis or scarring that interferes with normal liver function and is irreversible. In a small subset of cases, the level of fibrosis is so severe that the liver becomes cirrhotic and the tissue is no longer functional leading to liver failure and death unless they receive a liver transplant [50]. The progression of NAFLD has also been associated with hepatocellular carcinoma in later stages [50, 51].

1.2.3 Etiology of NAFLD

Several hypotheses have been proposed to explain the progression of NAFLD from simple steatosis to NASH. One of the most popular theories is known as the “two-hit” hypothesis [52]. Day and colleagues proposed that the first hit was the accumulation of
triglycerides in the liver during the onset of simple steatosis. The “second hit” was the onset of inflammation. This inflammation may originate from a number of sources including inflammatory cytokines from adipose tissue, oxidative damage within the hepatocyte due to lipid peroxides, or inflammation caused by bacteria-derived endotoxins. Another popular hypothesis is the of multiple parallel hits hypothesis [53]. Tilg et al. proposed that steatosis does not necessarily precede inflammation in the progression of NASH. They suggest that inflammation from other sources, such as TNF-α from dysfunctional adipose tissue or from endotoxin-induced intestinal inflammation may begin the inflammatory cascade in the liver prior to, or simultaneously with, the onset of steatosis. The exact cause of hepatic steatosis and NASH is still under investigation.

1.2.4 Lipotoxicity and Cell Death in NAFLD

Triglycerides stored in hepatocytes are inert and in fact, may protect the cell from the more cytotoxic free fatty acids (FFAs) [54]. As mentioned previously, FFAs in hepatocytes originate from the diet, de novo lipogenesis, and from adipose tissue [40]. Free fatty acids can lead to cell death and inflammation through a process called lipotoxicity. As free fatty acids accumulate in the hepatocyte, they induce endoplasmic reticulum (ER) stress resulting in the unfolded protein response (UPR) [55]. Protein kinase r-like ER kinase (PERK) is activated during the UPR [56]. During extended ER stress the continued upregulation of PERK leads to the transcription of C/EBP homologous protein (CHOP) [57]. CHOP begins a cascade that eventually leads to cell death by apoptosis. CHOP upregulates death receptor 5, which when activated leads to the activation of caspase-8 [30]. CHOP also promotes the activation of BH3-only BCL-2 family proteins [58]. These
proteins lead to the release of cytochrome c from the mitochondria and subsequent activation of caspase-9 and then caspase-3, resulting in cell death by apoptosis [59]. Although apoptosis is a normal cellular function, excessive apoptosis can lead to an inflammatory state. Apoptotic bodies release microvesicles that contain signaling proteins, ceramides, and micro-RNAs that may potentially activate inflammation in surrounding cells [58]. Inflammatory signaling resulting from lipoapoptosis may contribute to the progression from simple steatosis to NASH.

1.2.5 Treatment Options

The options for treatment of NAFLD are relatively limited. Treatments often focus on addressing symptoms and comorbidities such as the hypertriglyceridemia or insulin resistance that accompany NAFLD. Metformin is used to treat hyperinsulinemia and restores insulin sensitivity in the liver to some degree [60, 61]. Supplementation with vitamin E has also shown some promise. Vitamin E functions as an antioxidant in the liver and may help reduce the oxidative load that often leads to the progression of steatosis to NASH [62]. The earlier stages of the disease including hepatosteatosis and steatohepatitis can be resolved through weight loss by means of diet and exercise [63]. These approaches address the disease by treating obesity, which is likely the underlying cause.
1.3 Metabolic Endotoxemia

A condition closely intertwined with obesity, NAFLD, and inflammation is metabolic endotoxemia. Metabolic endotoxemia is a condition that arises from the infiltration of bacterial endotoxins from the intestinal lumen into the blood. A high-fat diet has been shown to alter the proportions of native bacteria in the gut to contain a greater number of Bacteroidetes and decrease the number of Firmicutes[64, 65]. Bacteroidetes are gram-negative bacteria that release endotoxins such as lipopolysaccharide (LPS) which can pass through the intestinal membrane and into the blood. The body mounts an immune response to the foreign LPS leading to inflammation [66]. LPS can be incorporated into chylomicrons and transported throughout the body causing systemic inflammation which can potentiate obesity, insulin resistance, and cardiovascular issues [67].

The liver can be greatly impacted by metabolic endotoxemia due to its function in nutrient processing, blood detoxification and anatomical proximity to the intestine. This relationship is referred to as the gut-liver axis [68]. Normally, the paracellular passage of LPS into the bloodstream is blocked by the tight junctions between colon cells, but these tight junctions begin to loosen with the ingestion of a high-fat diet, as is often the case in obesity [69-71]. Increased endotoxins in the blood promote a systemic inflammatory response, exacerbating preexisting inflammation in the adipose and liver. Gut permeability and the distribution of gut microflora are therefore important targets for the amelioration of NAFLD, obesity, and the associated inflammation.

Obesity, metabolic syndrome, NAFLD and metabolic endotoxemia are all important treatment targets as each one plays a role in the progression of the others. While medical treatments tend to treat a single symptom or set of symptoms, changes in lifestyle
can better address the underlying causes. Changes in dietary intake such as the addition of functional foods may mitigate the development of obesity-related pathologies. Functional foods are foods that provide health benefits beyond their nutritional value and may be a promising avenue of natural treatment for these metabolic disorders [72]. In this thesis we examine the functional food properties of soybeans and tart cherries.
1.3 Soybeans

1.3.1 Production and Economic Value

The soybean industry is a cornerstone of U.S. agriculture and has shown steady growth over the past several years. In 2015, 3.9 billion bushels of soybeans worth of over $34.5 billion dollars were harvested in the U.S. [73]. The crop is highly important in Pennsylvania. In 2015, Pennsylvania soybean farmers planted 580,000 acres of soybeans worth $209 million dollars [74]. Of all the soybeans planted in the U.S., 94% of them have been genetically modified to be herbicide resistant. These modified soybeans have paved the way for the development of other modified soybean varieties including the high-oleic soybean [75].

1.3.2 Nutrient and Phytochemical Content

Soybeans are a rich source of macronutrients and phytochemicals. Soybeans contain considerable amounts of protein, oil, fiber, and phytochemicals. In a 100g serving of soybean seeds, there is 36g of protein (33% of kcals), 20g of fat (40% kcal), 30g of carbohydrates (27% kcal) as well as 9g of fiber [76]. Traditional soybean oil contains a large percentage of poly-unsaturated fatty acids (PUFAs) (Table1-2). High-oleic soybeans, on the other hand, produce oil that is high in oleic acid, a monounsaturated fatty acid (MUFA) (Table1-2). Soybeans are known to contain relatively high amounts of the isoflavones daidzein, genistein, and glycitein [77]. Several components of soybeans including soy protein, soy fiber, and soy oil, appear to have biological activity beyond their caloric content when consumed by mammals.
1.3.3 The Health Benefits of Soy Protein

A number of studies have demonstrated that substituting soy protein may protect against the development of NAFLD [78-83]. A study by Ascencio et al. fed male Wistar rats a high-fat diet containing either soy protein or casein for 150 days [78]. Rats fed soy protein had significantly lower serum and hepatic triglycerides as well as decreased fasting insulin levels. Hepatic steatosis was less pronounced in the soy-fed rats compared to those fed casein. The authors believed the decrease is steatosis was due to reduced hepatic lipogenesis because of reduced expression of sterol regulatory element binding protein-1 (SREBP-1). SREBP-1 is a transcription factor responsible for the regulation of the lipogenic enzymes fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC). Similar

Table 1-2: Fatty acid profile of traditional and high-oleic soybean oils

<table>
<thead>
<tr>
<th>% Fatty Acid</th>
<th>Soybean Oil (SBO)</th>
<th>High Oleic SBO</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;C14</td>
<td>0.00</td>
<td>2.64</td>
</tr>
<tr>
<td>C14:0</td>
<td>0.04</td>
<td>0.03</td>
</tr>
<tr>
<td>C16:0</td>
<td>10.66</td>
<td>6.28</td>
</tr>
<tr>
<td>C18:0</td>
<td>4.00</td>
<td>3.85</td>
</tr>
<tr>
<td>C18:1</td>
<td>22.57</td>
<td>77.44</td>
</tr>
<tr>
<td>C18:2</td>
<td>50.30</td>
<td>7.25</td>
</tr>
<tr>
<td>C18:3</td>
<td>7.03</td>
<td>2.04</td>
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<td>C20:0</td>
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<td>0.29</td>
</tr>
<tr>
<td>C20:1</td>
<td>0.13</td>
<td>0.17</td>
</tr>
<tr>
<td>C22:1</td>
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<td>0.01</td>
</tr>
<tr>
<td>Other</td>
<td>5.03</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>100.00</td>
<td>100</td>
</tr>
<tr>
<td>%SFA</td>
<td>14.94</td>
<td>10.45</td>
</tr>
<tr>
<td>%MUFA</td>
<td>22.70</td>
<td>77.61</td>
</tr>
<tr>
<td>%PUFA</td>
<td>57.33</td>
<td>9.3</td>
</tr>
<tr>
<td>ω-6:ω-3</td>
<td>7.16</td>
<td>3.55</td>
</tr>
</tbody>
</table>
research by Shukla et al. found that short-term treatment of Sprague-Dawley fed a high-fat, high cholesterol diet, with soy protein isolate resulted in significant reduction of serum triglycerides and SREBP-1 expression [79]. Unlike the study by Ascencio and colleagues, there were no significant effects on insulin levels.

A majority of soy protein research concerned with NAFLD has found that soy protein in the diet results in the down-regulation of SREBP-1c [78, 79, 81, 84, 85]. SREBP-1c is a major regulator de novo lipogenesis in hepatocytes [86]. Reducing the production of lipids within the hepatocyte is thought to protect against lipotoxicity and the progression of NAFLD from simple steatosis to steatohepatitis [81, 85, 87].

Adipose tissue is another major source of free fatty acids that may contribute to lipotoxicity and the progression of NAFLD. Soy protein has been shown to positively alter metabolic functions in adipose tissue [80, 84, 88-91]. In a study by Torre-Villalvaz et al., rats were fed high-fat diets containing either casein or soy protein for 180 days [84]. They found that rats fed soy protein had significantly less visceral adipose compared to casein-fed rats. With soy protein treatment, the average white adipocyte size was significantly reduced, and the total number of adipocytes was significantly increased. This correlated with a decrease in leptin expression, an adipokine responsible for systemic lipid uptake. A reduction in adipocyte size may reduce adipose inflammation and prevent excess secretion of fatty acids to the liver. A genome-wide analysis of rats fed soy protein found similar benefits of soy protein in the regulation of adipose tissue [91]. Rats fed soy protein had smaller, more abundant adipocytes compared to casein-fed rats. A genome-wide analysis of adipose tissue revealed increased peroxisome proliferator-activated receptor (PPAR)-α and PPAR-γ in soy-fed rats indicating an increase in adipogenesis and fatty acid oxidation.
The authors concluded that soy protein was able to increase the metabolic activity of adipose and prevent the adipocyte hypertrophy responsible for the expression of inflammatory cytokines.

The exact mechanism by which soy protein effects these changes is relatively unclear. One study attempted to identify the mechanism by which soy protein alters pancreatic insulin secretion in rats using isolated pancreatic islets [92]. Islet cells were incubated with amino acids mimicking the patterns present in casein and soy protein both alone and co-incubated with soy-derived isoflavones. The soy amino acids, both in the presence and absence of isolated isoflavones, significantly reduced insulin secretion by islet cells. This research implies that the amino acid profile of soy protein is important for its biological activity. The soy isoflavones may also contribute to the benefits of soy protein, but a number of studies reviewed here used isoflavone-free soy protein and still observed similar protective effects.

Some posit that it is the amino acid profile that confers the putative health benefits of soy protein, while others believe that it may be bioactive peptides that result from the digestion products of soy protein. In one study, pepsin-pancreatin hydrolysates of soy protein were applied to RAW 264.7 macrophages, and their effect on inflammation was monitored. The hydrolysates significantly lowered the nitric oxide content of the macrophages compared to the LPS stimulated control. They also lowered the expression of inducible nitric-oxide synthase (iNOS), cyclooxygenase-2 (COX-2), IL-1B, IL-6, and TNF-a. The authors hypothesized that many of the peptides present in their digest have some degree of bioactivity giving them anti-inflammatory properties. The issue is whether
or not these peptides are present \textit{in vitro} and whether or not they reach the target cells intact in significant concentrations in order to elicit the same effect seen \textit{in vitro} [93].

\textbf{1.3.4 Health Benefits of Soybean Fiber}

Soybeans and certain soybean products contain significant amounts of fiber. Although research is limited, a few studies have indicated that soy fiber may have potential health benefits beyond the bulking properties of the dietary fiber. Soy fiber has limited solubility in water. Most insoluble fibers are not highly fermentable, but soy fiber is actually highly fermentable. One study digested various fiber sources using human fecal bacteria and measured the production of various short-chain fatty acids (SCFA) in the resulting digesta [94]. The researchers found that soy fiber fermentation resulted in high concentrations of SCFAs, most notably butyrate. Butyrate has been shown to be a crucial substrate for colonocyte metabolism. Deficiencies in butyrate or in the cells ability to metabolize butyrate have been linked to conditions such as inflammatory bowel disease [95].

A crossover study of dietary soy polysaccharide supplementation in 14 college-aged men showed little impact on the physiological markers examined [96]. Subjects were fed identical diets, but half were given soy fiber. No significant changes were observed in serum levels of total protein, albumin, globulin, lactose dehydrogenase, alkaline phosphatase, blood urea nitrogen, creatinine, uric acid, or bilirubin. Significant increases in wet fecal weight and decreases in fecal lipid concentrations were observed in subjects consuming the soy polysaccharide diet. Soy fiber had a small effect on postprandial hypoglycemia and may have reduced elevation in insulin levels after ingestion of glucose. Other articles indicate that supplementation with soy fiber may have a small effect on
decreasing total cholesterol [97, 98] and may reduce serum glucose levels in patients with T2DM [99, 100].

Soy fiber has been examined in a clinical setting as a treatment for diarrhea in infants. The study found that formula supplementation with soy fiber led to a decrease in the time of diarrhea [101]. The authors note that the fiber was highly fermented and led to an increase in the fecal bacteria content of the patients. They did not investigate the distribution of the bacterial populations but state that it may have contributed to the patient recovery. Other studies have noted changes in bacterial populations with highly fermentable fiber supplementation [102, 103].

A group studying the effects of a freeze-dried, whole soy pod powder, which included both the bean and the surrounding pod, in an obesogenic mouse model found that the supplement reduced weight gain, increased colonic SCFA content, and shifted microbiota population [102]. They observed increased fecal lipid concentrations which may account for the reduced weight gain. A human clinical study with isolated soy fiber found decreased fecal lipids in the treatment group. This may indicate that the antinutritive properties of the soy pod powder are not due to the fiber but to some other constituent of the whole soybean [96]. Soy fiber may offer many health benefits due to its fermentability, its ability to alter the distribution of the gut microbiota, and its potential to reduce markers of metabolic syndrome, but further research is necessary to determine its functional mechanism.
1.3.5 Health Benefits of High-Oleic Soybean Oil

Traditional soybean oil contains a large percentage of PUFAs. PUFAs are prone to oxidation and dramatically shorten the shelf-life of soybean. Soybean oil is partially hydrogenated to prevent oxidation and extend its shelf life. The process of hydrogenation results in the production of some trans fatty acids [104]. Trans fats have recently been banned by the USDA. Studies have found that consuming trans fats can contribute to the development of coronary heart disease. A prospective study of elderly men found that those that consumed trans fats (>2% total energy intake) were 28% more likely to develop coronary heart disease [105]. Trans fats have also been shown to cause dyslipidemia and inflammation (reviewed in [106]).

In an effort to produce shelf-stable soybean oil that does not have to be partially hydrogenated, a new genetically modified soybean variety was developed. The new soybean oil, referred to as high-oleic soybean oil, contains less than a fifth of the PUFAs found in traditional soybean and more than 3 times as much monounsaturated fat (MUFA) in the form of oleic acid. This was achieved by deactivating the delta-12 desaturase responsible for the conversion of oleic acid to linoleic acid in the soybean plants to make them produce a greater concentration of oleic acid [107]. Oleic acid is much less prone to oxidation, allowing for the soybean oil to be shelf stable without introducing trans fatty acids [104].

Although very little research exists on the health effects of consuming high-oleic soybean oil, other oils high in oleic acid, such as olive oil, have been evaluated previously. The Mediterranean diet is well known for its high-fat content derived mostly from olive oil. Similar to high-oleic soybean oil, olive oil contains approximately 75% oleic acid
The Mediterranean diet has shown to decrease the chance of cardiovascular disease, cancer, neurodegenerative diseases, and the overall incidence of mortality [109]. A recent review of olive oil consumption found that levels of total cholesterol, low-density lipoprotein cholesterol, very low-density lipoprotein cholesterol and blood pressure were all lowered significantly in subjects who consumed large amounts of MUFAs [110]. In a study by Hussein et al. NALFD was induced in Sprague-Dawley rats by feeding the animals methionine/choline deficient diets. The diets were supplemented with various types of dietary fat including olive oil, fish oil, and butterfat [111]. The rats that were given the olive oil had significantly lower hepatic triglyceride content and lower liver weights than the control group. Olive oil also caused an increase in peroxisomal β-oxidation and inhibition in triacylglycerol formation. It is important to note that in addition to its high-oleic acid content, olive oil contains many bioactive phytochemicals such as hydroxytyrosol, oleuropein, and lingstroseide [112]. These components have been shown to functions as antioxidants, preventing the oxidation of low-density (LDL) and inhibiting cyclooxygenase activity [113, 114]. It is unclear whether the health benefits of olive oil are due to its high-oleic acid content or its phytochemical content.

The potential benefits of MUFA-based diets are less obvious in high-fat models. One study gave mice a diet containing 60% kcal from fat enriched with MUFAs and PUFAs for 20-weeks [115]. These mice still developed markers of metabolic syndrome including obesity, insulin resistance, and hypercholesterolemia. Another study provided rats with high-fat diets containing either coconut oil (saturated fat), lard (mix of saturated and MUFA), olive oil (MUFA), or fish oil (PUFA) [116]. Rats fed the coconut, lard and olive oil all developed hepatic steatosis. Only the rats fed olive oil or lard developed insulin
resistance as measured by blood glucose response to an insulin challenge. The research appears to be split on whether or not diets high in MUFAs are protective against obesity-related pathologies. Further research is necessary to determine the role that total fat content, fatty acid distribution, and source of fat play in nutrition.

1.4 Tart Cherries

1.4.1 Production and Economic Value

Tart cherries (Prunus cerasus) are a rapidly growing sector of the fruit industry in the United States with substantial increases in production over the past decade. In 2015, U.S. cherry growers produced 251 million pounds of tart cherries worth $87 million dollars (USDA/NASS 2016). Each year more land is being utilized for the cultivation of tart cherries. Currently, around 38,000 acres of U.S. soil is devoted to the cultivation of this fruit, an increase of over 3,000 acres since 2007.

The popularity of tart cherries in the public sector is also on the rise. Tart cherry containing products are flooding the markets claiming numerous health benefits. Studies have shown the potential benefits of cherries in treating several health conditions including cardiovascular disease, T2DM, neurodegenerative diseases, and inflammatory diseases [117]. These health benefits have been attributed to the high concentrations of phenolic compounds in tart cherries.
1.4.2 Phytochemical Content

Tart cherries are rich in phenolics, mainly anthocyanins, and proanthocyanidins (PACs). Among anthocyanins, tart cherries contain greater amounts of cyanidin-3-rutinoside and its glycosylated form cyanidin-3-glucosylrutinoside [118]. The PAC content of tart cherry powder was analyzed by Capangola et al. and was found to be comparable to several other PAC-rich foods such as grape seeds and chocolate (Table 1-3)[119]. It was reported that the mean degree of polymerization (DP) of proanthocyanidins was relatively low for tart cherries (3.9±0.2) compared to other cherry cultivars such as Cornelian (62.9±3.1) and Laurel (45.2±2.1). Several other bioactive compounds have been identified in tart cherries including isorhamnetin rutinoside, kaempferol, quercetin, and melatonin [118]. It is clear from the existing body of research on cherry phenolic content that the content and distribution of tart cherry phytochemicals varies widely based on cultivar (Montmorency vs. Balaton), growing region, and type of processing.

Seeram et al. found that Balaton tart cherries had significantly greater anthocyanin content than Montmorency tart cherries although both had similar antioxidant and anti-inflammatory capacities [120]. Analysis of U.S. tart cherries revealed that most of the PACs had a DP>10mers while tart cherries purchased in Turkey contained PACs with an

<table>
<thead>
<tr>
<th>Source</th>
<th>Proanthocyanidins (mg/100g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tart Cherry</td>
<td>66</td>
</tr>
<tr>
<td>Cornelian Cherry</td>
<td>8</td>
</tr>
<tr>
<td>Laurel Cherry</td>
<td>218</td>
</tr>
<tr>
<td>Grape Seed</td>
<td>17-250</td>
</tr>
<tr>
<td>Dark Chocolate</td>
<td>31-37</td>
</tr>
<tr>
<td>Sweet Cherry</td>
<td>117</td>
</tr>
</tbody>
</table>

Table 1-3 Proanthocyanidin content of various
average DP of 4 [119, 121]. The freeze-dried tart cherry powder used in our research was found to have nearly twice the antioxidant capacity compared to other processed tart cherry products [118]. These differences highlight the difficulty of explaining the health benefits of a complex nutraceutical. Unless each study conducts a chemical analysis of its tart cherries or uses only isolated compounds, it will be difficult to draw a definitive conclusion about the health benefits and biological functionality of tart cherry bioactives. It is important to note that tart cherries contain a significant amount of sugar (Table 1-4). In dried tart cherries, sugar contributes 67% of the fruit’s mass and accounts for 80% of its energy content. These sugars primarily consist of approximately equal parts of the monosaccharides glucose and fructose [122, 123].
1.4.3 Bioavailability of Tart Cherry Bioactive Components

For any bioactive food component to exert a direct effect on an organ, it must first be absorbed in the intestinal tract and transported to the tissue. Otherwise, it must act locally in the intestine to have a systemic effect. A majority of anthocyanins are absorbed in the stomach mucosa immediately after ingestion, and absorption continues through the small intestine [124]. The systemic bioavailability is estimated to be 0.26-1.8% [124]. Kirakosyan et al. evaluated the tissue bioavailability of tart cherry anthocyanins in rats fed

<table>
<thead>
<tr>
<th>Table 1-4: Nutritional content of dried tart cherries</th>
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<tbody>
<tr>
<td><strong>Macronutrients</strong></td>
</tr>
<tr>
<td>Energy (kcal)</td>
</tr>
<tr>
<td>Protein (g)</td>
</tr>
<tr>
<td>Total lipid (g)</td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
</tr>
<tr>
<td>Dietary Fiber (g)</td>
</tr>
<tr>
<td>Sugars (g)</td>
</tr>
</tbody>
</table>

| **Minerals** | Mass per 100g | Mass per 40g serving |
| Calcium (mg) | 38 | 15.2 |
| Iron (mg) | 0.68 | 0.272 |
| Magnesium (mg) | 22 | 8.8 |
| Phosphorus (mg) | 36 | 14.4 |
| Potassium (mg) | 376 | 150.4 |
| Sodium (mg) | 13 | 5.2 |
| Zinc (mg) | 0.25 | 0.1 |

| **Vitamins** | Mass per 100g | Mass per 40g serving |
| Vitamin C, total ascorbic acid | 19.3 | 7.72 |
| Thiamin (mg) | 0.058 | 0.023 |
| Riboflavin (mg) | 0.101 | 0.040 |
| Niacin (mg) | 0.867 | 0.347 |
| Vitamin B-6 (mg) | 0.101 | 0.040 |
| Folate (µg) | 11 | 4.4 |
| Vitamin A (µg) | 141 | 56.4 |
| Vitamin E (mg) | 0.17 | 0.068 |
| Vitamin K (µg) | 5.1 | 2.04 |
tart cherry powder over 18 weeks [125]. Organ analysis revealed that the highest levels of anthocyanins were in the bladder, liver, and kidneys of the rats. Anthocyanins were also detected in brain and heart tissue, but none were detected in visceral adipose.

PACs are the other major bioactive component in tart cherries. PACs are flavan-3-ol polymers that are many times large than anthocyanins. Therefore, it is much more difficult for them to be absorbed during digestion. In vitro studies with intestinal monolayers have revealed that only PACs with a DP≤3 are able to absorb through the intestinal barrier [126]. This implies that long chain PACs can only act locally in the intestine as they are not systemically bioavailable.

1.4.4 In vitro Antioxidant and Anti-inflammatory capacity of tart cherries

Several studies have evaluated the antioxidant capacity of tart cherries by various methods [118, 121, 127, 128]. An investigation by Wang et al. found that anthocyanins isolated from tart cherries had anti-oxidant capacities similar to those of butylated hydroxyanisole and butylated hydroxytoluene, two commercially available antioxidant preservatives. The anthocyanins also had significantly greater antioxidant capacity than α-tocopherol [129]. Cyclooxygenase (COX)-1 and -2 are responsible for the production of inflammatory signaling molecules called prostaglandins and are the targets of anti-inflammatory drugs [130]. Tart cherry anthocyanins inhibit the activities of COX-1 and -2 in vitro [120, 131]. The capacity of TC anthocyanins to inhibit COX-1 were comparable to the non-steroidal anti-inflammatory drugs Naproxen and Ibuprofen and exceeded these drugs in their ability to inhibit COX-2 [120].
1.4.5 Effects of Tart Cherries in Animal Models

Previous animal research has found that tart cherries effectively reduce inflammation, obesity, insulin resistance, dyslipidemia, and the incidence of colon cancer. Studies by Seymour et al. have found that tart cherries mitigate the development of obesity and inflammation in rats [132, 133]. Zucker rats fed a high-fat (HF) diet containing 1% (w:w) tart cherry powder for 90 days exhibited significantly lower body weight, visceral adiposity, total cholesterol, triglycerides, fasting blood glucose, and insulin levels compared to HF controls [132]. Serum levels of TNF-α and IL-6 were significantly decreased due to reduced NF-κB nuclear translocation. TC activated PPAR-α, which is a transcription factor responsible for the activation of genes related to lipid metabolism.

Research using diabetic and insulin resistant animal models have found that tart cherries can mitigate insulin resistance and slow the progression of diabetes in rodents [134, 135]. Lachin and Reza induced diabetes in rats with Alloxan and then treated them with cherry extracts for 30 days [136]. Rats fed tart cherry extracts exhibited lower blood glucose, improved urinary creatinine excretion and decreased microalbumin excretion in urine indicating restored kidney function. The authors hypothesized that the cherry extracts would aid in the recovery of β-cell function in the pancreas. This hypothesis was supported by the work of Jayaprakasam et al. who demonstrated that cherry anthocyanins were capable of increasing insulin output of pancreatic β-cells in vitro [137]. It was also observed that cyanidin-3-glucoside, one of the anthocyanins present in tart cherries, was able to mitigate insulin resistance in leptin receptor-deficient mice through the inactivation of forkhead box (FOX)-O1 [135]. FOX-O1 is a transcription factor partially responsible for the development of insulin resistance in obesity.
The body of research exploring the action of tart cherries in the intestine is limited. A study by Kang et al. examine the effects of tart cherry anthocyanins on tumor development in ApcMin mice and the proliferation of human colon cancer cells in vitro [138]. Mice treated with tart cherries or tart cherry anthocyanins had reduced occurrences of cecal tumors compared to the control, but no effect was observed in the development of colon tumors. Tart cherry extracts reduced the proliferation of human colon cancer cells in vitro as well. The authors believed that COX-inhibitory capacity was responsible for the attenuation of tumor development as COX-activation is necessary for tumorigenesis. Currently, no research exists on the ability of tart cherries or tart cherry PACs to reduce intestinal inflammation related to obesity or inflammatory bowel disease.

1.4.6 Effects of Tart Cherries in Humans

The anti-inflammatory capacity of tart cherries makes them a potential target for treating post-exercise muscle damage and inflammation. Two studies evaluated the effects of tart cherry products on markers of inflammation and oxidation in marathon runners after a race [139, 140]. Both studies found that athletes treated with TC products had reduced levels of serum inflammation after an endurance race. Expression of C-reactive protein (CRP) was reduced in TC subjects in both studies. Howatson et al. examined the antioxidant status of the athletes and found that TC supplementation increased total antioxidant status and reduced serum concentrations of malondialdehyde compared to control [140]. A similar increase in the antioxidant status measured by ischemia/reperfusion-induced F2-isoprostane response was observed in elderly adults treated with tart cherry juice over 4-weeks [128]. Increased anti-inflammatory capacity by
tart cherry supplementation has the potential to mitigate damage related to inflammatory events such as exercise.

Tart cherries have also been examined for their potential to treat inflammatory diseases such as gout and osteoarthritis. Subjects were given tart cherry juice over a period of 2-days and levels of CRP, serum, and urinary urate were measured before and after treatment [141]. Subjects given tart cherry juice exhibited increased urate excretion and significant decreases in CRP levels. The authors suggested that tart cherry supplementation may be useful in treating gouty arthritis due to its ability to reduce circulating urate levels. A case-crossover study examining the efficacy of tart cherries in reducing gout severity identified tart cherry consumption to be significantly protective (Odds Ratio (OR) 95% Confidence Interval (CI) 0.50-0.85), especially when combined with prescription drug treatment (OR 95% CI 0.15-0.42) [142]. Similar results were observed in osteoarthritis patients given tart cherry juice over a period of several weeks [143]. Both CRP and osteoarthritis index scores were reduced in subjects consuming tart cherry juice.

Tart cherry supplementation has proven useful in treating symptoms of T2DM. Ataie-Jafari et al. provided female subjects who had T2DM with tart cherry juice concentrate for 6-weeks [144]. Women given the TC treatment experienced significant reductions in body weight, blood pressure, and glycated hemoglobin, a marker of sustained hyperglycemia. In vitro study of leukocytes from T2DM patients revealed that tart cherry extracts reduced the expression of inflammatory cytokines TNF-α and IL-8 and increased the expression of heme oxygenase-1, responsible for signaling anti-inflammatory pathways [145]. It remains unclear as to whether these observations are due to a simple reduction in inflammation or if they are a result of increased systemic insulin sensitivity. Further
research is necessary to determine whether tart cherries would be beneficial in combating other obesity-related pathologies in humans.
1.5 Purpose and Significance

Rates of obesity, metabolic syndrome, and NAFLD are on the rise both in the U.S and around the world. Both adults and children are now affected by these preventable diseases. These pathologies are a burden not only on the individuals who have them but also on the healthcare system that provides for their treatment. Treating these conditions with medication or invasive surgeries is costly, and these approaches pose potential risks themselves. A safer and more cost-effective course of action may be dietary approaches for the prevention and treatment of these conditions.

Current research indicates that there a number of functional foods that may have beneficial effects for the prevention and treatment of obesity and its related pathologies. Plant bioactive phytochemicals such as those from tart cherries have the potential to reduce weight gain and inflammation related to obesity. Although previous research has identified anthocyanins as the main active component in tart cherries, this food also contains significant amounts of PACs. We believe that PACs may work locally in the intestine to reduce inflammation in a high-fat diet.

Several bioactive macronutrients exhibit health benefits beyond their nutritional value. Research has established that soy protein may prevent the progression of NAFLD through a reduction in hepatic \textit{de novo} lipogenesis and through metabolic regulation of adipose tissue. Although research is limited, it appears that soy fiber has the potential to favorably alter the distribution of gut microbiota and reduce symptoms of metabolic syndrome. Diets high in MUFAs have been shown to lower cholesterol and increase fatty acid oxidation, although the effects of MUFAs in a high-fat diet are somewhat unclear.
The significance of this research is that it examines several unique approaches to treating obesity-related pathologies with bioactive dietary components. This thesis examines a multifaceted approach to combating obesity-related pathologies with several food bioactives including both phytochemicals and functional macronutrients. If successful, this research will demonstrate that multiple dietary changes with diverse biological targets are effective in achieving similar outcomes due to related underlying etiologies of obesity, metabolic syndrome, and NAFLD.
1.6 Hypotheses and Objectives

1.6.1 Soybean-derived Products

The existing body of evidence indicates that soy protein may help prevent the development of NAFLD by reducing *de novo* lipogenesis. Due to its high degree of fermentability, soy fiber may alter gut microbiota and has the potential to reduce inflammation. High-oleic soybean oil, although largely unstudied, may be protective against dyslipidemia and insulin resistance. I hypothesize that the combination of soy protein, soy fiber, and high-oleic soybean oil will effectively mitigate the progression of NAFLD and ameliorate obesity-related inflammation.

I propose the following objectives to test my hypothesis:

1. To determine if substituting a fiber-rich soy protein concentrate into a high-fat fed mouse diet prevents the development of obesity and NAFLD

2. To determine if substituting high-oleic soybean oil into a high-fat fed mouse diet prevents the development of obesity and NAFLD

1.6.2 Tart Cherries

Research has demonstrated that tart cherries are effective in ameliorating obesity and markers of NAFLD. The current mechanism revolves around the absorption and systemic action of tart cherry anthocyanins. We propose that tart cherry proanthocyanins may contribute to these effects through local anti-inflammatory action in the gut. I
hypothesize that supplementation with tart cherries in a high-fat diet will reduce markers of obesity, inflammation, and NAFLD due not only to the systemic actions of anthocyanins but also because of the local anti-inflammatory action of proanthocyanidins in the intestine.

I propose the following objectives to test my hypothesis:

1. To determine if tart cherries reduce the severity of obesity and its related inflammation in vivo

2. To determine if a tart cherry derived extract is capable of mitigating inflammation in TNF-α-stimulated human colonic epithelial cells
Chapter 2: Impact of Dietary High-oleic Soybean Oil and Soy Protein Concentrate on Obesity-related Pathologies in High-Fat-Fed Mice

2.1 Introduction

Obesity is defined as having a body mass index (BMI) greater than 30 kg/m². Rates of overweight and obesity are on the rise in the United States and the rest of the developed world. Around 35% of adults in America fall into the obese category [146]. Obesity is a major risk factor for insulin resistance and T2DM, hypertension, cardiovascular disease, and several other diseases [7].

Obesity is often accompanied by the onset of NAFLD [147, 148]. The exact underlying cause of NAFLD remains unclear, but research indicates that it is initiated by excess lipid deposition in the liver due to insulin resistance and high intake of dietary fat. The development of hepatosteatosis is the first hit in the pathogenesis of NAFLD [52]. The second hit in the progression of steatosis to NASH is the onset of inflammation linked to peroxidation of fat in the hepatocytes. Other hypotheses suggest that the progression of hepatosteatosis to steatohepatitis could be due to endotoxemia, lipotoxicity, adipose induced inflammation, and endoplasmic reticulum stress [53].

Several factors can contribute to the deposition of lipids in non-adipose tissues such as the liver. Hepatic steatosis can be caused by an excess influx of endogenous and exogenous fatty acids from adipose tissue and dietary fats, respectively, the synthesis of new lipids in hepatocytes through the metabolic process known as de novo lipogenesis, decreased beta-oxidation of fatty acids in hepatocytes, or the insufficient efflux of fatty acids to peripheral tissues in VLDL-triglyceride particles [53, 149]. The prevention or
reversal of hepatosteatosis is crucial in preventing the progression of NAFLD and has been the goal of many intervention studies.

While calorie restriction and exercise are effective in preventing and reversing hepatic steatosis, additional complementary treatments could help support positive lifestyle changes. Previous studies have shown that isocaloric manipulation of the protein source in the diet has proven to be effective in slowing the pathogenesis of NAFLD in rodent models. When soy protein was substituted for casein in the diet, it reduced steatosis, increased insulin sensitivity, restored lipid homeostasis, and increased fatty acid oxidation [78-81, 83, 84, 91, 98, 150-153].

Soy protein affects the expression of markers related to lipid homeostasis both in the liver and adipose tissue [78, 79, 91]. In adipose tissue, rodents fed soy protein in place of casein had reduced levels of leptin, an important adipokine involved in systemic lipid homeostasis as well as decreased expression of genes related to lipogenesis [91]. Obese rats fed soy protein had reduced adipocyte hypertrophy compared to rats fed casein, reducing inflammation in adipose tissue and the production of inflammatory cytokines [81, 91]. In the liver, soy protein downregulates sterol regulatory element binding protein-1c (SREBP-1c) [78, 79, 84, 85]. SREBP-1c is a transcription factor that encodes for many genes involved in lipogenesis [154, 155]. Downregulation of SREBP-1c is directly linked to reduced de novo lipogenesis, a major contributing factor in the development of hepatic steatosis. The systemic effects of soy protein make it an attractive tool in mitigating NAFLD.

Isocaloric modification of the fatty acid composition of the diet has also been shown to alter markers related to obesity and NAFLD [156-166]. Saturated fatty acids have been
shown, both in animal and cell culture models, to increase ER stress, caspase-3 activity and the production of TNF-α [157, 164-166]. Dietary fatty acids are a major contributor to hepatosteatosis and diets containing a greater amount of saturated fatty acids have been shown to induce ER stress and promote an inflammatory state in NAFLD [164]. Diets high in oleic acid, such as the Mediterranean diet, have been shown to protect against dyslipidemia and cardiovascular disease [109]. This present study was designed to elucidate the effects of a combined dietary replacement of casein (CAS) and lard (HF) with soy protein concentrate (SPC) and high-oleic soybean oil (HOS), respectively, in a diet-induced NAFLD mouse model. We hypothesize that the combined protective effects of a diet containing soy protein and high oleic soybean oil will be effective in preventing the severity and progression of NAFLD.
2.2 Materials and Methods

2.2.1 Materials

The experimental mouse diets were purchased from Research Diets Inc (New Brunswick, NJ, Table 2-1). All solvents and standards used in RNA isolation, protein isolation, and sugar analysis were purchased from Sigma-Aldrich (St. Louis, MO) and were of the highest grade available. The triglyceride quantification assay was purchased from Wako Chemicals (Richmond, VA). Caspase substrates were purchased from Cayman Chemical (Ann Arbor, MI). PCR primers were purchased from Integrated DNA Technologies (Coralville, IA, Table 2-2). Enzyme-linked immunosorbent assays were purchased from Mesoscale Discovery (Rockville, MD).

2.2.2 Animal Study

All animal studies were approved by the Institutional Animal Care and Use Committee at the Pennsylvania State University (IACUC protocol no. 45380). Four-week-old, male C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME) and housed 5 per cage on a 12-hour light/dark cycle for the duration of the study. The mice were provided *ad libitum* access to water and experimental diets. Mice were randomized into four experimental groups based on body weight. The mice were fed either a lard-based, high-fat control diet with casein (HF+CAS), a lard-based, high-fat, soy protein concentrate containing diet (HF+SPC), a high-oleic soybean containing diet with casein (HOS+CAS), or a diet containing both high-oleic soybean oil and soy protein concentrate (HOS+SPC) for 18 weeks (Table 2-1). The HF diets consisted of 60% kcal from lard, while the HOS diets contained 40% kcal from high-oleic soybean oil and 20% kcal from lard. The CAS
diets contained 100% of dietary protein from casein. The SPC containing diets replaced 12% of the diets by weight with 12% soy protein concentrate accounting for one-third of the final protein. The experimental diets were purchased from Research Diets Inc (New Brunswick, NJ).

Table 2-1: Composition of experimental soy diets

<table>
<thead>
<tr>
<th>Diet Ingredient</th>
<th>HF (gm)</th>
<th>HF+SPC (gm)</th>
<th>HOS (gm)</th>
<th>HOS+SPC (gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein, Lactic 30 mesh</td>
<td>200</td>
<td>127.2</td>
<td>200</td>
<td>127.2</td>
</tr>
<tr>
<td>Soy Protein Concentrate, Acron SJ</td>
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Food intake and body weights were recorded weekly. Fasting blood glucose was determined by tail blood draw every other week. Blood samples were collected in EDTA-coated tubes via submandibular puncture every four weeks and the plasma isolated by centrifugation at 10,000 xg for 10 min and stored at -80°C for analysis.

At the conclusion of the study, mice were fasted for 12h and then euthanized under anesthesia via cardiac puncture and cervical dislocation. Blood collected by cardiac puncture was centrifuged to isolate the plasma which was aliquoted at stored at -80°C. The liver, as well as the epididymal, retroperitoneal, and mesenteric depots of visceral adipose, were rinsed in sterile phosphate buffered saline (PBS), weighed, and stored at -80°C. A central section of each liver was fixed in 10% formalin for histological analysis.

2.2.2 mRNA isolation and PCR analysis

Hepatic mRNA was isolated using the TRI reagent according to manufacturer’s protocol. Resultant mRNA was quantified using the Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA) and stored at -80°C for future analysis. cDNA was synthesized from the diluted mRNA using the RT first Strand kit (Qiagen, Hilden, Germany) according to manufacturer’s instructions. RT-qPCR was conducted on samples of synthesized cDNA using a 7900HT PCR instrument at the Genomics Core Facility (Huck Institutes of the Life Sciences, Penn State University). All primers used were purchased from Integrated DNA Technologies (Table 2-2). Results of gene expression data were reported using the $2^{-\Delta\Delta CT}$ method representing the relative expression of the gene of interest compared to the expression of Gapdh.
2.2.3 Cytokine expression

Several cytokines were selected as markers to evaluate the degree of hepatic inflammation in the mice including IL-1β, IL-6, IL-10, TNF-α, and MCP-1. Samples of liver tissue were homogenized in phosphate buffered saline lysis buffer using a bead homogenizer (Next Advance). Homogenates were centrifuged at 10,000 xg for 5 min, and the protein-rich supernatant was collected. The samples were analyzed for cytokine expression by multiplex ELISA (Mesoscale Discovery, Rockville, MD) and results were normalized to total protein concentration as determined by the Bradford assay.

2.2.4 Liver histology

The degree of macrovesicular steatosis in the hepatocytes was determined through evaluation of the H&E stained liver section prepared from each mouse liver. Livers were
fixed in 10% buffered formalin, embedded in paraffin, and sectioned into 5μm thick sections at the Animal Diagnostic Laboratory (The Pennsylvania State University, University Park, PA). For each liver section, high-resolution images were taken of 5 individual microscopic fields (100x) by a member of the laboratory who was blinded to the identity of the samples. Each image was then converted to a high-contrast black and white image using the color select function in Adobe Photoshop CS6 (San Jose, CA). Lipid droplets remained white, while the surrounding hepatic tissue was black. These images were then processed using ImageJ software (National Institutes of Health, Bethesda, MD) to quantify the area occupied by macrovesicular steatosis. A computer macro was created that selected the lipid droplets based on the color threshold and then identified each discrete lipid droplet, quantified its area, and exported that data into Excel (Microsoft Co., Redmond, WA). Macrovesicular fat droplets were defined as any droplet occupying at least 1000 pixels. The area of all lipid droplets for each image was summed and divided by the total image area to determine the percent macrovesicular fat for each field. The degree of macrovesicular steatosis for each mouse was determined by taking the mean value of the percent area of macrovesicular fat from the 5 microscopic fields. The degree of macrovesicular steatosis could then be compared among the treatment groups.

2.2.5 Caspase activity

Caspase activity was used in this study as an indicator of apoptosis in hepatic tissue. The activities of both the initiator caspase-8 and the effector caspases-3 and -7 were determined. Liver samples from each mouse were gently homogenized in lysis buffer using a Dounce homogenizer. The homogenates were centrifuged at 10,000 xg for 10 minutes,
and the supernatants were collected. The concentration of protein (PROT) in each sample was determined using the Bradford assay. All samples were diluted in lysis buffer to a final concentration of 5 mg/mL. Caspase substrate consisting of four amino acid sequences recognized by the caspases (Ile-Glu-Thr-Asp for caspase-8 and Asp-Glu-Val-Asp for caspase-3/7) bonded to a fluorophore, aminomethylcoumarin (AMC), was diluted in assay buffer to a final concentration of 75 μM. Homogenate samples were incubated with 2 volumes of caspase substrate at 37°C for 1h. The fluorescence intensity of the liberated AMC (excitation λ: 380nm/emission λ: 460nm) was recorded for each sample, and the quantity of AMC was determined using an AMC standard curve. The caspase activity for each sample was reported as ngAMC/mg PROT/min.

2.2.6 Liver Triglycerides

Liver triglycerides were determined using a colorimetric endpoint assay. Liver samples were cut, weighed, and homogenized in a lysis solution of isopropanol:water:Triton X-100 (5:2:2) and the resultant homogenate was centrifuged at 10,000 xg for 5 min. The triglyceride concentration of the supernatant was determined using the triglyceride quantification assay according to the manufacturer’s instructions. Triglyceride content was expressed as a percentage of total liver weight based on the concentration of TGs in the weighed sample.

2.2.7 Gastrointestinal Permeability Assay

Increased gastrointestinal permeability has been associated with intake of high-fat diets [69, 167]. This can lead to an increase in circulating levels of lipopolysaccharide
which may contribute to hepatic inflammation and the progression of NAFLD [168-170].

Previous research in our lab has shown that soy protein concentrate reduces intestinal permeability in dextran sulfate sodium-induced colitis model [171]. A dual-sugar assay was used to evaluate intestinal permeability of mice fed experimental diets in vivo. This method was adapted from previous work [172, 173]. A mixture of L-rhamnose (3.33g/mL) and lactulose (6.66g/mL) was prepared in purified water and sterilized by filtration. A subset of mice (n=10 per treatment) was fasted for 12 h overnight with ad libitum access to water. Mice were given a bolus of the sugar solution by oral gavage. The dose was adjusted so that each mouse received 4mL/kg bodyweight. After 1h, a 200 μL blood sample was collected from each mouse via submandibular puncture. The blood was centrifuged to isolate the plasma fraction which contained the sugars. Plasma samples were extracted with methanol and dried according to established methods [174]. Dried metabolites were trimethylsilylated with a mixture of N,O-bis(trimethylsilyl)trifluoroacetamide: pyridine: trimethylchlorosilane (6:3:1) for 12h at 55°C in a sealed reaction vessel and then diluted (1:2) in hexanes. A DB5-MS capillary column (Agilent, Palo Alto, CA) was used with helium at a flow rate of 1.3 mL/min. The injector was set to 200°C, and the MS source at 250°C. The temperature profile consisted of injection at 65°C with a 2 min hold, a linear increase of 6°C/min to 300°C, and an isothermal hold at 300°C for 15 min [173]. All samples were run in splitless mode. The mass spectrometer was set to a scan of 50 to 650 Da at 1.27 scan/s. Silylated sugar concentrations were quantified using total ion current peak area compared to standards.
2.2.8 Statistical Analysis

Statistical analyses were performed using GraphPad Prism 6 software (La Jolla, CA). All data were analyzed using 2-way ANOVA with the main effects of protein source and fat source and the interaction of protein source by fat source. When main effects were significant in the absence of significant interaction, data were pooled. Significant main effects were analyzed by Student’s *t*-test as a post-test. Significant interaction effects were analyzed by Tukey’s post-test. Statistical significance was achieved at *p*<0.05. A non-significant trend was achieved at *p*<0.1.
2.3 Results

2.3.1 Body Weight and Visceral Adiposity

Both dietary protein and fat had significant effects on body weight and visceral adiposity (p<0.05, Figure 2-1). Mice that consumed HOS diets had significantly lower body weight gain (2.40 ± 0.05 fold-increase), measured as fold-increase in initial body weight, compared to mice fed lard-based (HF) diets (2.64 ± 0.03 fold-increase, p<0.0001). Mice fed soy protein (SPC) had less visceral adipose (8.51 ± 0.17%) than casein-fed (CAS) mice (9.44 ± 0.21%, p<0.001). Mice fed HOS had less visceral adipose (8.39 ± 0.19%) than mice fed the HF diet (9.51 ± 0.19%, p<0.0001). There was no significant interaction effect between protein source and fat source on body weight or visceral adiposity.
Figure 2-1: Body weights were measured weekly over the course of the study for all mice (n=15 per group) (A). Endpoint body weights were expressed as fold increase from initial weight for each mouse (B). Endpoint visceral adiposity was determined by the combined weight of epididymal, retroperitoneal, and mesenteric fat depots and expressed as a percentage of final body weight (E). Differences between treatment groups were determined by 2-way ANOVA. When there were significant main effects, data were then pooled by main effects of protein (C, F) and fat (D, G) and differences were determined by Student’s t test. Data presented as mean ± SEM, *p<0.05.
2.3.2 Blood markers of obesity-related pathologies

Blood glucose was measured over time. Mice fed SPC had significantly lower fasting blood glucose levels compared to mice fed CAS (p<0.01, Figure 2-2). The fat source had no effect on fasting blood glucose (p>0.05, Figure 2-2). Fasting blood glucose levels increased significantly over time independent of treatment (p<0.05, Figure 2-2). Circulating insulin levels were not measured, but previous work in our lab has shown that elevated fasting blood glucose is associated with increased serum insulin levels in the high-fat fed mouse model [175].
Plasma levels of alanine aminotransferase (ALT) were monitored as a marker of liver damage throughout the study. No significant differences were observed across treatments groups, but final ALT levels significantly increased from baseline levels for all mice (p=0.004, Figure 2-3). Endpoint concentrations of serum triglycerides and serum cholesterol were measured. No significant effects on serum cholesterol were observed between treatments (p>0.05, Figure 2-3). The levels of serum triglycerides were
significantly higher in mice fed SPC (p<0.05, Figure 2-3), but no effects of the dietary fat source or the interaction of fat and protein source were found (p>0.05, Figure 2-3).

**Figure 2-3:** Serum markers. Fasting serum alanine aminotransferase (ALT), cholesterol (CHOL), and triglycerides (TG) were measured for all mice (n=15 per group) at the conclusion of the study by colorimetric assay. Differences between treatment groups were determined by 2-way ANOVA with Tukey’s post-test. No significant differences were found in levels of ALT or CHOL. Mice fed HOS+CAS had significantly increased levels of fasting TGs compared to all other treatments. Data presented as mean ± SEM, letters indicate significant differences (p<0.05).
2.3.3 Gene Expression

The expression of several genes related to inflammation and inflammatory signaling were examined in the liver. IL-1β is an inflammation-promoting cytokine-activated by Caspase-1 which is related to increased hepatocyte steatosis, cell death, activation of other downstream inflammatory cytokines [176, 177]. There was no significant change in the expression of IL1b mRNA between treatment groups, but nonsignificant decrease in mRNA levels in the SPC treated mice compared to CAS-fed mice (p=0.06, Figure 2-4). There was a significant increase in the gene expression of Casp1, the protein responsible for the cleavage of pro-IL-1β in mice fed HOS (p<0.05, Figure 2-4). The gene expression levels of several other inflammatory cytokines were examined including IL-6, TNF-α, and MCP-1. IL-6 is a proinflammatory cytokine that plays a role in the recruitment of monocytes [178]. MCP-1 recruits monocytes to damaged tissues, and it is often upregulated in both alcoholic and nonalcoholic fatty liver disease [179, 180]. TNF-α is an important signaling cytokine in the upregulation of inflammation in several tissues throughout the body through a receptor-initiated cascade leading to cell death or the upregulation of inflammatory genes [29, 181]. No significant differences were found in the levels of gene expression of any of these inflammatory cytokines.
Hepatic protein concentrations of IL-1β, IL-6, MCP-1, and TNF-α were determined by ELISA. Levels of both MCP-1 and TNF-α were significantly higher in mice given HOS-based diets, while protein source had no effect (p<0.05, Figure 2-5). No significant changes in IL-6 or IL-1β were observed (p>0.05, Figure 2-5).

Figure 2-4: Inflammatory gene expression. Hepatic gene expression of Il-1b (A-C) and Casp1 (D-F) were determined for all mice at the conclusion of the study (n=15 per group). Differences between treatment groups were determined by 2-way ANOVA. When there were significant main effects and no significant interaction, data were then pooled by main effects of protein (B, E) and fat (C, F) and differences were determined by Student’s t test. Mice fed SPC had decreased Il-1b expression independent of dietary fat source compared to mice fed CAS (B). Mice fed HOS had significantly increased expression of Casp1 regardless of dietary protein source compared to HF-fed mice (F). Data presented as mean ± SEM, *p<0.05.
Signaling through death receptors is thought to be one mechanism behind hepatic inflammation and the progression of NAFLD [29, 30, 182-185]. We examined the expression of several genes related to these pathways including receptors, ligands and downstream targets in these pathways. Cysteine-aspartate proteases or caspases are a group of proteases that can both promote inflammation or induce apoptosis in the cell [186]. Upstream Caspases-1 and -8 are known as initiator caspases that act on other cellular targets. Analysis revealed that SPC containing groups had significantly lower levels of caspase-8 and caspase-8 associated protein-2 (Casp8ap2) mRNA compared to their SPC-free control diets (p<0.0002, Figure 2-6). Casp8 gene expression was significantly higher
in mice fed HOS compared to HF (p<0.05, Figure 2-6). Caspases-3 and -7 are effector caspases that are responsible for inducing apoptosis. No significant differences were observed in the expression of these two genes (p>0.05). The Fas ligand (FasL), Fas receptor and the Fas-associated death domain (Fadd) are a group of death receptor proteins involved in the activation of Casp8 [29, 187].

![Figure 2-6](image)

**Figure 2-6:** Death receptor gene expression. Hepatic gene expression of *Casp8* (A-C) and *Casp8ap2* (D-F) were determined for all mice (n=15 per groups) by RT-qPCR. Differences between treatment groups were determined by 2-way ANOVA. When there were significant main effects, data were then pooled by main effects of protein (B, E) and fat (C, F) and differences were determined by Student’s t test. Expression of Casp8 was lower in mice fed SPC compared to those fed CAS regardless of dietary fat source. *Casp8* expression was elevated in mice fed HOS compared to those fed HF regardless of dietary protein source. Expression of Casp8ap2 was significantly lower in mice fed SPC compared to those fed CAS regardless of dietary fat source. Data presented as mean ± SEM, *p<0.05.
The *Fas* gene expression was elevated in mice fed HOS, whereas the expression of *Fasl* was significantly lower in mice fed SPC (p<0.05, Figure 2-7). No significant differences between protein, fat, or their interaction were observed in the expression of *Fadd* mRNA (p>0.05).

**Figure 2-7**: Fas-related gene expression. Hepatic gene expression of *Fas* (A-C) and *Fasl* (D-F) were determined for all mice (n=15 per groups) by RT-qPCR. Differences between treatment groups were determined by 2-way ANOVA. When there were significant main effects, data were then pooled by main effects of protein (B, E) and fat (C, F) and differences were determined by Student’s *t* test. *Fas* expression was elevated in mice fed HOS compared to those fed HF regardless of dietary protein source. Expression of *Fasl* was significantly lower in mice fed SPC compared to those fed CAS regardless of dietary fat source. Data presented as mean ± SEM, *p*<0.05.
2.3.4 Caspase Activity

A fluorescence-based assay was used to determine the caspase activity in the liver tissues. The activity levels of the effector Caspases-3 and-7 were similar for all treatment groups, and no significant differences in activity were observed in activity when normalized to the total protein content of each sample (p>0.05, Figure 2-8). Similarly, there were no significant changes in caspase-8 activity, although there was a slight but nonsignificant decrease in the HF+SPC group compared to the HF+CAS control (p=0.07, Figure 2-8).

![Figure 2-8: Caspase activity. Hepatic caspase activity was determined in liver homogenates for all mice (n=15 per group) by fluorometric assay. No significant effects were observed. Data presented as mean ± SEM.](image-url)
2.3.5 Lipid regulation gene expression

Lipid metabolism can be altered in obesity, contributing to the deposition of triglycerides in hepatocytes leading to macrovesicular steatosis [91]. Several genes related to fat storage and metabolism were examined. Sterol regulatory element binding factor-1 (Srebf1) encodes for sterol regulatory element binding protein-1c, a transcription factor responsible for the regulation of fatty acid synthesis. Although not statistically significant, there was a decrease in the expression of Srebf1 in the SPC groups (p=0.06, Figure 2-9). Hepatic lipase (LipC) is responsible for breaking down stored triglycerides into non-esterified fatty acids. Gene expression of Lipc was significantly lower in mice fed SPC (p=0.05, Figure 2-9).
2.3.6 Hepatic Steatosis

The percentage of liver TGs were determined by colorimetric assay as an indication of total hepatic fat. Liver TG levels varied little between treatments with average TG content ranging from 5.36% to 5.66% across treatments. No significant differences in hepatic TG content were found among treatments (p>0.05). In contrast to liver TG content, significant differences in the degree of macrovesicular hepatic steatosis were found by histological analysis (Figure 2-10).
The average degree of steatosis defined by the percent area of macrovesicular fat droplets in the liver sections of soy protein-fed mice was 1.683 ± 0.340% (mean ± SEM), and increased significantly to 3.622 ± 0.681% in casein-fed mice (p=0.01, Figure 2-11). Perilipin-2 (Plin2) is a lipid droplet protein located on the surface of hepatic lipid droplets [188]. *Plin2* levels were slightly elevated in SPC treated mice (p=0.2, Figure 2-11).
Figure 2-11: Macrovesicular Steatosis. The degree of macrovesicular steatosis was determined for all mice (n=15 per group) by histological analysis (A-C). % images were taken of each liver slide and then analyzed for macrovesicular fat area using Image-J software. Plin2 gene expression was determined for all mouse livers by RT-qPCR (D-F) Differences between treatment groups were determined by 2-way ANOVA. When there were significant main effects, data were then pooled by main effects of protein (B, E) and fat (C, F) and differences were determined by Student’s t test. Mice fed SPC had significantly less macrovesicular steatosis compared to CAS-fed mice regardless of dietary fat source (B). No significant differences were found in the expression of Plin2 although mice fed SPC had slightly increased levels of expression compared to Cas-fed mice (E). Data presented as mean ± SEM, *p<0.05.
2.3.7 Gastrointestinal Permeability

Previous research by our lab demonstrated that soy protein concentrate reduces gastrointestinal permeability in a DSS-induced colitis mouse model [171]. The degree of relative gastrointestinal permeability was determined using a dual-sugar permeability assay as described previously [172, 173]. The lactulose to rhamnose (L/R) ratio was measured in the plasma collected from the mice. There were no significant differences between the L/R ratios for protein source, fat source or their interaction (p>0.05, Figure 2-12).

**Figure 2-12:** Relative gut permeability. Permeability expressed as the ratio between lactulose and rhamnose for all treatment groups. Mice (n=9 per group) were given a bolus of sugar solution and blood was collected after 1 hr. Lactulose and rhamnose concentrations were determined by GC-MS. No significant differences observed. Data presented as mean ± SEM.
2.4 Discussion

Obesity and NAFLD are on the rise in the world and place a massive burden on the healthcare system. Previous research with soy protein has indicated that it may protect against the progression of NALFD by regulating lipogenesis and mitigating inflammation [78, 81, 84, 152]. Oleic acid has been shown to ameliorate dyslipidemia and protect against obesity-related pathologies [108, 156, 189]. We hypothesized that together, soy protein and high oleic soybean oil would protect against obesity, its related pathologies, and NAFLD in a mouse model of diet-induced obesity.

The results of this study demonstrate the protective potential of soy protein concentrate in the progression of NAFLD and other obesity-related pathologies in a high-fat-fed mouse model compared to a casein-based diet. Soy protein concentrate significantly reduced the degree of hepatic steatosis and fasting blood glucose levels. This is likely related to an observed decrease in the expression of Srebf1 and hepatic lipase which play a vital role in hepatic lipogenesis. We also observed significant reductions in the levels of markers involved in inflammation and lipotoxicity including Casp1, Casp8, Il1b, and Fasl.

Despite previous evidence that oleic acid may be protective against NAFLD and markers of obesity, high-oleic soybean oil proved to be pro-inflammatory in this study. We found that mice fed HOS diets had elevated levels of inflammatory cytokines as well as proteins related to death receptor signaling and cellular apoptosis. These mice exhibited increased protein levels of TNF-α, and MCP-1 and increased gene expression of Casp1, Casp8, and Fas. HOS-based diets did reduce visceral adiposity and weight gain, but this had no effect on the severity of the previously mentioned inflammation or in the degree of hepatic steatosis.
We observed significantly lower fasting blood glucose levels in mice fed SPC. Foretz et al. demonstrated that increased glucose levels are required for the upregulation of genes, such as fatty acid synthase (FAS), required for de novo lipogenesis [86]. When increased levels of glucose are present in the hepatocyte, the transcription factor sterol regulating element binding protein-1c (SREBP-1c) activates transcription of FAS and acetyl-CoA carboxylase (ACC), stearoyl-CoA desaturase (SCD)-1, elongation of long chain fatty acids family member (ELOVL)-6. Previous research in our lab has found that increased fasting glucose levels are often correlated with insulin resistance and circulating insulin levels [175]. Although we did not measure levels of circulating insulin in this study our previous research would suggest that the lower fasting glucose levels in the SPC groups indicates reduced insulin resistance and circulating insulin levels. SREBP-1c is encoded for by the Srebf1 gene which is activated by an insulin dependent pathway [78, 154]. We observed significantly lower levels of Srebf1 mRNA in SPC-fed mice compared to CAS-fed mice. Although we did not examine SREBP-1c protein levels, our results are consistent with other studies that examined the effects of soy protein in the liver [78, 79]. Other studies have demonstrated that de novo lipogenesis contributes to the accumulation of lipids in steatohepatitis [83]. In the present study macrovesicular steatosis, a histopathological a marker of NAFLD severity [46], was reduced by 54% in soy protein-fed mice compared to casein-fed mice regardless of fat source. Soy protein downregulates the expression of lipogenic genes by reducing hyperglycemia and hyperinsulinemia. Our evidence indicates that by the reduction of hyperglycemia and by inhibiting the expression of genes related de novo lipogenesis, soy protein concentrate may protect against the development of macrovesicular steatosis.
Increased SREBP-1c signaling leads to increased production of long chain saturated fatty acids, mainly palmitic and stearic acid. These newly generated FAs are incorporated into triglycerides (TGs) which can be exported to other tissues via VLDL particles [190]. In obese subjects, leptin resistance can alter how these newly formed TGs are stored. In tissues, such as the liver, leptin resistance leads to storage of excess lipids within the cell rather than exporting them to adipose tissues or for use as energy. The reduced steatosis observed in the soy protein-fed mice is due, in part, to increased leptin sensitivity in addition to reduced lipogenesis in our model. We did not examine the impact of leptin in this model, but Torre-Villalvazo et al. observed that soy protein increased peripheral leptin sensitivity in rats and increased the expression of leptin receptors in the hypothalamus [84].

Free fatty acids from de novo lipogenesis have the potential to induce inflammation and cytotoxicity in the liver in a process known as lipotoxicity. Free fatty acids in the liver are derived from hepatic lipogenesis, via delivery from adipose tissue, or through the breakdown of stored hepatic triglycerides by hepatic lipase (LipC) [191]. We observed significantly lower expression of Lipc mRNA in soy protein-fed mice compared to casein-fed mice. This leads us to believe that mice fed soy protein have lower levels of hepatic free fatty acids. Hepatic free fatty acids have been shown to increase endoplasmic reticulum (ER) stress leading to the induction of the TNF-apoptosis inducing ligand receptor 2 (TRAIL-R2) [192-194]. TRAIL-R binding is responsible for Casp8 activation, the increased release of cytochrome c, the activation of Casp3 and hepatocyte apoptosis [30]. We observed higher expression of Casp8 and Casp8ap2 in CAS-fed mice compared to SPC-fed mice indicating potentially higher levels of apoptosis. Similarly, we observed
a trend toward increased in hepatic Casp8 activity in CAS-fed mice compared to SPC-fed mice, but the results did not achieve statistical significance.

Apoptosis can also be induced by the binding of the FasL, a cytokine released by activated macrophages, to Fas. We observed significantly lower levels of Fasl mRNA in SPC-fed mice compared to CAS-fed mice which may indicate a reduced degree of inflammation and help to explain our observations of reduced Casp8 expression.

Saturated free fatty acids can sensitize immune cells to proinflammatory signaling. Saturated free fatty acids have been shown to activate the Nod-like Receptor Protein-3 (NLRP3) inflammasome in vitro [195, 196]. The NLRP3-inflammasome can be activated by microvesicles released from apoptotic cells [197]. Although we did not observe any differences in the apoptotic activity of hepatic caspases, we did observe a significant reduction in the expression of Casp8 mRNA in mice fed soy protein concentrate. NLRP3-inflammasome activation is responsible for Casp1 activation [171, 195]. Casp1 is the protease that converts pro-IL-1β into its active form [198]. IL-1β is believed to contribute to obesity-induced T2DM [198]. We observed significantly lower expression of Casp1 mRNA which may indicate reduced activation of IL-1β. We also found reduced expression of Il1b in SPC-fed mice compared to CAS-fed mice. One limitation of this study is that we did not measure the protein levels of CASP1 or IL-1β. The protein concentrations and activities should be examined in future research to determine if transcriptional changes induced by SPC or HOS lead to changes in protein translation and activity. Activation of IL-1β by CASP1 requires activation of the NLRP3 inflammasome and future studies should examine the role of the inflammasome in this model [176].
One factor that may exacerbate NAFLD-associated inflammation is the onset of metabolic endotoxemia due to increased levels of circulating lipopolysaccharide [169, 199]. Although endotoxins can be absorbed through chylomicrons, another path of absorption is through weakened tight junctions in the intestine [67, 200]. Gastrointestinal permeability has been shown to increase in high-fat diets [167]. We have previously found that soy protein concentrate mitigates intestinal permeability *in vivo* using a DSS-induced model of colitis [171]. We did not observe this effect in our diet-induced obesity model in the current study. This may be due to differences in the mechanism of permeability. In colitis, the increased permeability may be due to excess inflammation in the gastrointestinal tract or in the DSS used in the mouse model. These factors were not present in the current study and may account for the lack of changes in intestinal permeability in SPC-fed mice. Gastrointestinal permeability is only one measure of metabolic endotoxemia. Future work should also examine plasma levels of lipopolysaccharide to determine if metabolic endotoxemia is contributing to the progression of NAFLD in the current model and whether or not the dietary interventions are having an effect.

One aspect of the present research that is unique compared to previous work on soy protein is that we employed a soy protein concentrate which contains a significant amount of soy fiber (~20%). Unlike the cellulose used in control diets, soy fiber is highly fermentable by gut bacteria [94, 99, 201]. Previous research has indicated that soy fiber may have systemic effects on obesity-related pathologies like insulin sensitivity and dyslipidemia [96, 99]. Fermentable fibers from other sources have been shown to alter the distribution of microbiota populations which may protect against inflammatory disease states such as inflammatory bowel disease and the absorption of endotoxins [171, 202-
Local changes in gut inflammation and toxin production have been shown to greatly affect liver function and inflammation [205, 206]. There is a possibility that some of the protective effects we see from SPC treatment are due to the high fiber content. This will be an active area for further study.

In contrast to HF-fed mice, HOS-fed mice had significantly reduced weight gain as well as reduced relative visceral adiposity regardless of protein source. This is consistent with other studies that have found that oils high in oleic acid, such as olive oil, can increase thermogenesis in adipose and lead to decreased adiposity [189, 207, 208]. The expression of factors related to thermogenesis in adipose tissue was not evaluated in this study and additional research is required to determine the mechanism of reduced adiposity in the high-oleic diet.

Despite its apparent benefit with regard to reduced weight gain and adiposity, several lines of data in this study indicate that HOS-fed mice had a greater level of inflammation than HF-fed mice. In fact, SPC and HOS appear to have opposing roles in the treatment of NALFD. We observed increased hepatic expression of several inflammatory genes including Casp1, Casp8, and Fas in HOS-fed mice while Casp8, Casp8ap2, and Fasl expression were decreased in SPC-fed mice. Hepatic protein expression of inflammatory macrophage activity markers TNF-α and MCP-1 were also increased in HOS-fed mice, while the gene expression of related marker Il1b was decreased in SPC-fed mice. These data indicate that HOS and SPC elicit their effects through similar pathways, although SPC acts as an anti-inflammatory agent and HOS works as a proinflammatory stimulus.
The HOS diets contained significantly less saturated fat compared to the HF diets. The link between long-chain saturated fatty acids and inflammation has been established in several different models including tissue culture, rodents, and humans [157, 161, 164, 166, 195, 209, 210]. Increased dietary saturated fat consumption has been linked to increased ER stress and inflammation in NAFLD, notably the release of TNF-α [164]. Other work has shown that saturated fatty acids can activate Toll-like receptor (TLR)-2 and -4 and induce ER stress and the upregulation of several key inflammatory cytokines involved in the progression of NAFLD [195]. The level of palmitic acid in our HF-diet was higher than the level in the HOS group (Table 2-3). Palmitic acid has been noted as highly inflammatory and is now used in conjunction with sucrose to induce NAFLD in some mouse models [211, 212].

Table 2-3: Fatty acid profiles of mouse diets and related fat sources. Values in table reported in percentages. All data taken from USDA National Nutrient Database for Standard Reference Release 28.

<table>
<thead>
<tr>
<th>% Fatty Acid</th>
<th>HF</th>
<th>HOS</th>
<th>Soybean</th>
<th>Canola</th>
<th>Flaxseed</th>
<th>Olive Oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;C14</td>
<td>0.12</td>
<td>1.80</td>
<td>0.00</td>
<td>0.00</td>
<td>0.02</td>
<td>0.00</td>
</tr>
<tr>
<td>C14:0</td>
<td>0.31</td>
<td>0.12</td>
<td>0.04</td>
<td>0.00</td>
<td>0.08</td>
<td>0.00</td>
</tr>
<tr>
<td>C16:0</td>
<td>20.94</td>
<td>11.17</td>
<td>10.66</td>
<td>4.30</td>
<td>5.11</td>
<td>11.29</td>
</tr>
<tr>
<td>C18:0</td>
<td>10.92</td>
<td>6.21</td>
<td>4.00</td>
<td>2.09</td>
<td>3.37</td>
<td>1.95</td>
</tr>
<tr>
<td>C18:1</td>
<td>34.03</td>
<td>62.97</td>
<td>22.57</td>
<td>61.74</td>
<td>18.32</td>
<td>71.27</td>
</tr>
<tr>
<td>C18:2</td>
<td>28.72</td>
<td>14.41</td>
<td>50.30</td>
<td>19.01</td>
<td>14.33</td>
<td>9.76</td>
</tr>
<tr>
<td>C18:3</td>
<td>2.04</td>
<td>2.04</td>
<td>7.03</td>
<td>9.14</td>
<td>53.37</td>
<td>0.76</td>
</tr>
<tr>
<td>C20:0</td>
<td>0.16</td>
<td>0.25</td>
<td>0.24</td>
<td>0.65</td>
<td>0.13</td>
<td>0.41</td>
</tr>
<tr>
<td>C20:1</td>
<td>0.59</td>
<td>0.31</td>
<td>0.13</td>
<td>1.32</td>
<td>0.00</td>
<td>0.31</td>
</tr>
<tr>
<td>C22:1</td>
<td>0.08</td>
<td>0.03</td>
<td>0.00</td>
<td>0.00</td>
<td>0.01</td>
<td>0.00</td>
</tr>
<tr>
<td>Other</td>
<td>2.08</td>
<td>0.69</td>
<td>5.03</td>
<td>1.76</td>
<td>5.27</td>
<td>4.24</td>
</tr>
<tr>
<td>Total</td>
<td>100.00</td>
<td>100.00</td>
<td>100.00</td>
<td>100.00</td>
<td>100.00</td>
<td>100.00</td>
</tr>
<tr>
<td>%SFA</td>
<td>32.34</td>
<td>17.75</td>
<td>14.94</td>
<td>7.04</td>
<td>8.68</td>
<td>13.66</td>
</tr>
<tr>
<td>%MUFA</td>
<td>34.62</td>
<td>63.28</td>
<td>22.70</td>
<td>63.06</td>
<td>18.32</td>
<td>71.58</td>
</tr>
<tr>
<td>%PUFA</td>
<td>30.84</td>
<td>16.48</td>
<td>57.33</td>
<td>28.14</td>
<td>67.71</td>
<td>10.52</td>
</tr>
<tr>
<td>ω-6:ω-3</td>
<td>14.06</td>
<td>7.06</td>
<td>7.16</td>
<td>2.08</td>
<td>0.27</td>
<td>12.83</td>
</tr>
</tbody>
</table>
The HOS diets also had a lower ω-6:ω-3 ratio than the HF diets. Several lines of research have suggested that the ideal ω-6:ω-3 ratio is 1:1 while the typical Western diet is approximately 15:1 [213]. Based on this evidence we believed that the lower saturated fat content and lower ω-6:ω-3 ratio would be protective in a high-fat diet. Our data do not support that hypothesis. HOS diets appeared to increase the expression of several of the inflammatory markers that were decreased by SPC. One possible explanation could be that the physiological effects of high oleic oils change in high-fat diets. A recent meta-analysis of high-fat diets indicated that diets containing over 40% fat with high ω-6:ω-3 ratios induced insulin resistance, a key factor in the development of hepatosteatosis, and inflammation, although no consistent predictor of insulin resistance has been identified in the fatty acid profiles of high-fat diets [214]. Tovar et al. also observed that the benefits of certain fatty acids are diminished in high-fat diets [163].

As we mentioned previously, dietary fatty acids contribute to the fatty acids present in the liver. Oleic acid has been shown to be protective against lipotoxicity in vitro although it may contribute to steatosis [215]. If oleic acid is not causing lipotoxicity in the liver, inflammatory signaling may be originating elsewhere in the body. Previous research has shown that changes in dietary fat can affect lipid regulation in adipose tissue [163]. Further investigation is needed to identify fatty acid profiles that induce or reduce inflammation in high-fat diets and to determine whether or not HOS alters lipid homeostasis in adipose tissue.
2.5 Conclusions

The present study provided evidence for the potential protective role of a fiber-rich soy protein concentrate in the pathogenesis of NAFLD. Mice fed soy protein concentrate in place of casein exhibited reduced hyperglycemia, visceral adiposity, and markers of inflammation. The data also indicate that soy protein concentrate may be protective against lipotoxicity which plays a major role in the progression of steatosis to NASH. Further research is necessary to investigate the exact mechanism by which soy protein mitigates lipotoxicity and to establish whether its effects are sufficient to prevent or slow the progression of liver disease in a NASH model. Soy protein concentrate also contains significant amounts of soybean fiber that is known to be highly fermentable by gut bacteria. Changes in gut microbiota due to the presence of fermentable soy fiber should be evaluated for their potential to mitigate obesity-related inflammation both in the gut and systemically.

This body of work has established that despite its oleic acid content, low saturated fat content, and relatively low ω6:ω3 ratio, high oleic acid soybean oil increased several inflammatory markers compared to the lard-based high-fat diet. The pro-inflammatory effects of HOS appear to oppose the anti-inflammatory effects of SPC. Several inflammatory markers mitigated by SPC were upregulated by HOS. Therefore, we can conclude that combined supplementation with HOS and SPC would not be effective in combating obesity or its related pathologies. Further studies are needed to evaluate these opposing roles in the context of the whole high-oleic soybean or in foods derived from the soybean to determine whether or not these effects are antagonistic in a more complex system. The effects of HOS should also be evaluated in the context of the products it is meant to replace: soybean oil and partially hydrogenated soybean oil.
Chapter 3: Efficacy of Tart Cherry Components for Mitigation of Obesity-related Inflammation

3.1 Introduction

Tart cherries (*Prunus cerasus*) are a rapidly growing sector of the fruit industry in the United States with substantial increases in production over the past decade. In 2014, U.S. cherry growers produced 298.4 million pounds of tart cherries worth $104 million dollars, more than triple the yield and double the crop value of tart cherries produced in 2012 (USDA/NASS, 2015). Each year more land is being utilized for the cultivation of tart cherries with around 38,000 acres of U.S. soil currently devoted to tart cherries. This is an increase of over 3,000 acres since 2007. Tart cherry containing products on the market claim numerous health benefits. Studies have shown the potential benefits of cherries in several health conditions including cardiovascular disease, diabetes, neurodegenerative diseases, and inflammatory diseases [117].

Obesity is a key contributor to the development of these diseases. Rates of overweight and obesity are on the rise in the United States and the rest of the developed world. Around 35% of Americans are classified as obese [1, 9, 216]. The healthcare cost of treating obesity and its comorbidities in the U.S. is near $200 billion dollars per year (CDC, [217]).

Many of the health issues related to obesity are caused by chronic inflammation linked excess adipose tissue. Inflammation in obese individuals is due, in part, to dysregulated lipid homeostasis in adipose tissue. Adipose tissue and responding immune cells release inflammatory cytokines or signaling proteins that cause a positive feedback
loop through the further recruitment of immune cells. This also contributes to a systemic inflammatory response by triggering inflammation signaling pathways in other organs such as the liver [218]. Studies have found relationships between obesity-related inflammation, insulin resistance, cardiovascular disease, NAFLD and other related pathologies [180, 219-222]. This inflammatory state is a primary target for treatment to combat the comorbidities of obesity and lessen the burden on the healthcare system.

A condition closely intertwined with obesity and inflammation is metabolic endotoxemia. A high-fat diet has been shown to alter the proportions of native bacteria in the gut to contain a greater number of Bacteroidetes compared to Firmicutes [65, 223]. Bacteroidetes are gram-negative bacteria that release lipopolysaccharide (LPS), an endotoxin, which can pass through the intestinal membrane and into the blood. The body mounts an immune response to the foreign endotoxin leading to inflammation in the gut and the liver [168, 203, 205, 206]. Bioactive compounds previously studied in our lab including proanthocyanidins from cocoa, green tea polyphenols, and soy protein derivates indicate that dietary components are capable of reducing local inflammation in the gut as well as systemic inflammation that may be linked to metabolic endotoxemia [171, 224, 225].

Existing research has begun to characterize the beneficial effects of tart cherries on obesity-related inflammation and other comorbidities [132, 133, 141, 226-228]. These studies have demonstrated tart cherries may be protective against some of the negative health consequences of obesity. For example, Seymour et al. found whole tart cherries reduced blood triglyceride levels, abdominal adiposity and inflammatory cytokines in rats [132, 133]. The anti-inflammatory effects of cherries have been attributed to the
anthocyanin content of tart cherries. This is supported by studies in similar anthocyanin containing berries that were found to be anti-inflammatory [229-232].

While anthocyanins may be responsible for many of the systemic effects of tart cherries, we propose that the high concentrations of proanthocyanidins present in tart cherries contribute to the anti-inflammatory impact of this berry by reducing inflammation locally in the intestine [121, 132, 133]. Due to the size of proanthocyanidin polymers, they have very low systemic bioavailability, so they must have some local anti-inflammatory effect. This hypothesis is based on findings previous research on proanthocyanidins rich foods [233-235]. Proanthocyanidins have been found to protect intestinal endothelial cells, reducing markers of colonic inflammation and increasing the expression of tight junction proteins [233, 235, 236]. The aim of this study was to evaluate the in vivo effects of a whole tart cherry powder on markers of obesity-related inflammation and to examine the in vitro effects of a tart cherry extract on markers of colonic inflammation.
3.2 Materials and Methods

3.2.1 Materials

The freeze-dried whole tart cherry powder was provided by the Cherry Research Committee (DeWitt, MI). All solvents and reagents used for tart cherry extraction were purchased from VWR (Radnor, PA). The experimental mouse diets were purchased from Research Diets Inc (New Brunswick, NJ, Table 3-1). TRI, Folin-Ciocalteu (Folin), MTT and Bradford reagents were purchased from Sigma-Aldrich (St. Louis, MO). Alanine aminotransferase (ALT) assay was purchased from Catachem (Oxford, CT). PCR primers were purchased from Integrated DNA Technologies (Coralville, IA, Table 3-2). Enzyme-linked immunosorbent assays were purchased from Mesoscale Discovery (Rockville, MD).
### Table 3-1: Composition of experimental diets

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>HF</th>
<th>HF+TC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein, Lactic 30 mesh</td>
<td>200</td>
<td>199.2</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Corn Starch</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Maltodextrin 10</td>
<td>125</td>
<td>124.6</td>
</tr>
<tr>
<td>Sucrose</td>
<td>68.8</td>
<td>63.6</td>
</tr>
<tr>
<td>Cellulose, BW200</td>
<td>50</td>
<td>49.3</td>
</tr>
<tr>
<td>Soybean Oil</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Lard</td>
<td>245</td>
<td>244.9</td>
</tr>
<tr>
<td>Mineral Mix S10026 (No Ca, P, K)</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Mineral Mix S10026A (No Ca, P, K, Na)</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Calcium Phosphate, Dibasic (29.5% Ca, 22.8% P)</td>
<td>13</td>
<td>12.5</td>
</tr>
<tr>
<td>Calcium Carbonate (40% Ca)</td>
<td>5.5</td>
<td>5.8</td>
</tr>
<tr>
<td>Potassium Citrate, Monohydrate (36.2% K)</td>
<td>16.5</td>
<td>16.5</td>
</tr>
<tr>
<td>Sodium Chloride (39.3% Na)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Vitamin Mix V10001</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Vitamin Mix V13002 No Vit A</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Choline Bitratrate</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Tart Cherry Powder</td>
<td>0</td>
<td>7.74</td>
</tr>
<tr>
<td>FD&amp;C Yellow Dye #5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>FD&amp;C Red Dye #40</td>
<td>0</td>
<td>0.05</td>
</tr>
<tr>
<td>FD&amp;C Blue Dye #1</td>
<td>0.05</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>773.85</strong></td>
<td><strong>774.40832</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>HF</th>
<th>HF+TC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Protein, gm</strong></td>
<td>179</td>
<td>179</td>
</tr>
<tr>
<td><strong>Carbohydrate, gm</strong></td>
<td>125</td>
<td>125</td>
</tr>
<tr>
<td><strong>Fat, gm</strong></td>
<td>78.8</td>
<td>78.8</td>
</tr>
<tr>
<td><strong>Fiber, gm</strong></td>
<td>270</td>
<td>270</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>
3.2.2 Animal Study

All animal studies were approved by the Institutional Animal Care and Use Committee at the Pennsylvania State University (IACUC protocol no. 45380). Four-week-old, male C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME) and housed 5 per cage on a 12-hour light/dark cycle for the duration of the study. The mice were provided *ad libitum* access to water and experimental diets. Mice were randomized into three experimental groups based on body weight. The mice were fed either a high-fat control diet (HF), a high-fat, tart cherry-containing diet (HF+TC), or a low-fat control diet (LF) for 18 weeks. The HF diets consisted of 60% kcal from lard, while the LF diet contained 10% kcal from lard. The tart cherry diet contained 1% (w:w) freeze-dried tart cherry powder. Food intake and body weights were recorded weekly. At the conclusion of the study, mice were fasted for 12-hours and then sacrificed under anesthesia via cardiac puncture and cervical dislocation. Blood collected by cardiac puncture was centrifuged to isolate the plasma which was aliquoted at stored at -80°C. The livers were weighed and stored at -80°C. Visceral adipose depots (mesenteric, epididymal, and retroperitoneal) were rinsed with PBS, weighed and stored at -80°C. Kidney and pancreas weights were recorded, and the organs were discarded. ALT levels were determined by colorimetric assay (Catachem, Oxford, CT).

3.2.3 mRNA isolation and PCR analysis

Hepatic mRNA was isolated from tissue samples with TRI reagent according to manufacturer’s protocol. Resultant mRNA was quantified using the Nanodrop 3000 spectrophotometer (Thermo Scientific, Waltham, MA) and stored at -80°C for future
analysis. cDNA was synthesized from the diluted mRNA using the RT first Strand kit (Qiagen, Hilden, Germany) according to manufacturer’s instructions. RT-qPCR was conducted on samples of synthesized cDNA using a 7900HT PCR instrument at Huck Life Sciences Core Facility (University Park, PA) and analyzed with SDS 2.4 Software (Applied Biosystems, Foster City, CA). Results of gene expression data were reported using the $2^{\Delta\Delta CT}$ method representing the relative expression of the gene of interest compared to the expression of glyceraldehyde-3-phosphate dehydrogenase (Gapdh).

**Table 3-2: RT-qPCR primers used for gene expression**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer (5'→3')</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>II1b</td>
<td>Forward</td>
<td>GCAACTGTTCCCTGAACCTCAACT</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ATCTTTTGGGCTCGGCTCAACT</td>
</tr>
<tr>
<td>Tnfa</td>
<td>Forward</td>
<td>TACTGAACCTTCCGTTGATTGTTCC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CAGCTTTGTCCTTGAAGAGAAC</td>
</tr>
<tr>
<td>Mcp1</td>
<td>Forward</td>
<td>ACCACAGTCCATGCCATCAC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TTGAGGTGGTTGTTGAAAAG</td>
</tr>
<tr>
<td>II6</td>
<td>Forward</td>
<td>CCGGAGAGGAGACTTCACAG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GGAATTGCGGTTAGGAAGGA</td>
</tr>
<tr>
<td>Ccr2</td>
<td>Forward</td>
<td>TGCCCATCATCCCCGGAGCCA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AGCACATGTGGAATCCAA</td>
</tr>
<tr>
<td>Gapdh</td>
<td>Forward</td>
<td>AGGTCGTTGGTAACCGATTG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TGTAGACCAATGTAGTTGAGGTCA</td>
</tr>
</tbody>
</table>
3.2.4 Cytokine expression

Several cytokines were selected as markers to evaluate the degree of hepatic inflammation in the mice including IL-1β, IL-6, TNF-α, and MCP-1. Samples of liver tissue were homogenized in T-PER lysis buffer (Thermo Scientific, Waltham, MA) using a bead homogenizer (Next Advance, Averill Park, NY). Homogenates were centrifuged at 10,000 xg for 5 min, and the protein-rich supernatant was collected. The samples were analyzed for cytokine expression by multiplex ELISA (Mesoscale Discovery, Rockville, MD) and results were normalized to total protein concentration as determined by the Bradford assay.

3.2.5 Tart Cherry Extract Preparation

To produce tart cherry extract (TCE), freeze-dried tart cherry powder (Cherry Research Committee) was extracted twice with 1L of solvent (80:20:0.1, acetone:water:acetic acid) for 12 h using a ratio of 1:10 (w/v) tart cherry to solvent. The supernatants were collected, combined, and dried under vacuum to remove the acetone. Sugars were removed from the remaining aqueous extract using an Amberlite XAD7 (Sigma-Aldrich, St. Louis, MO) packed column which selectively binds phenolic compounds, while allowing sugars and other impurities to be eluted. The phenol-rich TCE was eluted from the column using acidified methanol. The methanol was evaporated by rotary evaporation, and the remaining water was removed by freeze-drying to isolate the final TCE. The TCE was stored as a dry powder at -80°C.
3.2.6 Folin-Ciocalteu Assay

The Folin-Ciocalteu assay was used to quantify the phenolic antioxidants in the TCE. A 1 mg/mL solution of TCE in water:ethanol (90:10) was prepared. Gallic acid standards were prepared using gallic acid monohydrate dissolved in water:ethanol (90:10). The standards prepared were 0, 25, 50, 100, 250, and 500 mg/mL. Standards and samples were reacted with Folin-Ciocalteu reagent for 5 min, stopped with a saturated solution of sodium carbonate, and incubated a water bath for 30 min at 40°C according to previously established methods [237]. The absorbance of the samples was measured at 765 nm. A standard curve was generated based on the absorbance data of the gallic acid standards and was used to determine the antioxidant content of the TCE expressed as gallic acid equivalents (GAE, mg gallic acid/mg TCE)

3.2.7 Lipase Activity Assay

The inhibition of pancreatic lipase by the TCE was determined in vitro using established methods in our laboratory [238]. A 10mg/mL solution of type II porcine pancreatic lipase was prepared in deionized water. The solution was incubated at 37°C for 5 minutes and then centrifuged at 3000 rpm for 5 minutes. The supernatant was diluted to a final concentration of 200 μg/mL in a buffer solution containing 20mM tris-HCl, 150 mM NaCl, and 1.3 mM CaCl₂ at pH 8.0. The pancreatic lipase substrate, 4-nitrophenyl butyrate, was dissolved in DMSO to create a stock concentration of 11.4 mM. This was then diluted with deionized water to a final concentration of 0.904 mM. A concentrated stock of 40mg/mL TCE in DMSO was diluted to test concentrations of 0, 4, 20, 40, 80, 200, and 400 μg/mL in deionized water. To test lipase inhibition of the tart cherry, the
extract, substrate, and enzyme solutions were combined in a ratio of 1:1:2 in a 96-well plate. The solution was incubated for 10 minutes at 37°C, and the absorbance of each well at 400 nm was measured. Enzyme activity was expressed as percent activity relative to the uninhibited control that contained only water in place of TCE.

3.2.8 Anti-inflammatory effects of TCE in Cell Culture

TCE was evaluated for potential anti-inflammatory potential in cell culture. HT-29 cells stimulated with TNF-α were chosen to model inflamed human intestinal epithelial cells [239]. Approximately 5 x 10^3 cells were seeded in each well of a 96-well plate in McCoy’s 5A modified media. After the cells had reached 70% confluence, the media was aspirated and replaced with fresh media containing 5 ng/mL TNF-α for 6 hours. Cells undergoing co-treatment with TCE received media containing both TCE and TNF-α simultaneously. After 6h the media was aspirated and replaced with new media and incubated for an additional 24h recovery period. Cells receiving post-treatment with TCE received TCE-containing media at this time. After the final incubation, the cell supernatant was collected from all plates into Eppendorf tubes and centrifuged at 10,000 xg for 5 min to remove any cell fragments. The supernatant was collected, and the concentration of IL-8 was determined by ELISA (Mesoscale Discovery, Rockville, MD). The results were normalized to cell viability as determined by the MTT assay [240].

3.2.9 Statistical Analysis

Statistical analyses were performed using GraphPad Prism 6 (La Jolla, CA). Differences between HF and HF+TC treatments were evaluated using the Student’s t-test.
One-Way ANOVA with Dunnet’s post-test was used to identify differences in pancreatic lipase inhibition and a One-Way ANOVA with Tukey’s post-test was used to identify differences in IL-8 expression among TCE treatments. P-values less than 0.05 were considered statistically significant.
3.3 Results

3.3.1 Bodyweight

Tart cherry powder supplementation had no effect on weight gain. Mice fed the HF diet, and those fed the HF+TC diet gained significantly more weight than the LF control group (p<0.0001), but there was no significant difference in weight gain between the HF and HF+TC (p>0.05, Figure 3-1). The HF+TC diet had no effect on visceral adiposity with both high-fat diets hovering around 10% visceral adipose (p>0.05, Figure 3-1). The high-fat fed mice had significantly more adipose compared to the low-fat group in which mice had an average of 6% visceral adipose (p<0.0001, Figure 3-1). There was no effect observed of tart cherry on hepatomegaly or serum ALT concentration compared to the high-fat control diet (p>0.05, Figure 3-1).
Figure 3-1: Weight gain, visceral adiposity, and hepatomegaly. These markers were selected as physiological markers of obesity and fatty liver and measured in each mouse (n=12 for LF, n=20 for HF and HF+TC). Weight gain was measured as fold increase from initial body weight. Visceral adipose was determined by the combined weight of epididymal, mesenteric, and retroperitoneal depots expressed as a percentage of final bodyweight for each mouse. Liver weight is expressed as a percentage of final body weight. Alanine aminotransferase (ALT) was selected as a blood marker of liver damage. LF mice had lower bodyweight, adiposity, hepatomegaly and ALT levels. Significance was measured by ANOVA with Dunnet’s post-test with the LF group as the control. No significant differences were observed between HF groups. Data presented as mean ± SEM, *p<0.05.
3.3.2 Hepatic Gene and Protein Expression

The hepatic gene expression of several inflammation-related cytokines was examined. While significant decreases were observed in II1b, and C-C chemokine receptor-2 (Ccr2) in TC-treated mice, there was a significant increase in the gene expression of II6 in TC-treated mice (p<0.05, Figure 3-2). There was a slight, but nonsignificant increase in Tnfa expression with tart cherry supplementation (p=0.17, Figure 3-2). The mRNA levels of cytokines II10 and Mcp1 were unaffected (p>0.05, Figure 3-2). Protein expression was determined for IL-1β, IL-6, MCP-1 and TNF-α. While there appeared to be slight decreases in the levels of these proteins in TC-treated mice, the difference was non-significant (p>0.05, Figure 3-3).
Figure 3-2: Hepatic gene expression. Hepatic expression of genes encoding for inflammation-related cytokines II-1β, II-6, Tnf-α, II-10, Mcp-1 and the Mcp-1 receptor, Ccr2 for all mice (n=20 per group) determined by RT-qPCR at the conclusion of the study. Expression was normalized to GAPDH using $2^{-\Delta\Delta Ct}$ method. Expression levels of IIb and Ccr2 were significantly decreased in mice fed TC compared to those that were not. Expression of II-6 was significantly increased and Tnfa was non-significantly increased in mice fed TC. Data presents as mean ± SEM, *p<0.05.
3.3.3 Phenolic Content of Tart Cherry Extract

Total phenolic content of the TCE was determined to be approximately 0.473 ± 0.001 gallic acid equivalents per mg TCE (mean ±SEM, mg gallic acid/mg TCE, Figure 3-4). The TCE was evaluated for its ability to inhibit pancreatic lipase by colorimetric assay. At higher concentrations (100-400 μg/mL) TCE was able to significantly reduce lipase activity by 10-15% (p<0.05, Figure 3-4).

**Figure 3-3:** Hepatic cytokine content. Hepatic protein expression of cytokines IL-1β, IL-6, MCP-1, and TNF-α for all mice (n=20 per group) was determined using ELISA at the conclusion of the study. Expression reported as μg/mg total protein. Student’s t test was used to test for significant differences. No significant differences were observed. Data presented as mean ± SEM.
Figure 3-4: Pancreatic lipase inhibition and Folin assay standard curve. a.) Inhibition of pancreatic lipase *in vitro* by increasing concentrations of tart cherry extract as determined by colorimetric assay. Data were normalized to the activity of the TCE-free control and expressed as a percentage of control activity. ANOVA with Dunnet’s post-test was used to test for significant differences. *Indicates significant differences from TCE-free control (p<0.05). Data presented as mean ± SEM. b.) Sample gallic acid standard curve used to quantify phenolic content of TCE as gallic acid equivalents. TCE phenolic content was determined using the Folin-Ciocalteu assay.
3.3.4 In Vitro Inflammation

The TCE derived from freeze-dried tart cherries was evaluated in vitro for its potential as a local anti-inflammatory agent in the gut. IL-8 is a pro-inflammatory cytokine produced by human colonic epithelial cells when they are exposed to inflammatory stimuli such as TNF-α or endotoxin. Simultaneous treatment of HT29 cells with the pro-inflammatory stimulus TNF-α and the TCE (co-treatment) had no significant effect on reducing the expression of IL-8 at lower concentrations of TCE (p>0.05, Figure 3-5). Co-treatment with higher concentrations of TCE resulted in a slight decrease in IL-8, but the reduction failed to reach statistical significance (p=0.07). Treatment of cells with TCE that had previously been exposed to TNF-α (post-treatment) failed to reduce IL-8 expression (p>0.05, Figure 3-5).

Figure 3-5: HT29 IL-8 expression. Panel 1: Expression of IL-8 (pg/mg total protein) in TNF-α-stimulated HT29 cells co-treated with increasing concentrations of TCE. Cells were incubated with TCE and TNF-α for 6-hours and then allowed to recover for 24-hours before harvesting. Normalized to total supernatant protein content. Panel 2: Expression of IL-8 (pg/mg total protein) in HT29 cell supernatant treated with increasing concentrations of TCE after stimulation with TNF-α. Cells were incubated with CTE for 24-hours after TNF-α stimulation and then harvested. Normalized to total supernatant protein content (ng/mg total protein). All data presented as mean ± SEM. No significant differences were observed.
3.4 Discussion

Obesity and its comorbidities are a major health concern in the U.S. and the developed world. The high-fat content of obesogenic diets has been shown to initiate inflammation in the gut, contributing to the systemic inflammation observed in obesity. Previous research by our lab has shown that plant-derived phenolic compounds have the potential to reduce weight gain and mitigate both systemic and gut inflammation [175, 224, 225, 233, 238]. Tart cherries are a novel source of anthocyanins and proanthocyanidins; both of which have been shown to have anti-obesogenic and anti-inflammatory properties. Due to its high phenolic content, we hypothesized that dietary tart cherry powder would reduce weight gain, adiposity, and inflammation in vivo and mitigate the inflammatory response of colonocytes in vitro.

The data in this study indicate that tart cherry powder had a mild systemic anti-inflammatory effect on the expression of certain inflammatory cytokines. There was no significant effect on weight gain or visceral adiposity. While the TCE produced in this study had a high phenolic content (47.3 ±0.1%) and showed mild inhibitory effects on pancreatic lipase, it was unable to reduce expression of IL-8 in TNF-α-stimulated HT29 cells.

Previous research by Seymour et al. showed that tart cherry powder was capable of reducing the serum expression of IL-6 and TNF-α protein in high-fat fed rats as well as reducing bodyweight and visceral adipose [132]. Our data indicate that supplementation with tart cherry powder increased hepatic gene expression of Il6 and Tnfa, but these increases in mRNA expression did not lead to increases in protein expression. In fact, we observed slight decreases in hepatic inflammatory proteins including MCP-1, IL-1β, IL-6.
and TNF-α although they did not achieve statistical significance. It is possible that there was post-transcriptional regulation of IL-6 and TNF-α induced by tart cherry components. A majority of TNF-α and IL-6 is produced in adipose tissue by macrophages and adipocytes. Because both TNF-α and IL-6 are secreted cytokines, the decreased expression observed in the TC-fed mice may be due to changes in visceral adipose tissue rather than in the liver where they were measured. Therefore, it appears that tart cherry bioactives may effectively reduce inflammation in adipose tissue rather than in the liver [132]. Future research will utilize a systemic approach to evaluating inflammatory cross-talk between adipose and hepatic tissue with the aim of identifying the site of tart cherry activity.

We failed to see any differences in weight gain or visceral adiposity of the TC-fed mice compared to the HF-fed controls. Previous research in our lab demonstrated that procyanidin-rich foods such as cocoa could prevent weight through the inhibition of pancreatic lipase [241]. While TCE had a moderate inhibitory effect on pancreatic lipase, it was no enough to prevent weight gain.

We believe that matrix effects of the tart cherry powder may have played a role in the reduced efficacy of its bioactive components. Other groups have reported that whole fruit supplementation was less effective than isolated anthocyanins [242]. This may be due to reduced bioavailability or the inhibitory effects of another compound in the fruit. Use of fruit extracts in our lab has shown greater success in reducing inflammation and hepatic steatosis [243]. Tart cherries contain sugars in the form of fructose and glucose. In our experiment, we matched this sugar content using sucrose, but it is possible that the monosaccharides have a more deleterious effect than sucrose. One study found that monosaccharides induced more severe hepatosteatosis than an equivalent amount of
sucrose in rats [244]. Excess fructose has been linked to insulin resistance and the progression of NAFLD [245, 246]. The reduced efficacy of the tart cherry powder in this instance may be due, in some part, to the negative effects of dietary fructose. Further studies in the high-fat fed mouse model should utilize tart cherry powder from which dietary sugars have been extracted, or sugar content should be matched in the control.

We hypothesized that the tart cherry extract would effectively reduce colonic inflammation due to its procyanidin content. Neither co-treatment nor post-treatment of TNF-α-stimulated HT29 cells significantly reduced the expression of IL-8. Previous research in our lab demonstrated that long-chain proanthocyanidins were effective in reducing IL-8 in the HT29 model [225]. An evaluation of tart cherry proanthocyanidins by Capangola et al. revealed that the average degree of polymerization (DP) of tart cherry proanthocyanidins is only 3.9 ± 0.2 [119]. Our research indicates that proanthocyanidins with longer degrees of polymerization are more effective at reducing gut inflammation, mainly those with a DP>7 [225]. Therefore, it is possible that TCE was ineffective in reducing inflammation due to its lack of long chain proanthocyanidins. Further studies should compare TCE proanthocyanidins with those derived from other cultivars such as the Cornelian or Laurel cherry, which have been shown to have degrees of polymerization ranging from 40-60 DPs [119].
3.5 Conclusions

We found that tart cherry powder was moderately effective in reducing hepatic markers of inflammation, despite its inability to mitigate weight gain or visceral adiposity. This work was innovative in that attempted to identify the local anti-inflammatory capacity of tart cherries to explain the previously reported systemic effects. We hypothesized that the procyanidin-rich TCE would effectively reduce colonic inflammation, but no significant effects were observed. Other studies have demonstrated tart cherries to be beneficial in reducing inflammation [132, 133, 138, 141]. The possibility remains that our approach failed to account for matrix effects within the fruit powder or differences in experimental design. Future research in our lab will attempt to identify the key active components within the whole fruit that elicit an anti-inflammatory effect in order to circumvent any possible matrix effects or inhibitory interactions. Combating obesity and inflammation with dietary supplementation with tart cherries remains a promising avenue of research.
Chapter 4: Conclusions and Future Work

4.1 Conclusions and Limitations

We examined the effects of several bioactive dietary components on obesity and obesity-related pathologies.

4.1.1 Soybean Products

Previous research has indicated that soy protein, soy fiber, and high-oleic soybean oil have the potential to mitigate markers of obesity, inflammation, and NAFLD. We hypothesized that the combination of soy protein concentrate, soy fiber, and high-oleic soybean oil would effectively mitigate the progression of NAFLD and ameliorate obesity-related inflammation. We tested this hypothesis by completing the following objectives:

1. To determine if substituting a fiber-rich soy protein concentrate into a high-fat fed mouse diet prevents the development of obesity and NAFLD

The addition of 12% soy protein concentrate into an obesogenic diet appeared to have several benefits in reducing the severity of obesity and NAFLD. Mice fed soy protein concentrate had significantly less visceral adipose than mice fed a casein/cellulose-containing diet. Soy protein also lowered levels of fasting blood glucose. Several markers of inflammation were downregulated in SPC-fed mice including the gene expression of $\text{Il1b, Casp8, and Fasl}$. The degree of hepatic steatosis was markedly decreased in SPC groups. We believe this was due to the downregulation of $\text{Srebf1}$, the factor responsible for activating $\text{de novo}$ lipogenesis in the liver. This data led us to hypothesize that SPC would
have the effects of lipotoxicity, but no significant differences were observed in the activity levels of the caspases responsible for lipoapoptosis. Longer-term studies with SPC will aid in elucidating the protective mechanism of SPC in vivo as they would enable us to see the effects of SPC in more progressive stages of steatohepatitis.

The role of SPC in the mitigation of obesity-related pathologies needs to be investigated in humans. While previous studies have examined the effects of soy-based products on human health, the long-term effects of isoflavone-free soy protein on obesity and NAFLD have not been examined. In the current study, approximately one-third of the dietary protein was replaced by SPC. This level of soy-based protein in the diet may be difficult to replicate in the typical American diet. Currently, Americans get about 30% of their daily protein from plant sources, but a majority of that comes from grain-based products [247]. It would be a rather drastic step to implement such a large amount of soy protein into the diet. Soy protein may need to be incorporated into existing food products rather than introducing novel soy products into the existing Western diet.

2. To determine if substituting high-oleic soybean oil into a high-fat fed mouse diet prevents the development of obesity and NAFLD

The substitution of high-oleic soybean oil in a high-fat lard-based diet resulted in significantly reduced body weight and visceral adiposity in mice. Despite this reduction in weight gain, high-oleic soybean oil appeared to have an opposing role in reducing the severity of NAFLD and inflammation compared to SPC. We observed increased hepatic gene expression of the proinflammatory genes Casp1, Casp8, and Fas as well as increased
protein levels of TNF-α and MCP-1 compared to lard. Diets high in oleic, such as the Mediterranean Diet, have been shown to protect against obesity-related pathologies such as dyslipidemia and cardiovascular disease. It is possible that the benefit of high-oleic oils depends on the oil source or the total fat content of the diet. High-oleic soybean was created with the intention of replacing partially hydrogenated soybean oil, and it is possible that high-oleic soybean oil may be a healthier alternative. These hypotheses will need to be evaluated to determine the cause of inflammation observed in the present study.

4.1.2 Tart Cherries

Tart cherry bioactives have shown potential for the treatment obesity-related inflammation in previous research. We hypothesized that dietary supplementation of high fat-fed mice with tart cherries would reduce markers of obesity, inflammation, and NAFLD due not only to the systemic actions of anthocyanins but also because of the local anti-inflammatory action of proanthocyanidins in the intestine. We tested this hypothesis by completing the following objectives:

1. To determine if tart cherries reduce the severity of obesity and its related inflammation in vivo

We found that supplementation with 1% tart cherry powder had no effect on weight gain and did not prevent obesity in high-fat-fed mice. Although we did not observe any effect on body weight, previous research with high-fat-fed rats found that tart cherry
powder was able to reduce weight gain [132]. These differences could be due to differing phytochemical content in the tart cherry powder or because of differences in the experimental model. The research by Seymour et al. utilized obesity-prone Zucker rats while our research relied on diet-induced obesity in mice. We did, however, observe reductions in inflammatory markers. Protein expression of IL-1ß, IL-6, MCP-1, and TNF-α was moderately decreased in mice fed tart cherries.

2. To determine if a tart cherry derived extract is capable of mitigating inflammation in TNF-α-stimulated human colonic epithelial cells

Tart cherries contain high concentrations of proanthocyanidins [118, 119]. Previous research in our laboratory has demonstrated that food high in proanthocyanidins (e.g. cocoa, cranberries) are capable of reducing colonic and obesity-related inflammation [224, 225, 233]. We failed to observe significant reductions in the protein expression of IL-8 from TNF-α-stimulated HT29 cells treated with tart cherry extract. There was a decrease in inflammation at the highest dose of tart cherry extract tested (100μg/mL), but the effect failed to reach statistical significance. One explanation for this observed lack of anti-inflammatory effect could be the distribution of proanthocyanidin degree of polymerization in tart cherries compared to other foods. The average degree of polymerization of tart cherry proanthocyanidins was not determined in this study, but a previous analysis by Capangola and others found that tart cherry proanthocyanidins have an average degree of polymerization of only 4 [119]. Previous research from our group found that proanthocyanidins with a degree of polymerization greater than 7 were more efficient at
reducing colonic inflammation than those of lower degree of polymerization [225]. Further evaluation of the proanthocyanidin content of the TCE utilized in this study is necessary to test this possibility.

Several studies have examined the effects of tart cherry products in humans, but the effects of tart cherries on obesity and chronic inflammation have not been evaluated. Our mouse study used the human equivalent intake of 60 tart cherries per day and would provide around 150 kcal energy [133]. We believe that this is a practical serving size that could easily be incorporated into the daily diet by replacing about 1-2 servings of fruit. The tart cherries could be further processed into a tart cherry extract similar to the one we created for cell culture experiments. This extract would provide the tart cherry polyphenols without a large amount of sugar naturally found in tart cherries. The efficacy of both the fresh fruit and processed tart cherry products will need to be further evaluated in long-term human studies.

4.1.3 Significance and Implications

A growing body of evidence indicates that many foods have biological activities beyond their nutritional content. Here we demonstrated that SPC and tart cherries might protect against the development of obesity-related pathologies and inflammation while HOS may increase obesity-related inflammation. In our models, we examined these dietary constituents in purified diets in controlled experimental environments. By studying food components in controlled environments, we hope to identify key food products such as SPC or tart cherries that could easily be incorporated into an existing diet and function as a nutraceutical, providing some protective health benefit. The next step will be to
incorporate these components into the complex human diet and observe their effects on health. We hope that by identifying active food components we can mitigate an array of health issues and reduce the need for medical interventions.
4.2 Future Work

4.2.1 Soy Research

The underlying cause of the inflammatory changes observed in HOS-fed mice remains unclear. Any plant derived oil is a complex mixture of saturated, monounsaturated and polyunsaturated fatty acids, as well as non-lipid components. It is clear that different fatty acid profiles can have profoundly different physiological effects [159]. Some research, including ours, has indicated that fat sources may exert different effects on the body as the total fat content of the diet is increased. To determine whether or not this is the cause for the pro-inflammatory role of HOS in a high-fat diet, we will adjust the total fat content in HOS-based diets and measure the degree of inflammation and steatosis in the mice over time. Mice will be fed diets containing 10, 20, 30, 40, 50 and 60% kcal from fat (either lard or HOS) for 18-weeks. Markers of inflammation and hepatic steatosis will be measured to determine whether there is a level at which HOS is protective or if it is purely pro-inflammatory compared to lard. To determine if the plant source affects physiological outcomes we could conduct a study in which each high-fat diet contained a different source of high-oleic oil such as olive oil, safflower oil, sunflower oil, or high-oleic soybean oil. Mice would be fed these diets over the course of 18 weeks, and endpoint levels of proinflammatory cytokines would be determined. As high-oleic soybean was intended to replace partially hydrogenated soybean oil, we should also include the partially hydrogenated oil as a comparison to the high-oleic oils. It is possible that HOS is more inflammatory than lard but less detrimental than its partially hydrogenated counterpart.

Our work with soy protein concentrate yielded similar results to previous studies on soy protein, but a unique aspect of our work is the use of a soy protein concentrate that
contains relatively high levels of soy fiber [78, 81, 84, 152]. Soy fiber is an insoluble, but highly fermentable fiber that has been shown to alter the distribution of gut microbiota and produce elevated levels of butyrate during bacterial fermentation [94, 100, 201, 248]. Alterations in gut microbiota that reduce the prevalence of gram-negative bacteria may be protective against the onset of metabolic endotoxemia [168]. Previous research has shown that increased production of butyrate may mitigate the severity of inflammatory bowel diseases [202, 248, 249].

Further animal studies are necessary to determine whether or not these potential benefits of soy fiber contributed to the observed effects of soy protein concentrate. To examine this, we would design a mouse study in which mice would be given a high-fat diet with one of the following protein/fiber treatments: casein/cellulose (control), casein/soy fiber, soy protein/cellulose, or soy protein/soy fiber. This design would allow us to see the individual effects of soy protein and soy fiber as well as their interaction in a high-fat diet. The same markers examined in our previous study would be considered in addition to the genotyping of the gut microbiota, as well as measurement of short-chain fatty acids in the colon and of plasma endotoxin levels.

4.2.2 Tart Cherry Research

Although we observed a small reduction in a limited number of inflammatory markers in our tart cherry model, the effects were not as great as those observed by previous investigators using similar model systems [132]. It is possible that this reduced efficacy was due to matrix effects in the tart cherry powder we chose for our experiment. There are a number of ways that we could approach this differently in the future. One approach would
be to create a tart cherry extract similar to the one used in our *in vitro*. Both the cherry extract and tart cherry powder could then be analyzed for polyphenol content by HPLC and incorporated into mouse diets. The diets would be matched for phytochemical (*i.e.*, anthocyanin and proanthocyanidin content), but one would contain whole tart cherry powder, and the other would contain tart cherry extract. This would establish whether or not matrix effects in the tart cherry powder were preventing the activity of its bioactive components. A similar approach was used by Prior *et al.*, who found that berry extracts were more efficacious in reducing serum lipids compared to whole fruits [242].

Tart cherry sugars consist mainly of glucose and fructose monosaccharides. In our study, we matched the diets for sugar content using sucrose, but it is possible that the monosaccharides may elicit a different physiological response. Sanchez *et al.* found that rats fed glucose and fructose had increased levels of liver triglycerides and steatosis compared to rats fed an equivalent amount of sucrose [244]. Isolating the phytochemical extract and comparing its effects to the whole tart cherry powder would also allow us to see the effects of the sugars.

We could also isolate and individually test the anti-inflammatory properties of each of the individual anthocyanins, proanthocyanidins, and other phytochemicals identified by HPLC analysis to determine the key active components in tart cherry. This approach would be better suited to *in vitro* work with cell lines so that each element could be analyzed in a high-throughput manner at a range of concentrations.

One of the limitations of the tart cherry study was that it was only a single dose/single duration study. Future research should examine a range of tart cherry doses to establish whether a dose-response relationship exists between tart cherry concentration and
its anti-inflammatory effects *in vivo*. A longer-term mouse study may also reveal new information about the effects of tart cherries. The mouse model used in this research was intended to induce obesity and NAFLD over the course of 18 weeks. Maintaining this diet for 9-14 months has been shown to induce the inflammatory and fibrotic stages of NASH [250]. If tart cherries are effective anti-inflammatory agents *in vivo*, they should slow the progression or reduce the severity of NASH.
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