AN ABSTRACT OF THE THESIS OF

Marlena L. Sturm for the degree of Master of Science in Food Science and Technology Presented on July 20, 2018.

Title: Relative Health Effects of Intake of High-Fat Diets Enriched with Raspberry Fiber and Raspberry Polyphenol in C57B/J6 Mice

Abstract approved:

Neil F. Shay

BACKGROUND: Consumption of raspberry products have been observed to produce healthful effects and reduce weight gain and positively shift metabolism in mice fed obesigenic diets. In the past, our lab has successfully shown a reduction in weight gain for mice consuming RPC+HF vs. mice fed HF alone. Currently, there is not a lot of scientific literature characterizing specific mechanistic changes which occur in metabolism due to the consumption of raspberry products.

OBJECTIVE: This study investigates raspberry puree concentrate (RPC), RPC polyphenols (RP), and RPC fiber (RF) for their healthful and beneficial effects in mice consuming a high-fat western diet. Specific indicators of metabolic-related diseases were evaluated to see if RPC and RPC fractions attenuated the development of obesity, dyslipidemia, diabetes, hepatic steatosis, hypertension, and chronic inflammation to the same degree.

METHODS: The C57BL/6J (C57) mouse model was used for this study due to their propensity to easily develop metabolic disease when fed a high-fat diet. These metabolic conditions include obesity, high blood lipid and cholesterol levels, diabetes, fatty liver, hypertension, osteoporosis and chronic inflammation. Mice were fed a western-style diet containing 45% of calories from fat and 15% of calories from added sucrose. Diets included a low-fat control (LF), a high-fat control (HF), and supplemented HF diets (RPC+HF, RP+HF, and RF+HF). Mice had *ad libitum* access to these diets for 10 weeks, enough time for C57 to develop significant metabolic complications from HF consumption.

RESULTS: Based on our findings, fiber and polyphenols appear to work together to achieve the healthful effects observed when RPC is consumed. The unique properties and metabolic changes produced by these two fractions help us to better understand how RPC acts on the metabolism of C57BL/6J mice.

Relative Health Effects of Intake of High-Fat Diets Enriched with Raspberry Fiber and Raspberry Polyphenol in C57B/J6 Mice

By
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APPROVED:

Major Professor, representing Food Science and Technology

Head of the Department of Food Science and Technology

Dean of the Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes the release of my thesis to any reader upon their request.

Marlena L. Sturm, Author

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ABBREVIATIONS

°C	Degrees centigrade
A1C	Glycated hemoglobin
ACC	Acetyl CoA carboxylase
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
ATP	Adenosine triphosphate
AUC	Area under the curve
AUC_1	Inverse area under the curve
BMI	Body mass index
CARM1	Histone arginine methyltransferase 4
CDC	Centers for Disease Control
ChREBP	Carbohydrate-responsive element-binding protein
CRP	C-reactive protein
EA	Ellagic acid
ELISA	Enzyme-linked immunosorbent assay
FFA	Free fatty acids
FPG	Fasting plasma glucose
FPI	Fasting plasma glucose insulin
g	gram
GLUTs	Glucose transporters
h	hour(s)

HAT	Histone acetyltransferase
HDAC9	Histone deacetylase 9
HDL	High density lipoprotein
HF	High fat
HOMA	Homeostatic model assessment
HSL	Hormone-sensitive lipase
IDF	International Diabetes Foundation
LDL	Low density lipoprotein
LF	Low fat
L-PK	L-type pyruvate kinase
MCP-1	Monocyte chemoattractant protein
mg	Milligrams
min	minutes
mL	Milliliters
NAFLD	Nonalcoholic fatty liver disease
NCEP	National Cholesterol Education Program
NHANES	National Health and Nutrition Examination Survey
NIH	National Institute of Health
O-GlcNA	β-linked N-acetylglucosamine
OGTT	Oral glucose tolerance test
ORAC	Oxygen radical absorbance capacity
PP	Polyphenols
qPCR	Quantitative polymerase chain reaction

SAA	Serum amyloid A
QIIME	Quantitative Insights Into Microbiological Ecology
RF	Raspberry fiber
RJC	Raspberry juice concentrate
RK	Raspberry ketone
RNA	Ribonucleic acid
RPC	Raspberry puree concentrate
RP	Raspberry polyphenol
RSE	Raspberry seed extract
SEM	Standard error of the mean
SREBP-1c	Sterol regulatory element-binding protein -1c
TCA	Tricarboxylic acid cycle
TDF	Total dietary fiber
TNF	Tumor necrosis factor
UV	Ultraviolet
VLDL	Very low-density lipoproteins
w/w	Weight per weight
WHO	World Health Organization
Wt	Weight
μg	Microgram
μL	Microliter

DEDICATION

I wished to dedicate this body of work to my 6th grade science teacher Mrs. Charlotte Stein. Thank you for giving me the love of scientific discovery.

CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction:

According to the World Health Organization, metabolic disorders are disease states that are proposed as one of the primary drivers of chronic disease (WHO, 2017). Within the United States, it is estimated that the treatment of obesity and related metabolic conditions cost Americans between $147 billion to $210 billion annually (Cawley and Meyerhoefer, 2012). An additional $4.3 billion could be added if absenteeism and consequential decreased work production from obesity related illnesses or hospitalizations are included (Finkelstein et al., 2009). Aside from cost, The Centers for Disease Control and Prevention (CDC) estimated 300,000 people died of obesity related causes in 2010 (CDC, 2012).

Researchers are looking into metabolic disease associated with overconsumption and assessing potential therapies which may help remediate disease symptoms and increase metabolic function. Impaired metabolism can produce chronic inflammation, cause hormonal imbalances, and lead to excess body fat accumulation. It is of interest to the general public to come up with solutions to mitigate the obesity epidemic without requiring individuals to make major alterations to their dietary or lifestyle choices. Therefore, we are evaluating the potential of a polyphenol and fiber rich food, the red raspberry, to improve metabolism.

1.2. Lipid Metabolism

During states of excess energy consumption, an increase in body fat accumulation is typically observed. An increase in energy consumption paired with high saturated fat intake typically leads to an increase in circulating fatty acids and triglycerides. Lipoproteins carry lipids in circulation throughout the body and deliver them to peripheral tissues for uptake and use. Examples include chylomicrons, LDL, and HDLs. Lipoprotein particles contain non-polar fat, fat soluble vitamins, and cholesterol for transport within the circulatory system.

Lipids are synthesized in the liver tissue when glucose levels are high. Glucose enters hepatocytes via the GLUT-1, GLUT-2, GLUT-9 and GLUT-10 transporters (Karim, et al., 2012). Around 100g of glucose can be stored in the liver as glycogen for short term energy when blood glucose levels decline. After glycogen stores are repleted, excess glucose is then converted into fatty acids and transported for long term storage to adipose tissue.

The glycolytic pathway is utilized to convert glucose to glucose-6-phosphate. Glucose-6-phosphate then further catabolizes to form pyruvate. Pyruvate enters into the mitochondria and is acted on by pyruvate dehydrogenase to form acetyl CoA. Once acetyl CoA is formed it can enter into the TCA cycle, producing ATP. The first conversion within the TCA cycle forms citrate from acetyl CoA and oxaloacetate. Citrate can then be transported out of the mitochondria into the cytoplasm where oxaloacetate is cleaved off by ATP citrate lyase and acetyl CoA

is remade. In the presence of high insulin concentrations, ATP citrate lyase activity increases. Cholesterol and fatty acids can be synthesized from cytoplasmic acetyl CoA. These newly biosynthesized fatty acids are used in triglyceride synthesis.

Long chain fatty acids are made via acetyl CoA carboxylase (ACC) which forms malonyl CoA, the starting molecule for fatty acid synthesis. Malonyl CoA has two carbon units to which acetyl CoA is added. Additional acetyl CoA two carbon units are added as the fatty acid chain grows. Fatty acids are synthesized in the cytosol of the cell. These long chain fatty acids are then added to glycerol to create triglycerides. Adipocytes are the main tissue which stores body fat as triglycerides and diglycerides (Trayhurn and Bing, 2006). When lipid metabolism is working correctly, excess fatty acids will be stored in adipose tissue and not in muscle or hepatic tissues. Typically, fatty acids will be incorporated into triglycerides or diglycerides for long term storage within the adipocyte.

Conversely, β-oxidation is the catabolic process of breaking down fatty acids in the mitochondria for energy production. When serum insulin concentrations are high due to elevated serum glucose levels, the rate of β-oxidation decreases and triglyceride breakdown is inhibited. Insulin will affect different enzymes in the β-oxidation process in order to use glucose as a first source of energy. One example of the effect of insulin, on an enzyme of lipolysis, is its ability to inhibit hormone sensitive lipase function reducing triglyceride breakdown.

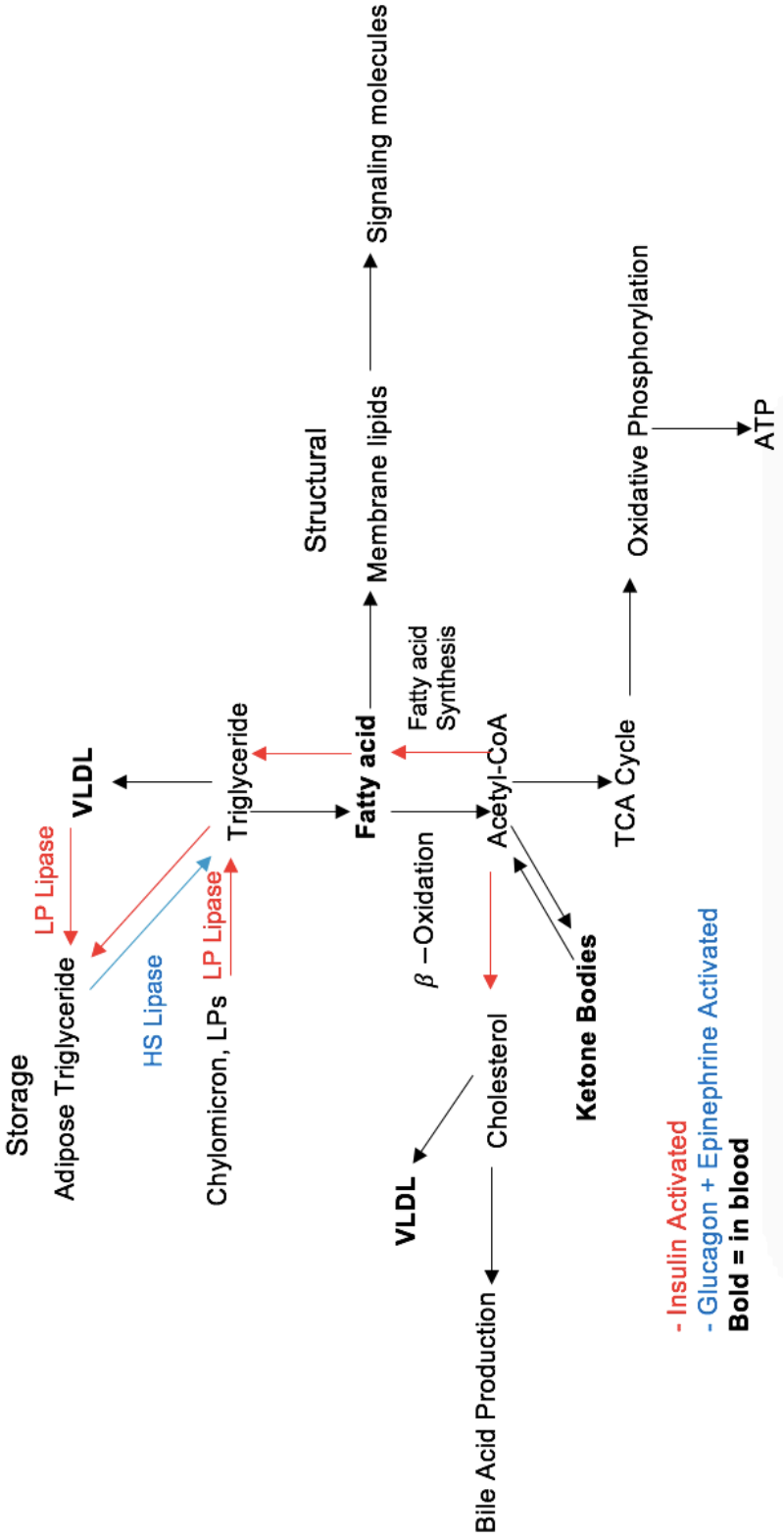


Figure 1.1 Lipid metabolism

1.3. Obesity and Related Metabolic Diseases:

Obese individuals have greater probability of developing diabetes, hepatic steatosis, chronic inflammation and hyperlipidemia. Metabolic dysregulation can cause individuals to develop one or more of these diseases.

Body mass index (BMI), is commonly used to estimate body composition and identify if a person is overweight or obese (25 - 29.9, >30, respectively) (National Institutes of Health, 1998). In obese individuals, accumulation of visceral fat is attributed to greater health risks and can increase pressure on internal organs and affect hormone regulation and function (Grundy et al., 2004).

Another predominant medical condition brought on by obesity and overconsumption is metabolic syndrome. Having three of the following symptoms constitutes a diagnosis of metabolic syndrome: 1) large waistline, 2) high plasma triglycerides, 3) low plasma HDL levels, 4) high blood pressure, and/or 5) a high fasting blood glucose (Grundy et al., 2004). More and more Americans are suffering from metabolic syndrome and people suffering from the disease are more likely to suffer from diabetes, heart attack or stroke.

Table 1.1 Conditions for metabolic syndrome[1]

Diagnostic Parameters	Men	Women
Waste Circumference	> 102 cm	> 88 cm
Plasma Triglycerides	≥ 150 mg/dL	≥ 150 mg/dL
HDL Levels	< 40 mg/dL	< 50 mg/dL
Blood Pressure	≥ 130 / ≥ 85 mg/dL	≥ 130 / ≥ 85 mg/dL
Fasting Blood Glucose	≥ 100 mg/dL	≥ 100 mg/dL

[1] Adapted from the revised 2005 NCEP ATP III criteria (Huang, 2009); metabolic syndrome is defined as having 3 or more of the following conditions. The IDF (2005) criteria requires central obesity of 94 cm (M) and 80 cm (F) and two additional conditions listed above (Alberti, et al., 2005).

1.3.1 Obesity:

Obesity is a growing epidemic and a severe health crisis affecting more than one-third of American adults (Flegal et al., 2012). A survey conducted by NHANES in 2012 showed 68% of American adults suffer from being either obese or overweight. Many obese individuals suffer from metabolic complications brought on by the disease (WHO, 2017).

Genetics can also play a role in determining whether or not a person will be more likely to develop obesity and related health problems, as seen in early twin studies (Standard, et al., 1986). However, genetic risk for obesity is unlikely to be

the explanation for the increase in obesity rates observed in the last few decades. This has led researchers to believe the current obesity epidemic is far more likely related to overconsumption paired with modern day sedentary lifestyles.

Different strains of mice were observed to have differential propensity to develop obesity when fed high-fat diets (West et al, 1992). One such obesity-prone mouse strain is the C57BL/6J (C57). Many metabolic studies, including ours, have used this strain due to their high susceptibility to diet-induced obesity and type II diabetes (Surwit et al.,1988).

Many overweight individuals develop insulin and leptin resistance which complicates weight loss. Certain foods have been shown to improve insulin and leptin sensitivity. Some studies suggest a calorie of a fat-rich food is not biologically equivalent to a calorie of vegetables even though they contain the same amount of energy. Certain foods will stimulate the release of insulin and/or leptin, while high fat diets tend to suppress insulin and leptin secretion (Van Heek, et al.,1997).

1.3.2 Diabetes:

Type II diabetes is a metabolic disorder caused by a genetic predisposition and unhealthy lifestyle and dietary choices. During the initial stages of pre-diabetes the peripheral tissues become insulin resistant which leads to prolonged high

levels of serum glucose. Pancreatic beta cells increase production of insulin in order to keep up with the rise in serum glucose levels. Eventually this causes beta cell hypertrophy and they become dysfunctional. Hyperglycemia results from loss of beta cell function in the pancreas and results in an inability to produce adequate amounts of insulin. Insulin is a hormone which helps the liver and other insulin sensitive tissues utilize blood glucose. Prolonged hyperglycemia can damage the kidneys, heart, eyes, and nervous system (American Diabetes Association, 2006).

As well as organ damage, high blood glucose will cause dysfunction in red blood cells as over-saturation causes sugar to bind hemoglobin rendering them useless for oxygen transport (Lima et al., 2009). The resulting inability for red blood cells to distribute oxygen causes cell respiration issues and potential cell death. Another key issue regarding high plasma glucose and hypertension is arteriosclerosis. The link between high glucose levels and hypertension may be a decreased production of nitric oxide, a vasodilator. Excess cellular glucose can cause an increase in O-GlcNA which competes with nitric oxide synthase for phosphorylation (Lima et al., 2009).

There are many tests to determine if someone has diabetes. One of the more popular methods is the fasting plasma glucose (FPG) test, which requires the participant to fast for 8 hours before measuring their blood glucose concentration (American Diabetes Association, 2014). A normal test would show less than 100

mL/dL glucose in the blood stream. Blood glucose levels of 100 to 125 mL/dL indicate prediabetes and 126 mL/dL or greater indicate diabetes (American Diabetes Association, 2014). Other methods of testing diabetes include glycated hemoglobin test (A1C), Oral Glucose Tolerance Test (OGTT), and the random plasma glucose test. Hemoglobin A1C estimates plasma glucose levels over the prior two to three months, with a value of 6.5% or higher indicating diabetes. A 5.7 to 6.4% value indicates a person is pre-diabetic and is at risk of developing the disease (American Diabetes Association, 2014). The OGTT measures how the body reacts to a controlled dose of glucose over two hours. Random plasma glucose testing will indicate diabetes if over 200 mL/dL blood glucose is found when randomly tested (American Diabetes Association, 2014).

1.3.3 Hepatic Steatosis

There are two kinds of common hepatic steatosis syndromes: alcoholic liver disease and nonalcoholic fatty liver disease (NAFLD). The prevalence of NAFLD within the U.S. population is somewhere between 14 and 24% and typically presents in adults rather than children (Browning and Horton, 2004). Broadly speaking, insulin resistance and resulting hyperinsulinemia will cause an increase in hepatic glucose and lipid synthesis, and a decrease in glucose excretion. This leads to hepatic steatosis which can result in reduced liver function.

Mechanistically, insulin resistance will cause up-regulation of hepatic sterol regulatory element-binding protein 1 (SREBP-1c) and carbohydrate-responsive element-binding protein (ChREBP) which in turn increases transcriptional activation of lipogenic genes (Elkatrwy, 2011). An up-regulation of ChREBP will also increase activation of L-type pyruvate kinase (L-PK). (Elkatrwy, 2011) These changes in hepatic gene expression causes glucose to be converted to free fatty acids (FFA) which are readily used in hepatic lipogenesis. Excess FFA will also cause a rise in malonyl CoA which inhibits carnitine palmitoyltransferase I, a key enzyme in fatty acid transport though the mitochondrial membrane, thus favoring lipogenesis (Browning and Horton, 2004). Adipose is also affected by hyperinsulinemia which acts to up-regulate hormone-sensitive lipase (HSL) activity, increasing adipose triglyceride lipolysis and elevating production of FFA flux to hepatic tissue (Browning and Horton, 2004). These regulatory changes in hepatic and adipose tissues will lead to a metabolic shift which promotes lipid accumulation in hepatic tissue.

1.3.4 Chronic Inflammation

Many of the metabolic problems seen in obese individuals are due to chronic inflammation brought on by high-calorie diets and low physical activity. Metabolic diseases may be caused by chronic low-grade inflammation causing tissues to become metabolically dysregulated and inflamed. Chronic inflammation is related to homeostatic imbalances within tissues which leads to an increased propensity

for disease, decrease in tissue repair, and decreased ability for tissues to adapt to stressors (Xu et al., 2014).

Inflammation is associated with elevated C-reactive protein (CRP) and serum amyloid A (SAA). Normal serum CRP levels are below 3.0 mg/dL (Berlin and Lisovaya, 2014). Immune cells, liver and adipose tissue are all sources of CRP. As a pro-inflammatory marker and an active agent in the inflammation process, high levels of CRP can indicate an increased risk of heart disease, obesity and diabetes (Berlin and Lisovaya, 2014). Another pro-inflammatory marker is SSA which is elevated in people with atherosclerosis. Atherosclerotic plaques and increased risk for cardiovascular disease are also elevated in those with high serum SAA levels (Berlin and Lisovaya, 2014).

Chronic inflammation can cause cell membranes to breakdown causing an imbalance in electrical currents kept by concentration gradients through membrane compartmentalization (Xu et al., 2014). Also, an increase in cell death or apoptosis is expected with resulting poor membrane stability with chronic inflammation. The potential to select foods which have an anti-inflammatory effect would be likely to improve metabolism.

1.3.5 Hyperlipidemia

Hyperlipidemia is a condition where abnormally high concentrations of blood lipids exist in the circulatory system for extended periods of time. According to

the National Institute of Health, problematic levels of serum total cholesterol begin at ≥ 200mg/dL (National Cholesterol Education Program, 2001). The NIH also recommends keeping LDL blood level below 100mg/dL and for HDL blood levels to be above 60mg/dL for optimal health (National Cholesterol Education Program, 2001). Total blood triglyceride level is recommended to be kept below 150mg/dL for optimal health (National Cholesterol Education Program, 2001).

People who suffer from hyperlipidemia are at a greater risk of developing arteriosclerosis, coronary heart disease, myocardial infarction, stroke, and renal failure. Fat and cholesterol accumulates in the circulatory system when there is an excess of triglyceride and cholesterol synthesis in body tissues (Xu et al., 2014). After a meal, blood lipid and cholesterol levels increase, which becomes a significant issue when these concentrations increase past recommended levels or remain elevated for extended periods of time.

1.4 Functional Foods and Metabolic Health

Consumption of functional food is a popular way of attempting to improve metabolic function. Functional foods contain healthful compounds or ingredients which have a medicinal effect. Our goal is to understand how functional foods and their components work to improve metabolism. Popular functional foods include tree nuts, berries, brassicas, and whole grain foods. These recognized functional foods contain healthful compounds such as omega-3 fatty acids, polyphenols, isothiocyanates, and fiber.

1.4.1 Fruit and Vegetable Intake

Dietary Guidelines for American 2015-2020 recommends a vegetable intake of at least 2.5 cups per day for adults based on a 2000 Calorie daily diet. The recently published guideline recommendation also suggests a daily fruit intake of around 2 cups. Based on these 2010 recommended values, only 57.8 percent of US citizens are meeting the guidelines for fruit and vegetable intake, leaving 42.2 percent deficient for these two food categories.

Increased consumption of healthful foods can benefit metabolic function and decrease one's risk of developing metabolic related diseases. Tree nuts contain high levels of omega-3 fatty acids which impacts cardiovascular, immune and inflammatory pathways. Berries are a rich source of polyphenols which are reducing agents that help decrease oxidative stress, improving metabolic function and lessening inflammation. Cruciferous vegetables have high levels of glucosinolates, which upon hydrolysis become biologically active isothiocyanates capable of regulating enzymes involved in metabolism, inflammation and elimination of xenobiotics. Fiber is a well-recognized functional food component of whole grain foods. Consumption of high fiber foods can reduce risk for type II diabeties and increase cardiovascular and gut health (Slavin, 2013).

1.4.1.1 Anti-inflammatory properties

Among the bioactive compounds in berries are salicylic acid, gallic acid, quercetin, raspberry ketone, catechines, kampferol, cyanidins, carotenoids, and pelargonidins. Many of these compounds can act to decrease levels of inflammatory cytokines. These compounds may be capable of shifting metabolism and alleviating metabolic concerns brought on by poor diet choices.

1.4.1.2 Anti-oxidant properties of Red Raspberry

Many bioactive molecules are relatively abundant within raspberries and promote free radical scavenging and increase metabolic health and productivity. Some of these anti-oxidant promoting molecules include quercetin, raspberry ketone, and ellagic acid. Anti-oxidants can alleviate some of the issues associated with catabolic processes which require oxygen. A reduction in the concentration of free radicals will also provide protection against mutations to DNA and tumor growth.

1.4.2 Nutrient Content of Red Raspberry

Red raspberry is commonly sold commercially as a hybrid of *Rubus ideaeus* and *Rubus strigosus* (Fernández-Fernández et al., 2011). Approximate composition of red raspberry is as follows: 80.6% water, 15.7% carbohydrate, 1.5% protein, 0.6% fat, and 1.6% ash (Jay et al., 2008). The USDA National Nutrient Database for Standard Reference estimates one cup of whole red raspberry contains 80 kcal of energy, 2 g of protein, 16 g of carbohydrate, 6 to 8 g of dietary fiber, 6 g of

sugar and 1 g of lipid. Whole red raspberry is also a good source of minerals and vitamins and is also known for being one of the highest fiber-containing fruits (Dodevska, Šobajić, and Djordjević, 2015). One cup of whole red raspberries contains 20 mg of calcium, 1.08 g of iron, and 23.9 of vitamin C (USDA National Database). Red raspberry does not contain appreciable levels of sodium, vitamin A, saturated or trans fats (USDA National Database). Anthocyanin content of red raspberries varies with region and season harvested (2.43 to 1113.1 mg/100 g) (Probst, 2015).

1.4.2.1 Red Raspberry and Fiber

Red Raspberry in particular is exemplary in providing dietary fiber, more than most whole foods on the market today (USDA National Database). One serving (100g) of whole red raspberries contains 6 to 8 g of dietary fiber (USDA National Database).

Table 1.2 shows the fiber and polyphenol content of one portion of whole red raspberry as analyzed by Dodevska in 2015. They found the soluble and insoluble fiber content of red raspberry to be approximately equal, 2.6 to 2.9 g respectively. Cellulose, as determined from the insoluble fraction, was present at a much lower rate within red raspberry 1.2g per 100g.

Table 1.2 Fiber content of one portion (100g) of whole red raspberry*

Fiber parameters	Red raspberry
Total fiber (g / 100g)	5.5
Soluble fiber (g / 100g)	2.9
Insoluble fiber (g / 100g)	2.6
~ Cellulose (g / 100g)	1.2

* Adapted from (Dodevska et al., 2015).

~ Subcategory of the previous row

In a second study, Jakobsdottir et al. measured soluble and insoluble fiber along with digestible polysaccharides, in dried whole red raspberries. They found a higher content of insoluble compounds, although their reported values included non-fiber compounds.

1.4.3 Red Raspberry Health Benefits

Whole raspberries have been examined as a potential food candidate that could remediate metabolic disease concerns brought on by consumption of a western diet. Bioactive compounds of red raspberry have been studied for their ability to regulate gene and protein expression within the body. Addition of red raspberry flavonoids and polyphenols to the diet have been studied and potential functional biomolecules within red raspberry have been identified (Burton-Freeman et al., 2016).

Table 1.3 Phytochemical compounds in frozen red raspberry*

Phytochemical Compounds	mg/100g
Anthocyanidins	
Cyanidin	22.6
Delphinidin	0.02
Pelargonidin	1.60
Flavones	
Apigenin	0.01
Luteolin	0.02
Flavonols	
Kaempferol	0.01
Myricetin	0.03
Quercetin	1.10

*Adapted from (Burton-Freeman et al., 2016).

Effects of red raspberry polyphenols are less studied than many other berries for their relative health benefits.

1.4.3.1 Red Raspberry and Polyphenols

A large portion of the polyphenols within raspberries are anthocyanin compounds. Anthocyanins are responsible for the pigmentation of many fruits including raspberries and have antioxidant capabilities which can reduce inflammation. Within plants, anthocyanins are used to reduce the amount of free radical species created during conditions of high UV light and increased rates of photosynthesis, as well as protect plants against potential plant pathogens

(Paterson et al., 2013). These compounds are less well studied for mechanistic effects on physiology than other phenolic compounds.

Table 1.4 Composition of polyphenolic compounds in 300g of red raspberry*

(Poly)phenolic Compounds	μmol/ 300g
Cyanidin-3-O-sophoroside	175 ± 6
Cyanidin-3-O-(2-O-glucosyl) rutionoside	56 ± 2
Cyanidin-3-O-glucoside	37 ± 1
Cyanidin-3-O-rutinoside	20 ± 1
Cyanidin-3-O-(2-O-xylosyl) rutinoside	2.7 ± 0.1
Pelargonidin-3-O-sophoroside	1.2 ± 0
Pelargonidin-3-O-glucoside	1.1 ± 0
Cyanidin-3,5-O-diglucoside	trace
Total anthocyanins	**292 ± 10**
Ellagic acid-O-pentoside	3.2 ± 0.2
Ellagic acid-O-hexoside	3.1 ± 0.2
Total ellagic acids	**6.3 ± 0.3**
Sanguiin H-6	195 ± 7
Sanguiin H-10	5.7 ± 0.4
Lambertianin C	50 ± 1
Total ellagitannins	**251 ± 3**
Ferulic acid	1.2 ± 0.1
4-Hydroxybenzoic acid	2.3±0.1
Total (poly)phenols	**553±19**

*Adapted from (Ludwig et al., 2015). (Poly) phenolic compounds identified and quantified by HPLC-MS.

Ellagic acid is another phytochemical which may be responsible for improved metabolic function upon consumption of red raspberry. Raspberries contain one

of the highest levels of ellagic acid (Landete, 2011). Around sixty percent of the phenolic content in red raspberries are ellagitannins which are hydrolyzed to ellagic acid during metabolism. (Landete, 2011) The three main benefits of ellagic acid consumption are their anti-inflammatory, antioxidant and antimicrobial functions. Once ellagic acid reaches the microbiota within the gut, it is then metabolized into a family of compounds called urolithins (Kang et al., 2016).

Current research suggests urolithins are modulators of the gut microbiome. Urolithins may influence epigenetic regulators through DNA methylation, histone modifications and mRNA expression, though more evidence is needed to support the potential causal relationship (Gerhauser, 2018). Microbial metabolism of ellagic acid contributed to an ecological change in the microflora promoting the growth of *Bifidobacterium* spp. and reducing inflammation. Two enzymes involved in adipogenisis, histone deacetylase 9 (HDAC9) and histone arginine methyltransferase 4 (CARM1), are inhibited in the presence of ellagic acid. Ellagic acid also, helps to reduce TNFα-induced inflammation through inhibition of histone acetyltransferase (HAT) activity in monocytes (Kang et al., 2016).

A strong antioxidant, quercetin, is found abundantly in many fruits and vegetables. The abundance of quercetin in red raspberry is shown in Table 1.3. Quercetin has a catechol structure, with two hydroxyl groups on the b-ring phenol, which gives it superior free radical scavenging ability. Quercetin has

been shown to improve pancreatic islet cell function, enhancing insulin secretion, which increases the body's ability to process food and respond to metabolic stresses (Natarajan et al.,2017). Supplementation with quercetin has also shown a protective antimutagenic and antiviral effect. Quercetin can protect against tumor growth and promotion as it scavenges free radicals which could cause DNA damage and has been shown to stop mutagenic cells at the G1 stage of cell division (Wei et al., 1994).

1.5 Fiber and Health

Fiber is of particular interest as it has been connected to better metabolism and increased colon health (Anderson et al., 2009). Americans on average do not eat enough fiber in their diet (Dietary Guidelines for Americans 2015-2020), which causes heath complications and increases absorption of glucose into the epithelial cells of the small intestine (Anderson et al., 2009). Inadequate fiber intake can also damage the gut lining causing leaky gut, a shift in normal microbiome, cause laxation issues, diverticulosis, and increase cancerous polyp development (Madhu et al., 2017).

Adults are advised to consume 20-35 g/day of fiber and children 5 g/day, these levels are typically not met by the most US citizens (Dietary Guidelines for Americans 2015-2020). Consumption of fiber is associated with both glucose and cholesterol reductions in blood and increased gut microbial heath. These effects are related to the viscous properties of fiber. Viscous fiber creates a physical

barrier to bile acid reabsorption within the gut, which promotes excretion of bile acids in the feces. Enhanced bile excretion causes cholesterol to be used by the liver to replace the lost bile acid, therefore lowering overall concentrations of LDL-cholesterol within the blood (Anderson et al., 2009).

1.5.1 Fiber and the Microbiota

Consumption of a high fiber diet will promote a healthier microbiota and increase gut health. The gut contains the greatest number of immune cells in the body and is responsible for mitigating the exposure to multiple antigens from diets, commensal bacteria, and pathogens (Sun et al., 2015). Short chain oligosaccharides, contain two to ten sugar units, also promote the formation and colonization of *bifidobacteria* within the microbiota which are dependent on fiber as an energy source (Anderson et al., 2009). Pathogenic bacteria cannot use short chain oligosaccharides as an energy source and will decrease in number and slough off into fecal matter when short chain oligosaccharide fiber content is high (Anderson et al., 2009). The physiological impact of incorporating longer chain fiber into the diet is not fully understood at present (Benítez-Páez et al., 2016). Fiber studies have often given inconsistent or inconclusive results, likely due to the persistence and resistance of certain gut microbes (Kieffer et al., 2016).

1.6 Previous Red Raspberry Studies

Our lab has conducted two previous studies examining the overall health benefits provided by consumption of raspberry products. The first study evaluated effects of supplementation of whole red raspberry juice concentrate (RJC), whole red raspberry puree concentrate (RPC), raspberry seed extract (RSE), raspberry fruit extract (RF), ellagic acid (EA), raspberry ketone (RK), and ellagic acid with raspberry ketone (EA+RK) in a high-fat western style diet provided to mice. In study II the scope was narrowed to assess only the health benefits, as seen in study I, of RJC and RPC. In Study I, these supplements were given at the equivalence of four serving per day and in study II the dietary dose was lowered to one-serving per day.

1.6.1 Study I

A study conducted by the Shay laboratory in 2015 examined the development of obesity in mice fed a high fat diet with or without the supplementation of different raspberry products. The diet groups in this study included a low-fat control, a western diet control, and high-fat western-style diet supplemented with the seven raspberry-derived products mentioned above. The macronutrient content of the western diet was ~45% fat, 35% carbohydrate, and 20% protein, by energy. Concentrates and powders were supplemented at 20% of total energy and purified compounds were supplemented at 0.2% (w/w). RPC and RJC was provided at the equivalence of four servings a day. Six-week-old C57 mice were acclimated for 2 weeks and then were divided into their respective diet groups

and fed for 10 weeks. Mice were maintained in an animal care facility with a 12h light/dark cycle and were allowed to eat and drink *ad libitum*. Body weight, food consumption, and spillage were measured weekly and blood glucose was tested 6 hours prior to sacrifice at the end of week 10. (Luo et al., 2016)

There was a decrease in weight gain for mice supplemented with RPC, RJC and EA+RK compared to western diet alone. Serum resistin, a marker of insulin insensitivity, was decreased in mice consuming the RPC and RJC diets (Luo et al., 2016).

1.6.2 Study II

The Shay laboratory conducted another study providing a single daily serving of RPC and RJC in order to observe specific changes in metabolic syndrome parameters. Four groups of C57 mice (n=8) were fed: low-fat diet (LF), high-fat western-style diet, or a high-fat western-style diet was supplemented with either RPC or RJC (Luo, et al., 2017). Mice were placed on the diets for 10 weeks, and allowed to eat and drink *ad libitum* in a 12-hour light/dark controlled environment, maintained at 24°C (Luo, et al., 2017). The results from Study II are highlighted in Table 1.5.

Table 1.5 Study II experimental results*

Metabolic Parameters Compared to HF-diet alone	Treatment groups (2.5% of total energy, ~1 serving / day)	
	HF+ RPC	**HF+RJC**
Decrease in Hepatic Lipid Accumulation	15% ± 6%	17% ± 3%
Decrease in Final Body Weight	15% ± 6%	17% ± 3%
Insulin Sensitivity	Increased	Increased
Baseline Blood Glucose	No difference	No difference
HOX1 up-regulation	Yes	Yes
Lipe up-regulation	Yes	Yes

* From (Luo et al., 2017)

CHAPTER 2
PREPARATION OF FIBER AND POLYPHENOL - RICH FRACTIONS FROM RASPBERRY PUREE CONCENTRATE

2.1 Introduction

The consumption of polyphenols and fiber have been shown to ameliorate some of the negative health impacts of consuming a high-fat and high-sugar western-style diet. For this reason, this experiment has undertaken a study to deduce the relative health benefits associated with the fiber and polyphenol fractions of red raspberry. This project will produce a better understanding of the health benefits observed with consumption of red raspberry fiber and polyphenols. The purpose of our study is to observe the relative effects of raspberry derived polyphenols and fiber when fed to mice consuming a “western-style diet.”

Chapter two describes how these food fractions were derived from the whole food (RPC). This chapter also includes a characterization of the whole food (RPC) and the individual fractions, red raspberry polyphenol-enriched (RP) and red raspberry fiber-enriched (RF). The analysis includes the relative fiber content, moisture content, and specific polyphenolic compounds within each treatment group.

We hypothesize the red raspberry polyphenols and fiber are unique in their metabolic activity and may also work synergistically to produce the health effects observed in our earlier studies (Luo et al., 2016 and Luo et al., 2017).

2.2 Materials and Methods

2.2.1 Storage Condition

Raspberry Puree Concentrate (RPC) was donated by Milne Fruit Products (Prosser, Washington). The RPC was stored at -20°C in the dark in air-tight containers until needed. After-storage, RPC was thawed for 24 h at room temperature followed by additional time, if needed, at 4°C. Any time during processing when the product was not being actively handled, it was stored at 4°C in the dark. Nutritional content of RPC is found in Table 3.1. Diet composition of the RPC-containing rodent diet is found in Table 3.2.

2.2.2 Moisture Content

Moisture content of RPC was determined using an oven heating protocol. Two empty aluminum metal drying containers (70 mm diameter x 32 mm height with a tight-fitting lid) were cleaned and dried at 100°C for 15 minutes and placed in a desiccator. Special care was taken to handle them with gloves as to not transfer any oil to the surfaces. Each aluminum metal drying container with its corresponding lid was weighed and recorded. The scale was tared and around 5g of product was weighed out directly into each container.

A vacuum oven is connected to a pump which maintains a partial vacuum (≤ 25mm Hg) and is connected to a H_2SO_4 gas-drying bottle. For this procedure, the vacuum oven was set to 70°C. Once the desired temperature was reached, the sample-filled containers were transferred to the oven. Lids were placed on

the containers unsealed in order to allow moisture to escape during the heating process. Both samples were heated at 70°C overnight (16 h) and then removed from the oven. After retrieval, the sample-filled containers were cooled in a desiccator until they reached room temperature, after which they were weighed. The new weights were recorded and moisture content was calculated. Samples were dried for an additional three hours in the vacuum oven to ensure a final dry weight was achieved.

The equations used were

Wet Sample wt. - Dry Sample wt. = Moisture Lost During Drying

Wt of Container & Sample After Drying - Empty Drying Container = Dry wt

(Moisture Lost / Wet Sample wt.) x 100 = Moisture Content Wt. Basis %

2.2.3 Fiber Content of RPC

Fiber was measured using a modification of AOAC Official Method 991.43 (Total, Soluble and Insoluble Dietary Fiber in Foods) and a combination of enzymatic (pancreatin) and gravimetric analysis. A preliminary analysis of the puree used a dietary fiber kit (Sigma TDF-100A) to determine total fiber content. The manufacturer's recommended protocol was followed.

A second analysis used pancreatin in place of the dietary fiber kit. Fiber used in the feeding study was obtained using the pancreatin substitute as the dietary

fiber kit uses celite, which could cause dietary distress in mice even if provided in trace amounts.

2.2.3.1 Preparation of Buffer

A potassium buffer was used to achieve optimal conditions for the porcine pancreatin. An acetate buffer (pH 4.0) was mixed with a phosphate buffer (pH 7.0) to create a pH 6.8 buffer which was then used for the enzymatic digestion with pancreatin.

2.2.3.2 Preparation of Pancreatin Digest

Pancreatin was added at 2 g for every 100 g of RPC converted (amylase activity: minimum 25 USP u/mg, protease activity: minimum 25 USP u/mg, lipase activity: minimum 2 USP u/mg) according to specification. The porcine pancreatin was sourced from Alfa Aesar (Tewksbury, MA). The mixture was placed in a water bath at 40°C overnight (~16 h) to simulate conditions within the porcine digestive track.

2.2.3.3 Clarification of Fiber

After retrieving the digest from the water bath the contents were cooled to room temperature. The digest then underwent centrifugation at 6,000 rpm or 6037xg (Rotor GSA, Centrifuge RC5c) for 10 minutes and the supernatant was collected. The supernatant obtained from the first centrifugation was used to produce a polyphenol-rich fraction via hydrophobic chromatography (2.2.3.4). The pellet

obtained was then re-homogenized, washed, and centrifuged twice with 80% and 95% ethanol to further clarify the enriched fiber fraction. After washing, the pellet was dried using a vacuum oven at 70°C for 5 h and stored at -20°C until needed.

2.2.3.4 Amberlite Polyphenol Extraction

Amberlite® FPX-66 (Rohm Haas, Philadelphia, PA) was used to produce a polyphenol-rich fraction from the supernatant obtained after centrifugation. FPX-66 are adsorbent polymer beads which bind to hydrophobic polyphenolic compounds in the supernatant through hydrophobic interactions. The mixture of amberlite® beads and the digest was agitated for 1 h on a shaker table resulting in a clear supernatant which was then discarded. Amberlite® beads with the bound polyphenols were then eluted with a 95% w/w ethanol wash until the wash ran clear and all of the colored supernatant was collected.

2.2.3.5 Drying of Extracted Polyphenols

After collecting the eluate of the amberlite® bead wash, the volume was reduced under vacuum while heating in a water bath at 50°C. This product was stored at -20°C until further concentration. The subsequent concentration utilized a food dehydrator at 74°C until a thick paste was produced. This polyphenol-rich material was then used for rodent diet production.

2.2.3.6 Polyphenol Content Determination

Polyphenol content of RPC and RP was conducted by Mr. Robert Durst, Oregon State University, using the Folin-Ciacatten method (Ainsworth and Gillespie, 2007).

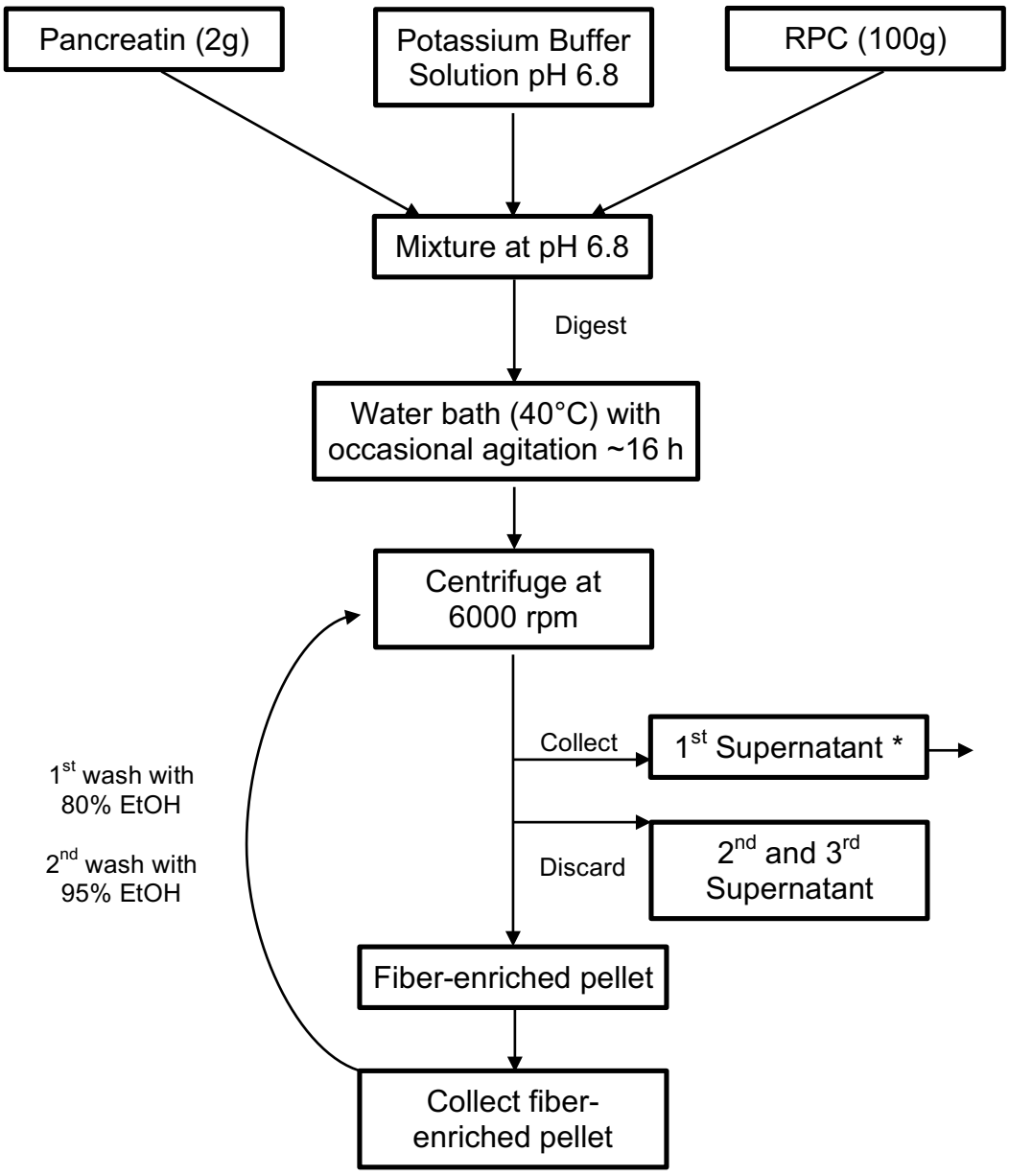


Figure 2.1 Flow chart of the fiber extraction of using a modified AOAC official method 991.43 with pancreatin.

* Indicates fraction used for polyphenol extract preparation.

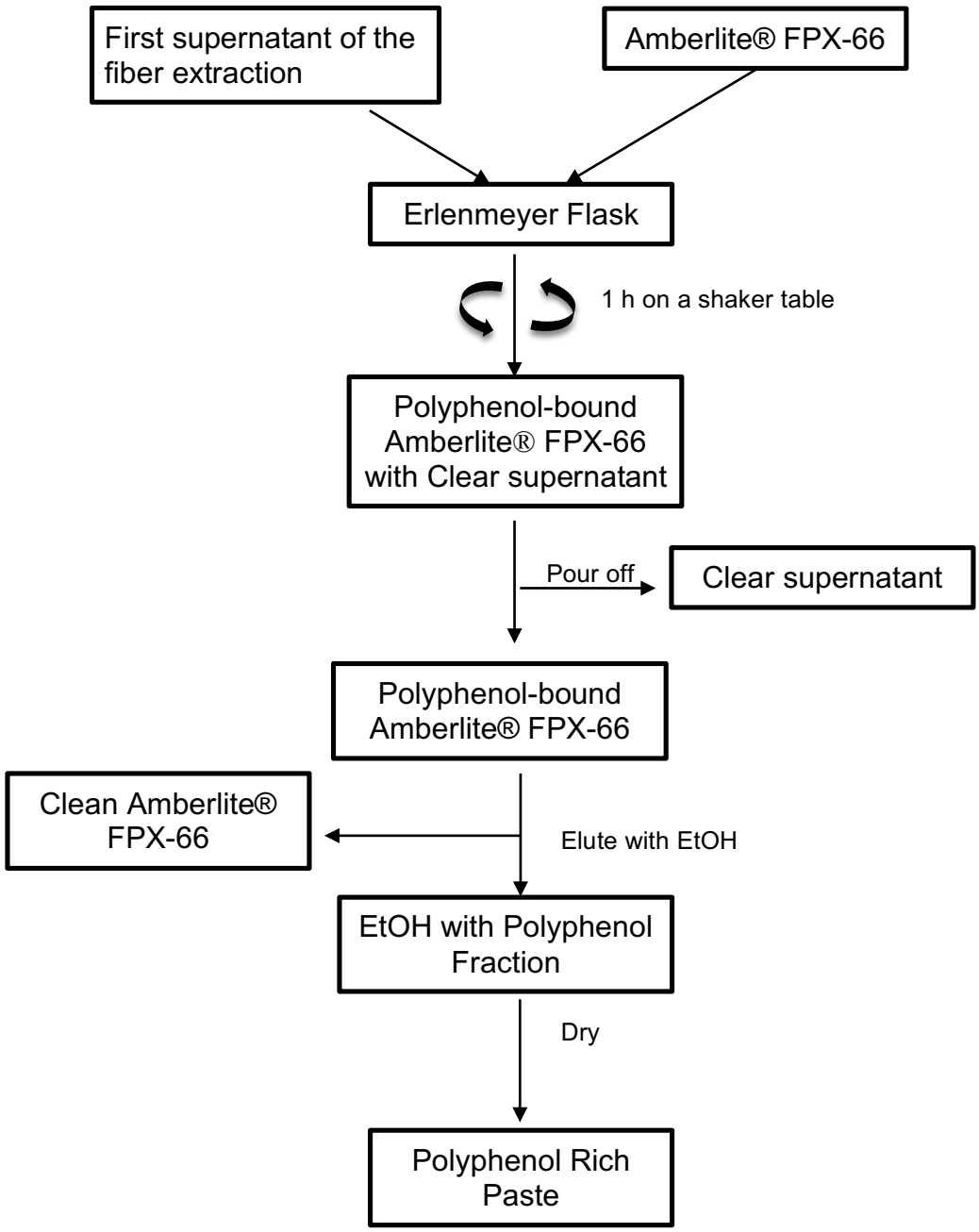


Figure 2.2 Flow Chart of Amberlite® FPX-66 extraction of polyphenol-rich fraction.

2.3 Results

2.3.1 Moisture Content

Table 2.1 Moisture content measurements

	1st Container *	2nd Container *
Empty Container(g)	28.719	29.488
Wet Sample RPC (g)	5.032	5.078
RPC + container after 16 h of drying (g)	30.173	30.944
Dried Sample (g)	1.454	1.456
Water lost (g)	3.579	3.622
Water Content (%)	71.10	71.33

* measurement was preformed in duplicate

2.3.2 Fiber Content of the Red Raspberry Puree Sample

Using a dietary fiber kit (Sigma TDF-100A), we measured a fiber content of 13% in the dried raspberry puree sample. This method resulted in a much higher fiber content than in the manufacturer's nutrient content data sheet for the RPC.

A modified AOAC Official Method 991.43 (Total, Soluble and Insoluble Dietary Fiber in Foods) substituted pancreatin instead of individual enzymes, and was used during the fiber fractionation. We obtained a value of 2.8% fiber content, which was quite close to the given manufacture value.

2.3.3.7 Fiber Content of the Enriched Product

The RF fiber content was measured using AOAC method 934.06 by Rtech laboratories, Anden Hills, MN

2.4 Lab Analysis and Enrichment Calculations

2.4.1 Fiber Enrichment of RPC*

The fiber content of the whole food RPC was 2.0% based on wet weight. Dry fiber content was calculated using the percent moisture (73.4%). Table 3.1.

Table 2.2 Fiber enrichment of RPC*

Material	Mass (g)	Fiber Concentration (g / 100g)	Fiber Content (g)	Yield (%)	Fold Enrichment
RPC (wet)	2000	2.0	40	100	1.0
RF (wet)	316	9.45	29.9	74.8	4.73
RPC (dry)	532	7.5	40	100	1.0
RF (dry)	66.9	44.6	29.9	74.8	5.95

*RF fiber content was measured using AOAC method 934.06 Rtech Laboratories, Anten Hills, MN.

2.4.2 Enrichment of Phenolic Compounds

Table 2.3 Phenolic enrichment of RPC*

Material	Mass (g)	Polyphenol Concentration (mg / 100g)	Polyphenol Content (mg)	Yield (%)	Fold Enrichment
RPC (wet)	2000	131.7	2634	100	1.0
RP (wet)	204	488.2	996	37.8	3.8

* Polyphenol content was analyzed by Folin-Ciacatten method

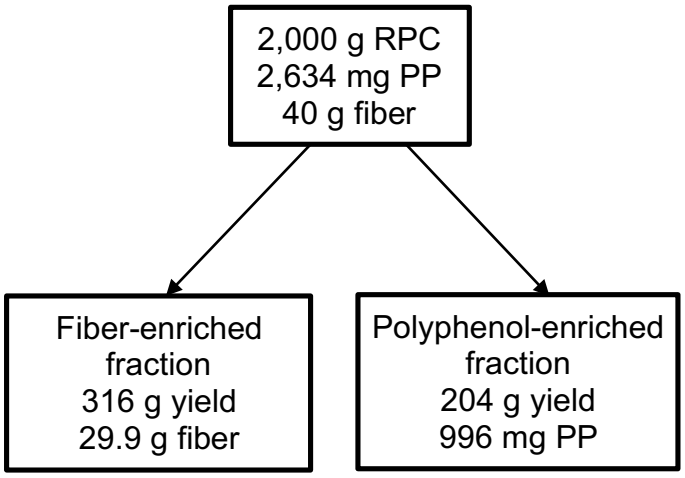


Figure 2.3: Red raspberry fiber-enriched and polyphenol-enriched fraction yields

2.5 Discussion

One of the ways to combat obesity is through the use of functional food products in the diet. In order to best use functional foods, we need to understand their effects on metabolism and through this exploration, if possible, increase the nutritional properties of food products. One way to enhance beneficial bioactivity of food is through fractionation to increase the abundance of healthful ingredients.

In this chapter, two methods of fractionation were utilized. For the red raspberry fiber fraction, we used a modification of AOAC Official Method 991.43 (Total, Soluble and Insoluble Dietary Fiber in Foods) with pancreatin. For the red raspberry polyphenol fraction, we used a hydrophobic affinity column fractionation technique. These two techniques enabled us to concentrate the desired bioactive fractions from the whole food, which allowed us to assess the relative metabolic effect of these two fractions in a 10-week feeding study conducted using C57 mice (Chapter 3).

Our measured moisture content of the whole food (RPC) was comparable to the moisture content provided by the manufacturer (~71 vs. ~73.4%, respectively). Fiber obtained through the modified AOAC Official Method 991.43 with pancreatin was also consistent with the nutritional label (2.8% to 2.0%, respectively). These findings indicated that the food shipped was representative of the nutrition label given and represented in Table 3.1.

However, the fiber content found when using the dietary fiber kit (Sigma TDF-100A) was much higher than represented on the nutrition label (~13 to 2.0%, respectively). These findings might support the evidence that the fiber content found in the analysis of the fiber fraction could be over-estimated. Often, foods rich in polyphenols, will assay higher in dietary fiber content in comparison with their actual concentration (personal communication with Dr. James Podolske, Director of Innovation K. Rettenmaier USA-LP, School craft, MI.) This is what we observed when testing the RPC food through the dietary fiber kit method and then again in fiber analysis conducted by an outside service lab.

One limitation of our fiber-enrichment procedure is possible loss of soluble fiber during the RPC enzymatic digestion in a water based buffer. Our fiber-enrichment procedure yielded 75% of the original RPC fiber concentration. We expect the fiber-enriched product has more insoluble fiber than original RPC product (2:1 instead of 1:1 insoluble to soluble fiber, respectively).

Analysis showed the phenolic enriched-fraction contained a 3.8-fold increase in phenolic content from the original RPC product. The fiber enriched-fraction contained a 5.95-fold increase in fiber content (dry weight) from the RPC product. Both of these results indicate the success of our fractionation techniques and the activity of the pancreatin used in the digest.

CHAPTER 3
METABOLIC EFFECTS OF A HIGH-FAT DIET SUPPLEMENTED WITH POLYPHENOL - AND FIBER - RICH FACTIONS PRODUCED FROM RASPBERRY PUREE CONCENTRATE

3.1 Study Introduction

This study assessed the relative contribution of red raspberry polyphenol- and fiber-enriched fractions of RPC in promoting metabolic health, as observed with our earlier studies of whole RPC (Luo et al., 2016 and Luo et al., 2017). An obesigenic high-fat western-style diet was used to promote the development of metabolic disease in male C57 mice. Many different parameters associated with metabolic disease were assessed to better clarify the differences produced by consumption of the red raspberry polyphenol- and fiber-enriched fractions.

Raspberry puree concentrate (RPC) was used to produce two fractions: a polyphenol-enriched fraction (RP) and a fiber-enriched fraction (RF). Fractions were produced via amberlite® affinity hydrophobic chromatography and enzymatic methods, respectively (Chapter 2). The whole food RPC, RP and RF fractions were fed for 10 weeks to C57 male mice fed a high-fat, high-cholesterol, and high-sucrose diet (HF). A second control group was fed a LF diet. Body weight and food intake were measured weekly and a glucose tolerance test and an insulin sensitivity test was administered in weeks 9 and 10, respectively. After 10 weeks mice were sacrificed and tissues and serum samples were collected for further analysis.

3.2 Materials and Methods

3.3 C57 Mouse Model and Diets

The C57 mouse is an excellent model to mimic human metabolic disease when provided a high fat diet. As a commonly used murine animal model, the C57 mouse develops diet-induced metabolic syndrome shortly after being placed on a HF diet. The C57 mice will develop obesity, hyperinsulinemia, hyperglycemia, hypertension, and become more susceptible to atherosclerosis when fed a HF diet, whereas if the same mice are provided a LF diet they will eat normally and not develop these debilitating metabolic conditions. After around four weeks, C57 mice fed a HF diet will show a significant increase in body weight gain compared to LF-fed control mice. Male mice more readily develop diabetes in comparison to their female counterparts and are typically used in metabolic studies (Ingvorsen et al., 2017).

Forty, six-week-old, male C57 mice were purchased from Jackson Laboratory (Bar Harbor, ME) and allowed to acclimate for four weeks prior to experimentation. They were gradually introduced to the LF diet over the first two weeks by incrementally adjusting the diet every two to three days from common lab chow to the semi-purified LF diet in around 20% steps. By the end of the two-week adjustment period, mice were consuming 100% LF. Animals then were maintained on the LF diet for an additional two weeks until the experimental diets were introduced. Purified LF and HF diets, along with the experimental red

raspberry-enriched diets were produced by Research Diets, Inc. (New Brunswick, NJ, USA) (Table 3.2).

Mice were housed four each to a plastic shoebox-style cages with standard bedding and were allowed to drink water *ad libitum*. They were kept in the controlled environment of our animal facility at 20°C. The animal room had an automated 12h light/dark cycle. Mice were assigned to different diet groups (n=8), the two control groups were placed on either a LF diet which contained 10% fat and 70% carbohydrate (by energy) or a HF diet which contained 45% energy from fat, 35% energy from carbohydrate and 1% (w/w) cholesterol. The other three groups were placed on HF diets enriched with RPC (HF+RPC), RP (HF+RP), or RF (RF+HF). The HF+RPC diet contained 20% energy from RPC. Both the RP and RF treatments were added to the diet according to the relative amount obtained from RPC. The RPC, RP, and RF were all provided at the equivalence of four serving / day.

During the ten-week study, body weight, diet intake, and diet spillage were measured each week. All mice were closely monitored during the study and acclimated bi-weekly to routine handling and exposed to a restraining tube used during glucose and insulin tolerance testing. Training was used to acclimatize mice to the procedure and helped further mitigate false insulin and glucose spikes related to stress. Tubes were cleaned between training exposures.

3.4 Glucose Tolerance and Insulin Sensitivity Testing

Two tests were conducted to measure glucose homeostasis: the glucose tolerance test and the insulin sensitivity test. Baseline glucose and changes in glucose levels were measured during both tests to determine the response to an intraperitoneal injection of glucose or insulin.

Both glucose tolerance and insulin sensitivity tests were conducted one time each on all mice. Testing was performed during the second half of the light cycle (12:00 to 18:00 h). All animals were fasted for 5 h prior to testing.

3.4.1 Glucose Tolerance Test

Glucose tolerance protocols generally followed those used in our lab previously and other established protocols (Andrikopoulos et al., 2008, Ayala et al., 2010, Heikkinen et al., 2007, Christensen et al., 2009). Glucose was measured from blood obtained from a small nick of the lateral tail vein produced by a sterile scalpel after brief anesthetization by isoflurane inhalation. A small drop of blood was then acquired through a milking technique to read the serum glucose levels using a glucometer (Contour Next *EZ*, Bayer HealthCare LLC, Mishawaka, IN) at 0, 15, 30, 60, 90 and 120 min after a bolus glucose injection. A 20% glucose solution was used to provide a 2g glucose / kg body weight dose injected intraperitoneally (10μL of glucose solution per gram body weight). Mice were continually observed to ensure the surgical nick had clotted and that mice were

alert. Area under the (glucose) vs. time curve (AUC) was calculated using the trapezoidal rule. Baseline glucose concentration was set as the glucose reading at time 0.

3.4.2 Insulin Sensitivity Test

Insulin sensitivity was tested in a similar manner to the glucose tolerance test. Human Recombinant Insulin (MP biochemical IC0-219390010) was used. Blood glucose levels were measured at time 0, 15, 30, 60 and 90 min after bolus insulin injection (1 IU/kg insulin administered at 7.2 μL per g b.w.) Mice were continually observed to ensure the surgical nick had clotted and that mice were alert. Insulin sensitivity was then determined. Baseline glucose concentration was set as the glucose reading at time 0 calculating area above the curve (AUC_1). If the glucose level exceeded baseline, the values above were set to baseline to ensure AUC_1 values only correspond to the reduction in glucose levels from the dose of insulin.

3.5 Necropsy

At the end of the ten-week study, all mice were sacrificed and blood and tissues collected for further analysis. Mice were anaesthetized using inhaled isoflurane. To ensure depth of anesthesia, mice were tested using the pedal withdrawal test on both hind legs. If flinching was observed when the foot pad pinch was administered, isoflurane inhalant was increased until no response was observed. Careful attention was paid to ensure depth of sedation throughout the cardiac puncture, and exsanguination procedure.

3.6 Plasma Biomarkers

Blood collected via cardiac puncture was immediately put on ice for a 30-60 minute incubation period before centrifuging at 1,000 g for 15 min at 4°C to isolate serum. After centrifugation, serum was stored on dry ice and then at -20°C until needed.

Serum insulin and monocyte chemoattractant protein 1 (MCP-1) levels were measured using ELISA kits (88-7391 and EMINS, respectively) following the manufacturer's procedures. For serum insulin determination, the ELISA kit from the Invitrogen Corporation (Camarillo, CA, USA) was used. For serum MCP-1 determination, the ELISA kit from (Thermo Scientific, Frederick, MD, USA) was used. A Wallac 1420 Victor2 Microplate Reader (PerkinElmer life and analytical sciences, Turku, Finland) was used to read the 96-well plates.

3.7 Hepatic Tissue Histology

Hepatic tissue pieces (~100 mg) were collected post-mortem and formalin-fixed overnight. Liver tissue was fixed in 10% buffered formalin and embedded in paraffin. To quantify fat droplet accumulation, two 5-µm thick sections of each animal's liver were transferred to numbered slides. Tissue processing, cutting and staining were conducted by Nationwide Histology (Spokane, WA). Liver sections were then stained with hematoxylin-eosin. Images were acquired using a Nikon Eclipse E400 microscope (Nikon Co., Tokyo, Japan) equipped with an

Infinity 1-3C camera (Lumenera Corporation, Ottawa, ON, Canada). Lipid droplet percentage (the ratio of white color area pixel to the total area pixel) was obtained with Adobe Photoshop 7.0, as described by Dahab et al. (2004). Liver sections of each mouse were evaluated at random locations (n=3) and averaged to represent each mouse. Care was taken to not include biliary tubules during analysis, as inclusion could increase the percentage of white color area.

3.8 HOMA-IR and HOMA-%B

Using insulin and glucose values, a mmol/L glucose concentration was obtained through the homeostatic model assessment for insulin resistance and percent beta cell function (HOMA-IR and HOMA-%B) calculations. (Heikkinen et al., 2007).

Calculations used to determine HOMA-IR and HOMA-%B are shown in the appendix. Fasting plasma insulin (FPI) was obtained from serum collected at necropsy. Fasting plasma glucose (FPG) was taken as an average of the two baseline values obtained on the glucose tolerance test day (ninth week) and the insulin sensitivity test day (during the tenth week).

3.9 Microbial Ecology in the Cecum

Whole ceca were collected from each C57 mouse for microbiome analysis. Quantitative polymerase chain reaction (qPCR) was conducted on all samples to acquire the representative DNA sequencing of the existing microbiological

population. After isolation of DNA in each cecum and sequencing, a microbiome profiling open source pipeline, quantitative insights into microbial ecology 2 (QIIME 2), was used to determine the specific bacterial species comparing the DNA sequences obtained against a large genomic library. Matched DNA sequences allowed for the determination of the relative abundance of different bacterial species. Average values of each control and treatment group were calculated in order to determine the relative bacterial populations.

Table 3.1 Nutrition information for the raspberry puree concentrate*

28 BRIX Analysis Name	Results	Limit of Quantitation	Units
Moisture (Vacuum Only)	73.4	0.01	% by wt.
Total Carbohydrate	22.2	0.1	% by wt.
Estimated Caloric Value	106	2	Cal/100g
The Estimated Caloric Value has been calculated according to the deÀnition found in the nutrition labeling regulations printed on January 6, 1993 in CFR Part 101.9, where: Calories/100g = 4 (% protein) + 9 (% fat) + 4 (% carbs)			
Vitamin C	32	1	mg/100g
Total Dietary Fiber	2.0	0.1	% by wt.
Protein (modiÀed Dumas)	2.3	0.1	% by wt.
The % protein was calculated from % nitrogen using a factor of 6.25.			
Fat (Acid Hydrolysis)	0.9	0.1	% by wt.
Beta Carotene	80	20	IU/100g
Lycopene	0	0.5	mg/100g
Total Sugar	16	1	% by wt.
Fructose	8.0	0.1	% by wt.
Dextrose	7.7	0.1	% by wt.
Sucrose	0.2	0.2	% by wt.
Maltose	< 0.3	0.3	% by wt.
Lactose	< 0.5	0.5	% by wt.
The total sugar result does not include trisaccharides or tetrasaccharides.			
Ash	1.29	0.01	% by wt.
Calcium	38.3	0.5	mg/100g
Sodium	23.9	0.2	mg/100g

* Produced by Milne Fruit Company Disclaimer: This nutritional information does not represent lot specific testing and is not to be considered a specification.

Table 3.2 Red raspberry study composition of experimental diets

	Diet Groups				
	LF	HF	RPC[1]	RP[2]	RF[3]
Ingredient	g	g	g	g	g
Casein	200	200	190	200	200
L-Cystine	3	3	3	3	3
Corn Starch	452.2	72.8	57	72.8	72.8
Maltodextrin 10	75	100	100	100	100
Sucrose	172.8	172.8	112.8	172.8	172.8
Cellulose, BW200	50	50	42.5	50	50
Soybean Oil	25	25	25	25	25
Lard	20	177.5	174.3	177.5	177.5
Mineral Mix S10026	10	10	10	10	10
DiCalcium Phosphate	13	13	13	13	13
Calcium Carbonate	5.5	5.5	5.5	5.5	5.5
Potassium Citrate, 1 H20	16.5	16.5	16.5	16.5	16.5
Vitamin Mix V10001	10	10	10	10	10
Choline Bitartrate	2	2	2	2	2
Cholesterol	0	8.5	8.5	8.5	8.5
RPC Whole Food	0	0	375[1]	0	0
RPC Polyphenol Fraction	0	0	0	28.3[2]	0
RPC Fiber Fraction	0	0	0	0	59[3]
Total	1055	866.6	1145.1	894.95	925.6
Total (dry basis)	1055	866.6	869.9	880.8	925.65
kcal%					
Protein	20	18	18	18	18
Carbohydrate	70	36	36	36	36
Fat	10	46	46	46	46
kcal / gm	3.77	4.59	4.57	4.29	4.29

[1]RPC (Milne Fruit): 106 Cal /100g = 4 servings; 20 % of total kcal
[2]Raspberry Polyphenol Enriched Fraction: 4 servings; (6.4% w/w) 0 kcal/serving
[3]Raspberry Fiber Enriched Fraction: 4 servings; (3.16% w/w) 0 kcal/serving

A)

	Week of Study									
Measurement /Test	1	2	3	4	5	6	7	8	9	10
Body Weight	X	X	X	X	X	X	X	X	X	X
Diet Consumption	X	X	X	X	X	X	X	X	X	X
Baseline Glucose									X	X
Glucose Tolerance Test									X	
Insulin Sensitivity Test										X
Necropsy										X

B)

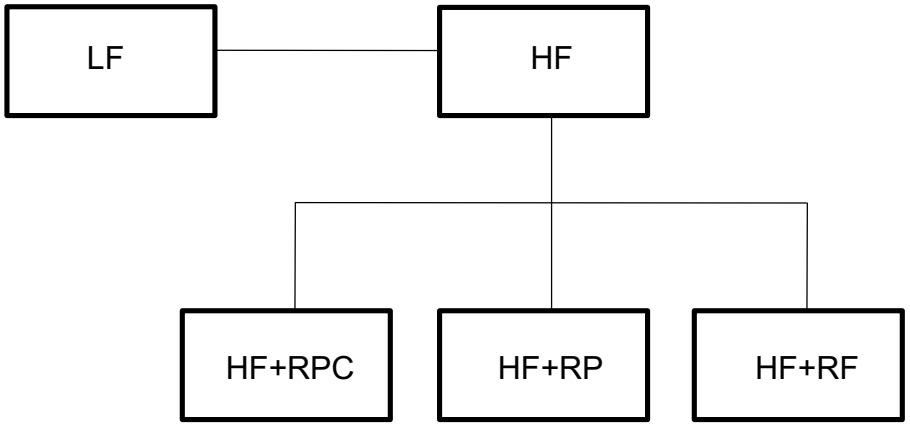


Figure 3.1 (A) Mouse study schedule, (B) study groups Low fat (LF), a high fat (HF) diet alone, HF plus: raspberry puree concentrate (RPC), enriched raspberry polyphenol (RP), or enriched raspberry fiber (RF)

3.10 Results

3.10.1 Body Weight and Net Weight Gain

No initial body weight differences were observed between the groups at the start of our study. Mice consuming the LF remained lean as the study progressed. However, animals fed HF gained weight readily throughout the ten weeks. No differences in net weight gain were observed in the red raspberry puree (RPC), red raspberry polyphenols (RP), and red raspberry fiber (RF) groups compared with the HF-diet groups (Figure 3.2A and 3.2B).

3.10.2 Energy Intake and Food Efficiency

The HF fed mice consumed more energy than mice fed the LF diet ($p < 0.05$). The energy intake of mice consuming RPC, RP, and RF diets were not reduced compared to HF-fed mice. In fact, the RPC-fed mice consumed more energy than the HF-group ($p < 0.05$) (Figure 3.2C)

Food efficiency for HF-fed mice was greater than the LF-fed group ($p < 0.05$). However, the RPC and RP groups were statistically indistinguishable from both the LF and HF-fed mice ($p > 0.05$). This indicates a reduced accumulation of body weight (g) for the same energy of ingested food (in joules) in these two treatment groups as compared to HF-alone. This beneficial food effect was not, however, observed in the mice fed the RP treatment, which like the HF-fed group was greater than the LF-group ($p > 0.05$).

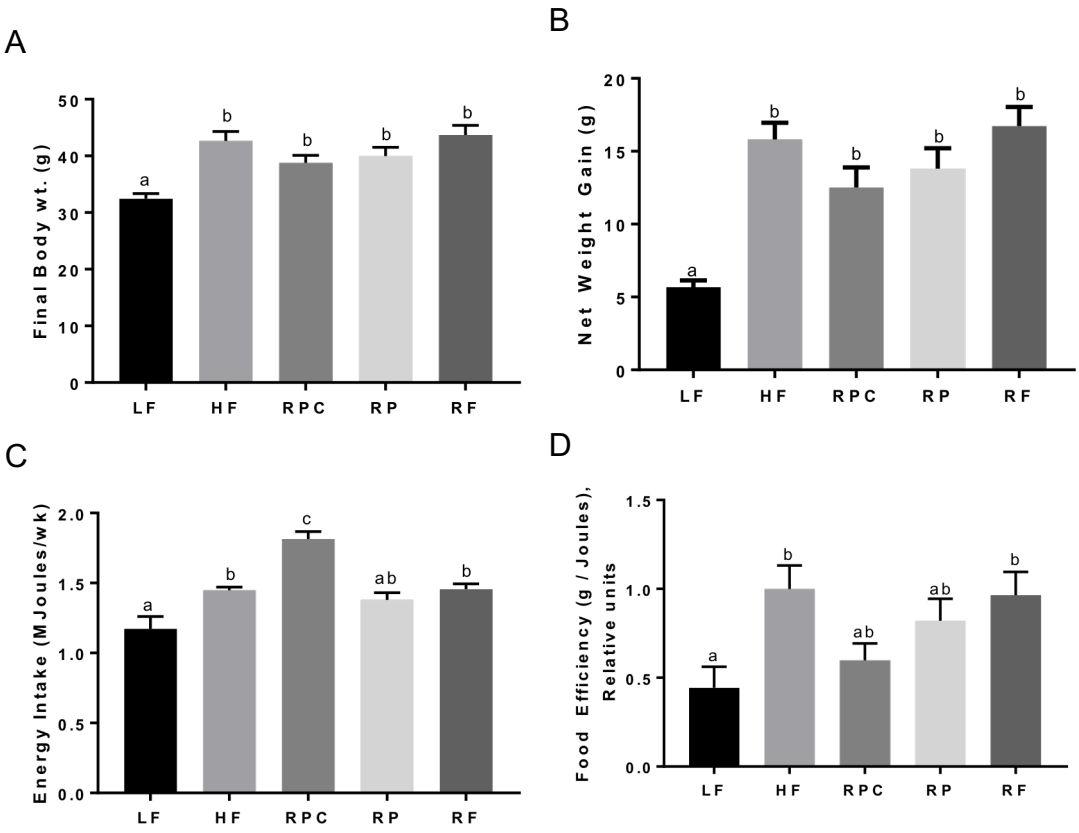


Figure 3.2: (A) Final body weight, (B) net body weight gain (C) weekly energy consumed (in joules), and (D) food efficiency of diets in C57 male mice fed a low fat (LF), a high fat (HF) diet alone, or HF plus red raspberry puree concentrate (RPC), enriched red raspberry polyphenol (RP), or enriched red raspberry fiber (RF) during 10 weeks feeding. (B) Results obtained with the subtraction of day one weight from the end necropsy weight of each animal. Values are mean $\pm$ SEM (LF and HF controls n=12, otherwise n=8). Values sharing the same superscript are not significantly different from each other. One way ANOVA indicated significant differences between diet groups ($p < 0.05$) Values that do not share a letter differ ($p < 0.05$).

3.10.3 Organ to Body Weight Ratio

The HF-fed mice had greater liver mass than animals fed the LF-diet ($p < 0.05$). All red raspberry treatment groups were comparable to the liver weight observed in the HF-fed mice, with only the RPC group being statistically indistinguishable from the LF fed mice ($p > 0.05$) (Table 3.3).

A difference in liver to body weight ratio was also observed between the two control groups with the HF-fed mice exhibiting a larger ratio than animals fed on LF alone ($p < 0.05$). All red raspberry treatment groups were comparable to the ratio observed in the HF-fed mice, with only the RPC group being statistically indistinguishable from the LF fed mice ($p > 0.05$) (Table 3.3).

None of the kidney weights were statistically different. (Table 3.3). None of the red raspberry treatment groups changed the kidney to body weight ratio compared to HF-fed mice. However, mice in the RPC group also exhibited a kidney to body weight ratio that was not statistically different than mice consuming the LF-diet.

No statistical differences in accumulation of inguinal adipose tissue were observed in the RPC, RP and RF treatment groups compared with the HF-fed mice. However, all treatment groups showed an improvement in their corresponding adipose to body weight ratios. All three red raspberry products were comparable to both the high and low fat-fed mice.

Table 3.3 Organ tissue weight and weight as percentage of final body weight of male C57 mice

	Diet Groups				
Organ Tissue Weights	**LF**	**HF**	**RPC**	**RP**	**RF**
Liver (g)	1.1±0.08[a]	2.24±0.25[b]	1.69±0.06[ab]	1.98±0.18[b]	2.38±0.27[b]
Liver/Body Weight (g/g %)	3.37±0.17[a]	4.89±0.35[b]	4.49±0.09[ab]	4.88±0.32[b]	5.34±0.41[b]
Kidney (g)	0.20±0.01	0.21±0.01	0.21±0.01	0.19±0.02	0.17±0.02
Kidney/Body Weight (g/g %)	0.67±0.04[c]	0.51±0.03[ab]	0.54±0.02[bc]	0.47±0.05[ab]	0.38±0.04[a]
Adipose Tissue (g)	0.69±0.08[a]	1.25±0.09[b]	1.10±0.09[b]	1.17±0.10[b]	1.14±0.06[b]
Adipose/Body Weight (g/g %)	2.08±0.22[a]	3.26±0.32[b]	2.62±0.10[ab]	2.88±0.17[ab]	2.65±0.18[ab]

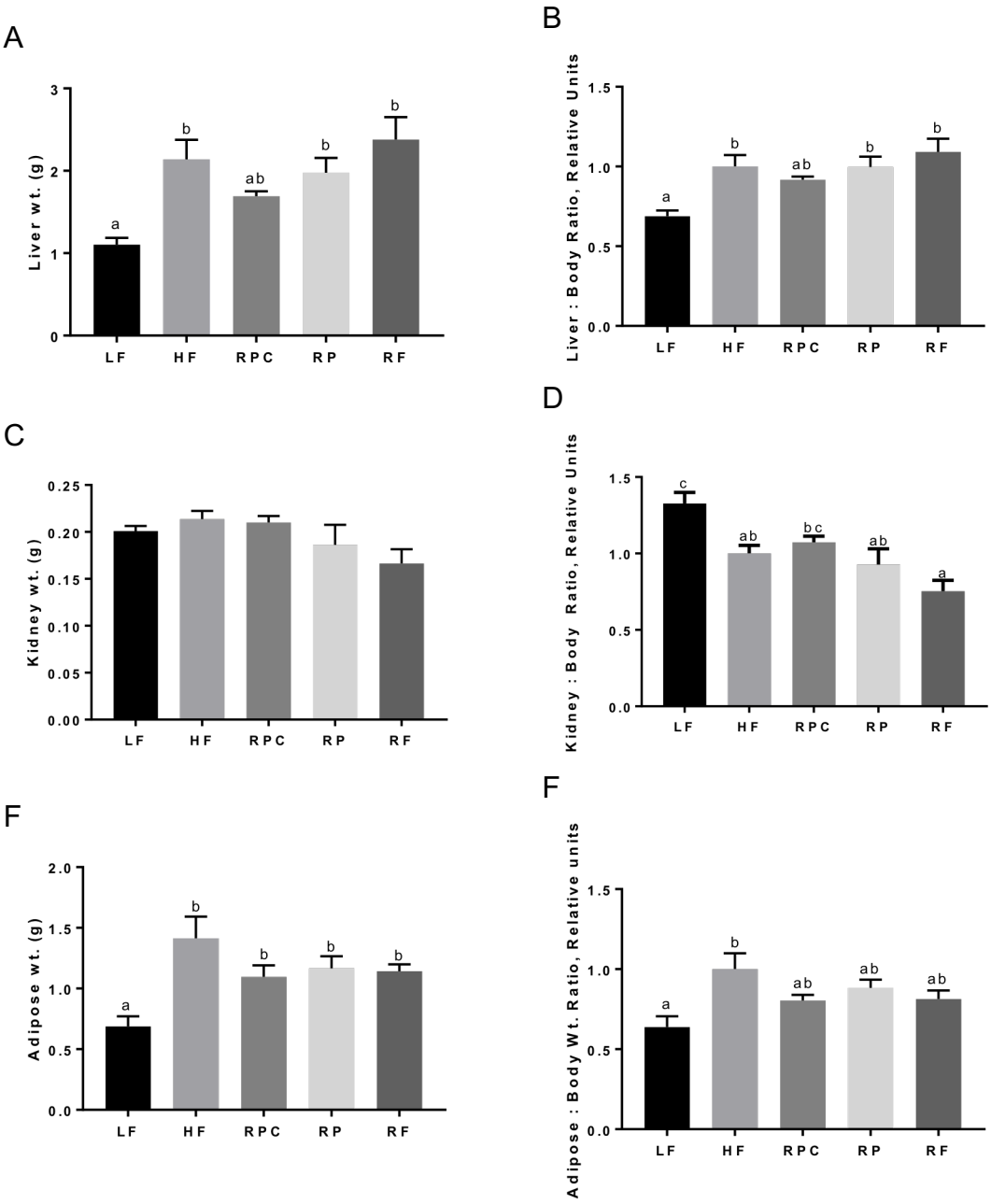
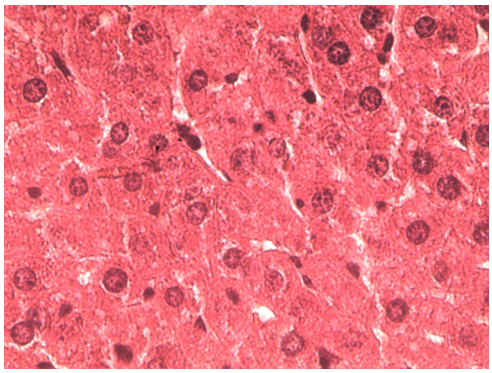


Figure 3.3: Organ weights and organ to body weight ratios of liver, kidney and adipose tissue (A) liver weight, (B) liver to body weight ratio, (C) kidney weight, (D) kidney to body weight, (E) adipose weight, and (F) adipose to body weight. Values reflect organ and organ to body weight ratios of C57 male mice fed a low fat (LF), a high fat (HF) diet alone, or HF plus red raspberry puree concentrate (RPC), enriched red raspberry polyphenol (RP), or enriched red raspberry fiber (RF). Bars are mean ± SEM LF (n=12) and HF (n=11), otherwise n=8). Values sharing the same superscript are not significantly different from each other. One way ANOVA indicated significant differences between diet groups ($p < 0.05$). Values that do not share a letter differ ($p < 0.05$).

3.10.4 Hepatic Fat Content

Liver fat content was measured using histological image analysis (Figure 3.4). All images shown in Figure 3.4. Quantification of hepatic lipid content and the corresponding ANOVA is shown in Figure 3.5. Among HF-fed groups, only in the RPC-fed mice was there a decreased amount of lipid accumulated within hepatic tissue, so that it is statistically indistinguishable from both the LF and HF animals ($p > 0.05$).

LF



HF

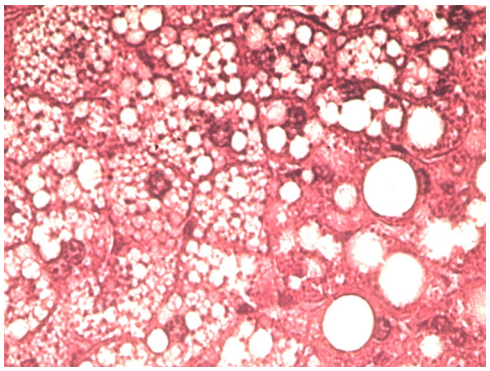


Figure 3.4: Representative hematoxylin and eosin stained liver sections from C57 Mice fed a low fat (LF), a high fat (HF) diet alone. Slides were observed under 40x magnification using a Nikon Eclipse 50i microscope (Nikon Corporation, Japan; Serial # 211880) fitted with an Infinity1-3C camera (Lumenera Corporation, Ottawa, ON, Canada; Serial #; 0186995). Scale 0.5'' = 50 μm

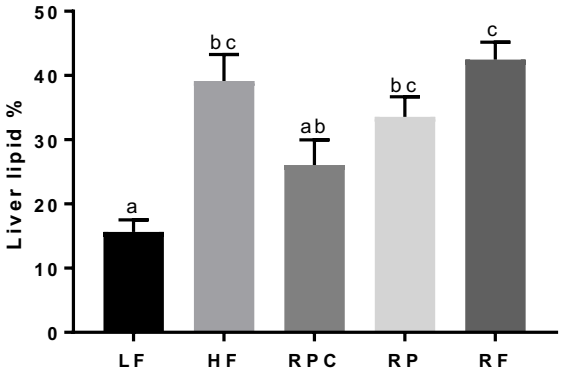


Figure 3.5 Hepatic fat content. Male C57 mice were fed a low fat (LF), a high fat (HF) diet alone, or HF plus red raspberry puree concentrate (RPC), enriched red raspberry polyphenol (RP), or enriched red raspberry fiber (RF). One way ANOVA indicated significant differences between diet groups ($p < 0.05$) Values that do not share a letter differ ($p < 0.05$).

3.10.5 Fasting (baseline) Glucose

Baseline glucose (t=0) was determined in LF, HF, RPC, RP, and RF-fed mice. Two measurements were taken for each animal, once for the glucose tolerance test and once for the insulin sensitivity test. Baseline glucose values are shown in Figure 3.6A. As seen, HF-fed mice had an increased blood glucose level compared to LF-fed mice ($p < 0.05$). The RPC group exhibited a reduction in baseline glucose values which was statistically indistinguishable from both the LF and HF groups.

Serum insulin concentration was determined for all treatment and control groups. Serum insulin level was greater in HF-fed vs. LF-fed mice ($p < 0.05$). Both the RPC and RF treatments showed a reduction in serum insulin levels with both treatments being statistically indistinguishable from LF controls ($p > 0.05$) and significantly lower than HF controls ($p < 0.05$). The RF treatment reduced plasma insulin concentration but the reduction did not reach statistical significance.

The insulin resistance test (HOMA-IR) showed differences between both controls with HF exhibiting a higher IR value than LF ($p < 0.05$). Both RPC and RP showed a reduced HOMA-IR values so that they were indistinguishable from LF (Figure 3.6C). Beta cell function (HOMA-%B) did not reveal any statistical differences in any of the treatments or control groups. However, a trend difference ($p < 0.10$), was observed between both controls with HF having an increased HOMA-%B.

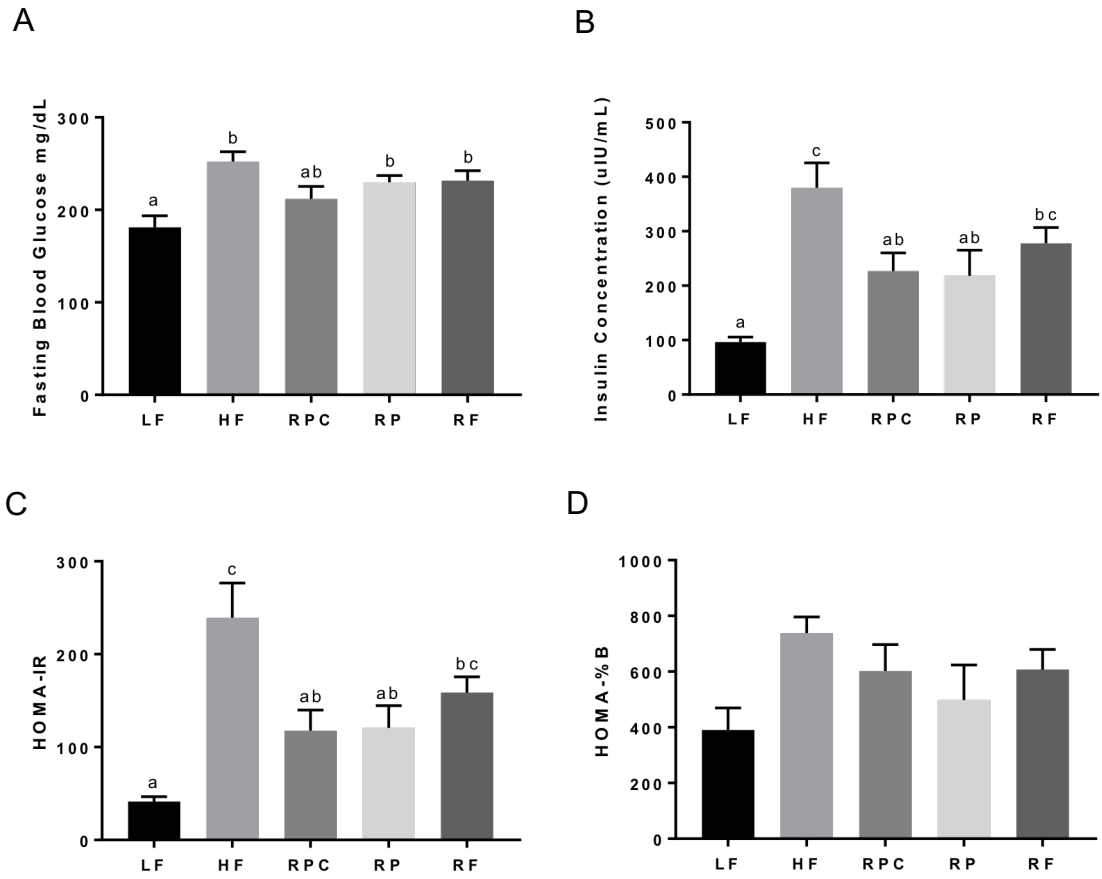


Figure 3.6: (A) Fasting glucose values, (B) insulin concentration, and (C) homeostatic model assessment of basal insulin resistance (HOMA-IR), and (D) beta cell function (HOMA-%B) for C57 male mice fed a low fat (LF), a high fat (HF) diet alone, or HF plus red raspberry puree concentrate (RPC), enriched red raspberry polyphenol (RP), or enriched red raspberry fiber (RF) during the beginning of week 10. Bars are mean $\pm$ SEM (LF (n=12) and HF control (n=11), otherwise n=8). Values sharing the same superscript are not significantly different from each other. One way ANOVA indicated significant differences between diet groups ($p < 0.05$) Values that do not share a letter differ ($p < 0.05$).

3.10.5.1 Glucose Tolerance and Insulin Sensitivity Test

Area under the curve (AUC) was calculated from a glucose tolerance test conducted on all animals during the ninth week of the study. The AUC was greater in HF- vs. LF-fed mice ($p < 0.05$). However, no statistical difference was seen between the HF control AUC and those of the RF and RP experimental treatments ($p > 0.05$). Mice fed RPC had low AUC values, such that they were statistically indistinguishable from both the LF and HF control animals (Figure 3.7.A)

The log of the slope between 15 and 30 minutes for the insulin tolerance test is shown in Figure 3.7B. Mice on the LF diet have a larger slope than those on the HF diet, though it was not statistically significant. Though no significance was shown, mice on the RP and RF diets also appear to have a larger slope than the HF mice ($p > 0.05$). A trending difference ($p < 0.10$) was observed between the HF and RF fed mice, with mice consuming the RF diet having a larger decrease in blood glucose between 15 and 30 minutes than animals consuming the HF diet.

Total AUC_1, between 0 and 90 minutes, showed no statistically significant differences between any of the treatment or control groups ($p > 0.05$). No statically significant differences were found in the AUC_1 associated with the 15 to 30-minute time frame either for any of the treatment or control groups. Results for total AUC_1 and AUC_1 from 15 to 30 minutes are not represented in ANOVA

figures below. No trending data was observed between control and treatment groups ($p > 0.10$)

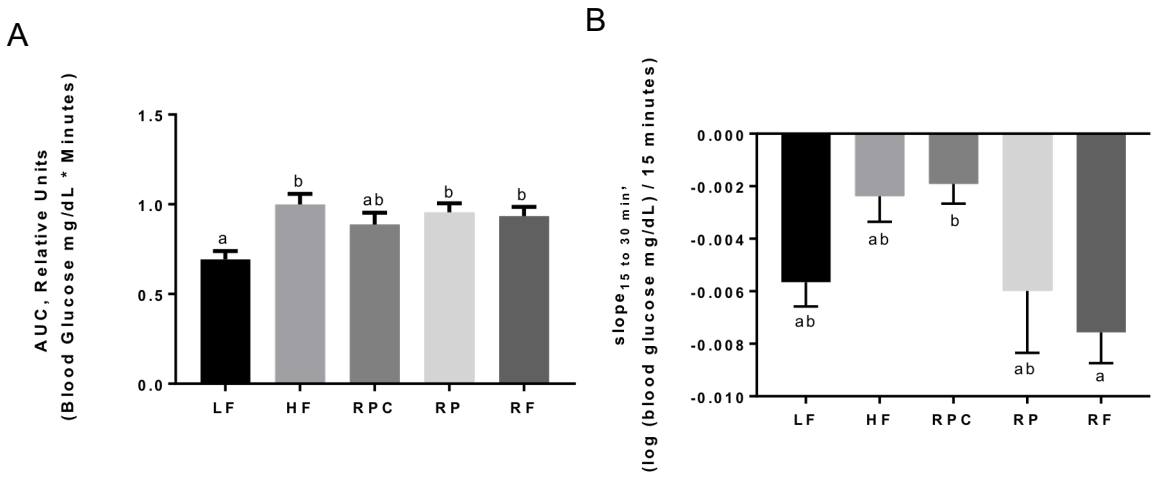


Figure 3.7: (A) Area under the curve generated from the glucose tolerance test, (B) slope generated from the insulin sensitivity test, for C57 male mice fed a low fat (LF), a high fat (HF) diet alone, or HF plus red raspberry puree concentrate (RPC), enriched red raspberry polyphenol (RP), or enriched red raspberry fiber (RF) during the beginning of week 9 and 10. Bars are mean $\pm$ SEM (LF (n=12) and HF (n=11), otherwise (n=8). Values sharing the same superscript are not significantly different from each other. One way ANOVA indicated significant differences between diet groups ($p < 0.05$) Values that do not share a letter differ ($p < 0.05$).

3.10.6 Monocyte Chemoattractant Protein-1

No statistically significant differences were observed in serum levels of monocyte chemoattractant protein-1 among controls or treatment groups ($p > 0.05$).

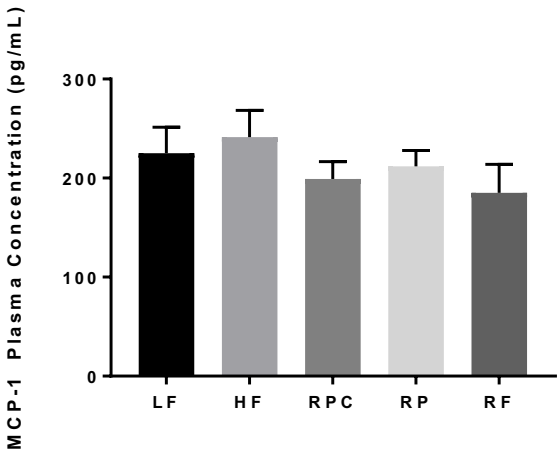


Figure 3.8: Serum concentration of monocyte chemoattractant protein 1 in C57 male mice fed a low fat (LF), a high fat (HF) diet alone, or HF plus red raspberry puree concentrate (RPC), enriched red raspberry polyphenol (RP), or enriched red raspberry fiber (RF) obtained through ELISA. Bars are mean ± SEM (LF and HF controls n=12, otherwise n=8).

3.10.7 Microbiome Ecology in the Cecum

The relative microbiome ecological differences were determined for each group. The seven most abundant bacterial species are reported in Figure 3.9 and Table 3.4. A decrease in the relative abundance of Bacteroidales S24-7 compared to LF was seen for all animals consuming a HF diet regardless of treatment ($p < 0.05$). Mice consuming the RPC had a decreased relative abundance of *Akkermansia muciniphila* and reflected the bacterial population of mice consuming the LF alone. The population of Bacteroidaceae *Bacteroides* was increased only in mice consuming the RF-diet ($p < 0.05$).

The rest of the bacteria found were of the order Clostridiales. A difference in DNA sequencing was determined for four groups of Clostridiales reported below (Figure 3.9 and Table 3.4). Three of these four Clostridiales populations are vague in name due to unknown information on the sequence within the Greengene database default in QIIME. To keep these three-reported bacterial populations separate, their respective color codes from Figure 3.9 will be reported alongside values or significant findings (grey, orange, and blue). No statistical difference was found in the relative abundance of Clostridiales *Ruminococcaceae* (orange) ($p > 0.05$) for any of the diet groups. However, differences in Clostridiales *Ruminococcaceae* (blue) populations were found. Mice consuming the LF diet had less blue Clostridiales *Ruminococcaceae* (blue) than HF, with RPC and RP exhibiting similar population frequencies to LF ($p < 0.05$). Clostridiales populations (grey) were significantly higher in mice

consuming the RPC and RF diets than mice consuming LF alone. The relative frequency of Clostridiales *Lachnospiraceae* was much higher in animals consuming the HF diet compared to LF. Mice consuming the RPC and RF diets had similar Clostridiales *Lachnospiraceae* populations to mice on the LF diet. However, the Clostridiales *Lachnospiraceae* population of mice on the RF diet was indistinguishable from both the LF and HF-fed mice.

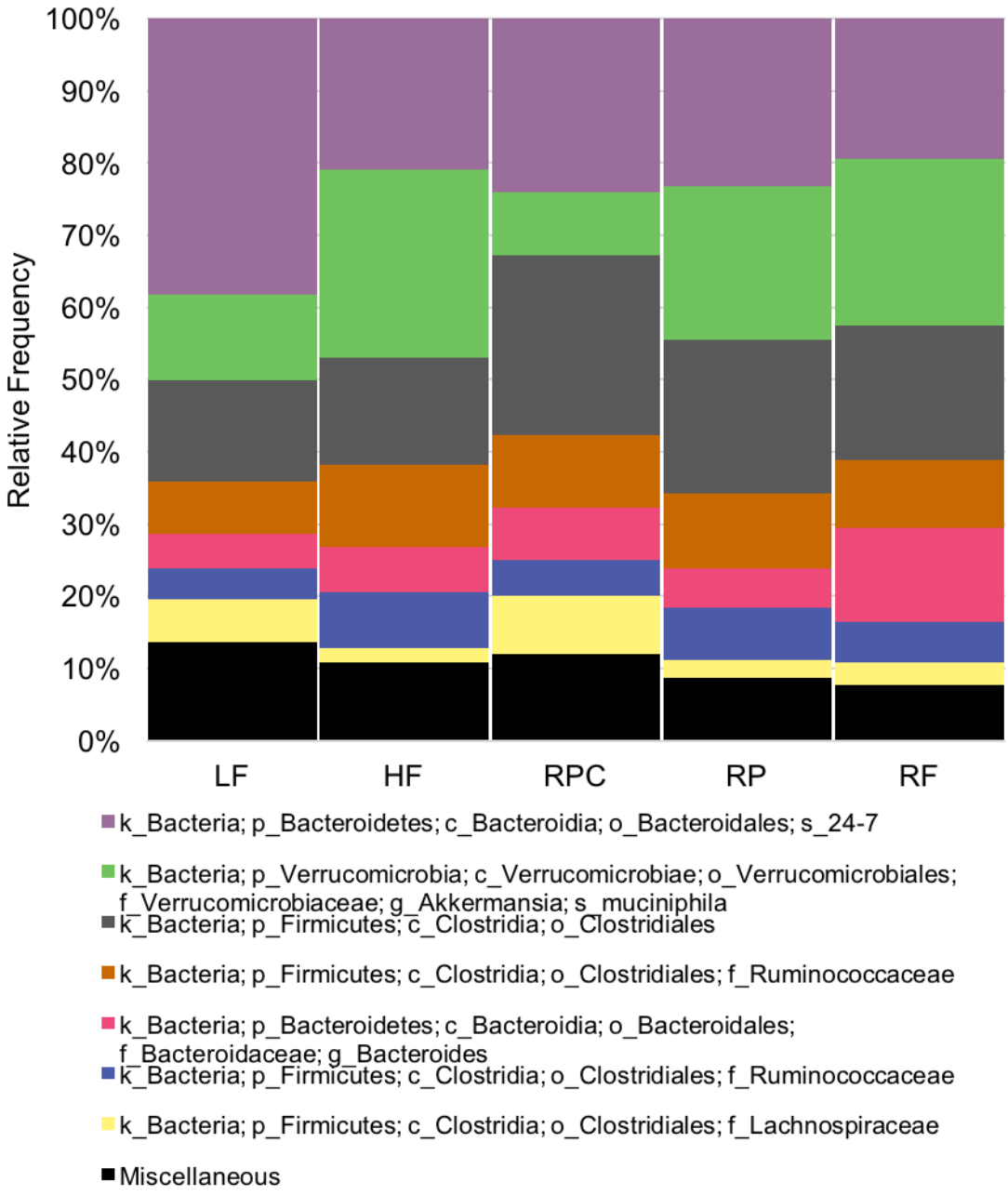


Figure 3.9: Cecal microbiome ecology from C57 male mice fed a low fat (LF), a high fat (HF) diet alone, or HF plus red raspberry puree concentrate (RPC), enriched red raspberry polyphenol (RP), or enriched red raspberry fiber (RF).

Table 3.4 Ceca microbiome in mice fed LF, HF, RPC, RP and RF*

	Diet Groups				
Bacteria Type	LF	HF	RPC	RP	RF
o_Bacteroidales f_S24-7	38.2±3.2^{b}	20.9±1.2^{a}	24.1±2.3^{a}	23.3±2.0^{a}	19.5±0.8^{a}
g_*Akkermansia*; s_*muciniphila*	11.9±1.6^{a}	26.1±2.5^{b}	8.7±1.6^{a}	21.3±2.7^{b}	23.0±1.1^{b}
o_Clostridiales[1]	14.1±2.0^{a}	14.8±0.8^{ab}	25.0±2.1^{c}	21.3±1.6^{bc}	18.7±1.3^{abc}
o_Clostridiales; f_*Ruminococcaceae*[2]	7.3±1.3	11.4±1.3	10.1±1.5	10.4±1.4	9.3±0.9
f_*Bacteroidaceae*; g_*Bacteroides*	4.8±0.6^{a}	6.3±0.6^{a}	7.3±0.4^{a}	5.3±0.7^{a}	13.1±1.1^{b}
o_Clostridiales; f_*Ruminococcaceae*[3]	4.3±0.5^{a}	7.7±0.7^{c}	4.8±0.8^{ab}	7.3±0.6^{bc}	5.6±0.3^{abc}
o_Clostridiales; f_*Lachnospiraceae*	5.9±1.2^{bc}	2.0±0.2^{a}	8.1±0.5^{c}	2.4±.2^{a}	3.2±0.4^{ab}

* One way ANOVA indicated significant differences between diet groups ($p < 0.05$) Values that do not share a letter differ ($p < 0.05$).
[1] DNA sequencing for the grey Clostridiales in Figure 3.9
[2] DNA sequencing for the orange Clostridiales *Ruminococcaceae* in Figure 3.9
[3] DNA sequencing for the blue Clostridiales *Ruminococcaceae* in Figure 3.9

3.11 Discussion

Study I, the Shay Laboratory confirmed the health benefits observed with the supplementation of the equivalent of four servings a day of RPC in the HF diet. Study II, it was shown the healthful effect observed in Study I could be attained at the lower level of one serving per day. In the present study, Study III, as outlined in this thesis, we provided information on how fiber and polyphenol fractions of RPC impact health. Overall, we conclude that that the health effects observed with red raspberry consumption in HF-fed mice is most likely provided by both the fiber and polyphenol fraction.

The whole food, RPC, alleviated more of the metabolic parameters associated with metabolic syndrome than either of the enriched fractions (Table 3.5). Beneficial effects of whole red raspberry products was seen even though the energy intake of mice on RPC was significantly higher than either the LF or HF-fed mice. Care was taken to account for spillage in order to accurately reflect consumption, however, the RPC pellets were indeed softer than any of the other diet pellets, which may explain the increase in consumption seen within the RPC-mice. Unrecovered fine spillage on the bottom of the cage may account some of the high intake seen in the RPC treatment groups. Another possible explanation for the increase in intake is enhanced consumption of the softer feed, due to increased access from feed which fell to the bottom of the cage.

Mice consuming the RPC and RP diets had a decreased food efficiency, which corresponds to a lower conversion rate of ingested energy in to body mass. This improvement was not significantly reflected in the final body weight or net weight gain of these animals over the ten-week study. Based on our findings for food efficiency, more significant weight differences would be observed between HF-fed mice and the two treatment groups (RPC and RP) if the mice had consumed these diets for a longer duration than ten-weeks.

Typically, as mice gain weight their livers increase proportionally, thus the organ to body weight ratios should remain unchanged. Only the RPC group had significantly lower liver weights at necropsy, signifying a benefit related to lower liver fat content. This effect of the whole food on the liver was not seen in either the polyphenol or fiber fraction. The findings for hepatic fat content were consistent between photomicrograph assessment and liver weight measurements. During both of these tests RPC showed a remediation of hepatic fat accumulation and liver weight gain, so that values were indistinguishable from LF. This indicates a reduction of liver lipid percentage within the RPC-fed mice. The liver specific health benefits of RPC might only be achievable through a symbiotic relationship between the two food fractions or possibly an unknown and unexplored component of RPC.

Organ to body weight ratios were affected differently depending on the treatment mice consumed, with RPC exhibiting beneficial effects on all ratios observed. All

three diets improved the adipose to body weight ratio so that they were indistinguishable from the LF fed mice.

The kidneys do not accumulate fat as mice gain weight and therefore organ to body weight ratio decrease as the mice gain weight. Only RPC improved kidney to body weight ratio and liver to body weight ratio so that they were indistinguishable from mice fed LF-alone. Neither of the food-fraction treatments showed beneficial improvement to either kidney or liver body weight ratios.

Along with organ to body weight ratios, only the RPC group was seen to significantly lower baseline glucose values in mice fed a high-fat high-sugar western-style diet to a level similar to LF. Improvements to baseline glucose, reduces health risks associated with continual high levels of glucose in the blood. One such risk is an increase in A1C which could cause aggregation of platelets and potential vascular blockage.

Both the RPC and RP fed mice had serum insulin concentrations which were statistically indistinguishable from the LF-fed mice. This indicates less pancreatic beta cell stress for mice fed these two diets. This data is supported by the trend observed in HOMA-%B, which indicates an increase in beta cell function for mice consuming the LF vs. HF diet. An enhanced secretion is often observed in the early stages of diabetes as the beta cells hypertrophy and overproduce insulin in order to keep up with the increasing demand, The HOMA-IR also reflected these

findings, indicating less resistance within tissues to bind and respond to insulin. Less insulin needs to be produced by the beta cells when insulin sensitive tissues are able to used insulin effectively to cause an uptake of excess glucose from the blood. A reduction in insulin production and insulin resistance indicates better ability of these mice to maintain glucose homeostasis.

Both the RP and RF treatments showed an improvement in the ability to reduce glucose levels significantly between 15 and 30 minutes after administration of insulin during the insulin sensitivity test.

Introducing fiber-rich foods into the diet will decrease the colonic pH due to an increase in starch fermentation by intestinal bacteria. Prebiotic fibers enter into the ileum and colon where they are broken down into monosaccharide units which are fermented by various microorganisms into short-chain fatty acids such as propionic acid, acetic acid, and butyric acid (Stipanuk and Caudill, 2013). The resulting pH change impacts the ecology of the microbiota favoring bacteria tolerant to low pH environments.

Diet significantly influenced the microbiome ecology of all animals and the seven most abundant bacteria are listed in Figure 3.9 and Table 3.4 above. The whole food RPC was the best treatment for remediating the ecological shifts observed within the cecum of mice consuming a HF diet. Mice consuming RPC had bacterial populations which closely resembled the microbiome of mice on the LF

diet. The next best diet for remediating the bacterial shifts observed with HF-food consumption was RF, with RP only showing positive effects on one bacterial population. Enriching the diet with RF was more effective than RP at remediating potentially detrimental bacterial shifts in the microbiome of C57 mice. The RF diet encouraged the growth of beneficial bacteria and hindered the bacterial shifts associated with poor diet choices.

No positive dietary effect was observed in increasing Bacteroidales S24-7 levels to that in the LF fed mice. Higher levels of *Akkermansia* were observed in animals fed the HF, RP and RF diets. A decrease in Clostridiales, shown in grey (Figure 3.8), was observed in mice consuming the RF compared to other treatment groups. The relative frequency of *Bacteroides* was increased in RF well above all other treatment or control groups. *Ruminococcaceae,* (shown in blue), was also significantly decreased in the RPC and RF treatments, so that they were indistinguishable in frequency to the mice consuming LF-alone. *Lachnospiraceae* populations were increased in the cecum of mice consuming the LF compared to HF diets. Treatment with RPC and RF remediated *Lachnospiraceae* population loss observed with HF consumption.

Currently, little is known about the activity and function of Bacteroidales S24-7 within the mice microbiome. However, many studies have reported observing high numbers of these bacteria within the gut of diabetes-sensitive mice fed a high-fat diet (Ormerod, et al., 2016). More is known about the effects of

Akkermansia on murine health. Studies have observed a reduction in obesity related inflammation and the prevalence of diabetes in animals with higher *Akkermansia* populations (Everard et al., 2013). We see different data, our study shows an increase in the *Akkermansia* populations with high-fat consumption

One possible explanation for our different microbiological findings is that our animals were fasted before tissue collection. A five hour fast may have allowed for slight microbiological shifts due to lack of nutrients. Cyclical changes occur in the gut microbiome during natural feeding and fasting rhythms. Feeding patterns and time of harvest should be considered when comparing the microbiomes of two different studies (Zarrinpar et al., 2014).

Clostridiales are often gram-positive bacteria which can form spores. Some of the best-known Clostridium species are *Clostridium perfringens* and *Clostridium botulinum*, both of which can cause foodborne illness. Some of the Clostridium class, however, have a more positive impact on health. *Ruminococcaceae* and *Lachnospiraceae* are two families of the class Clostridium which are obligate anaerobes. In humans, a low abundance of these two bacterial families (*Ruminococcaceae* and *Lachnospiraceae*) is associated with the condition known as Crohn's disease (Morgan et al., 2012). Other studies have shown a high abundance of *Lachnospiraceae* populations are associated with protection against colon cancer (Wang et al., 2012)

A summary of all findings is provided in Table 3.5.

Table 3.5 Highlights of significant findings for raspberry treatment groups

	Diet Groups		
Statistically Indistinguishable from LF	RPC	RP	RF
Energy Intake (MJoules / wk)	N	Y	N
Food Efficiency (g / Joule)	Y	Y	N
Liver Weight (g)	Y	N	N
Liver to Body Weight (%)	Y	N	N
Kidney to Body Weight (%)	Y	N	N
Adipose to Body Weight (%)	Y	Y	Y
Liver Lipid (%)	Y	N	N
Averaged Fasting Glucose (mg/dL)	Y	N	N
Insulin Concentration (μIU/mL)	Y	Y	N
HOMA-IR	Y	Y	N
AUC (mg/dL * minutes)	Y	N	N
$Slope_{15\text{-}30}$ (log (mg/dL) / 15 minutes)	Y	Y	Y
Akkermansia Relative Frequency (%)	Y	N	N
Clostridiales Relative Frequency (%)	N	N	Y
Bacteroides Relative Frequency (%)	Y	Y	N
Ruminococcaceae Relative Frequency (%)	Y	N	Y
Lachnospiraceae Relative Frequency (%)	Y	N	Y

CHAPTER 4
SUMMARY AND FUTURE DIRECTIONS

4.1 Summary

In this study, the metabolic improvements and changes in phenotype and two serum markers (MCP-1 and insulin) were measured to see if the intake of RF or RP had a significant impact. Mice fed the HF diet became obese and developed the hallmark phenotypes of metabolic syndrome. The HF-fed mice had higher baseline glucose values, higher insulin levels, and a reduced ability to handle glucose or insulin challenges. The diets with the enriched fiber and polyphenols did not perform as well on remediating metabolic syndrome as the whole-food RPC group. However, a significant reduction in adipose to body weight ratio was observed for both RP and RF diet groups. The RP-fed mice also exhibited a significant reduction in food efficiency and HOMA-IR, comparable in value to LF-fed mice. Red raspberry enriched fiber (RF) mice showed a reduction in serum insulin concentration, comparable to LF.

Food phytochemicals, or their metabolites, are absorbed by the body and delivered to the liver and peripheral tissues. After absorption into the cells, phytochemicals may interact with nuclear hormone receptors and other regulatory factors to modulate carbohydrate and lipid metabolism. In the future, we would like to explore the nuclear hormone effects of both the polyphenol- and fiber-enriched red raspberry products. This would give us a clearer understanding of exactly how these two fractions impact different cellular mechanisms to produce the phenotypic results observed in the mouse study discussed in this thesis.

This dietary supplement study shows that different healthful fractions of red raspberry impact health parameters in different ways. When consumed together, as RPC, these two fractions were better able to remediate the detrimental health effects of HF diet consumption.

4.2 Future Directions

Due to the nature of our findings, it would be interesting to conduct a study looking at the delivery of polyphenols into the body. It became apparent in our study that polyphenol exposure alone is not as beneficial as consuming this fraction within the whole food matrix. To better understand this phenomenon, it would be interesting to look at different slow delivery techniques. It is possible that the fiber plays a role in slowing the absorption of polyphenolic compounds, to allow these compounds to be introduced more gradually into the enterocyte.

The effects of fiber in the microbiome should also be an exciting future study. Metabolism of the polyphenolic compounds are somewhat dependent on the composition of the microbiota in the gut. Increased consumption of fiber-rich foods allows *Bifidobacteria* and *lactobacilli* to proliferate more readily. As *Bifidobacteria* increase in number, the demographics of microorganisms in the gut change to accommodate. *Bifidobacteria* are strongly influenced by the presence of polyphenolic compounds and flavanols are active inhibitors of gram positive bacteria (Gwiazdowska et al., 2015). Both *Bifidobacteria* and *lactobacilli*

can exert a hypocholesterolemic effect by increasing bile acid deconjugation (Kaczmarczyk et al. 2012).

One limitation of the study design is of the fiber extraction technique, which was only able to isolate insoluble fiber. Soluble fiber was probably dissolved in the pancreatin digest, which was used to rid the RPC of sugar, fat and protein. This limitation likely caused a reduction in the health effects observed with mice consuming the RF diet. Soluble and insoluble fiber impact the physiology of the gut differently.

Another limitation of this study is the limited knowledge available about the bacteria that colonize the mice gut microbiome. Information related to strains of murine gut bacteria is not presently abundant, and much is still unknown about the interaction between these bacteria and their host's physiology. These major limitations can hinder the investigative efforts behinds the exploration of how diet affects bacterial populations and the subsequent physiological effects.

Future studies to observe the synergy between red raspberry polyphenols and red raspberry fiber are warranted. Secondary metabolites of polyphenols are affected by the ecology of the microbiome (Marín et al., 2015). Inclusion of soluble and insoluble fibers into the purified feed will increase the growth of beneficial bacterial populations (*lactobacilli* and *bifidobacteria*) and increase the diversity of bacterial populations (Slavin, 2013) (Kaczmarczyk et al., 2012).

Increased utilization of polyphenols by microbial populations would be expected to occur. Care must be taken in studies observing the effects of polyphenols on health to allow their best action.

A clinical trial could be undertaken evaluating the effects of red raspberry intake on pre-diabetic obese individuals. This study will allow for a more direct measurement of health benefits observed in human subjects.

Studies like ours will add to the available literature and allow for an increase in knowledge about the diversity and function of different microbiota members and how diet affects colonization. Hopefully in time, the persistent investigative efforts of the microbiome will lead to a universal understanding of how different bacterial strains affect metabolic function. An increased knowledge of how diet affects these bacterial populations will lead to meaningful health breakthroughs related to diet. Our study adds to the available knowledge and helps in addressing the obesity epidemic facing America today.

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Appendix

Preparation of buffers

pH 4 buffer:

Solution A glacial acetic acid 0.2M

Solution B potassium acetate 0.2M

pH 7 buffer:

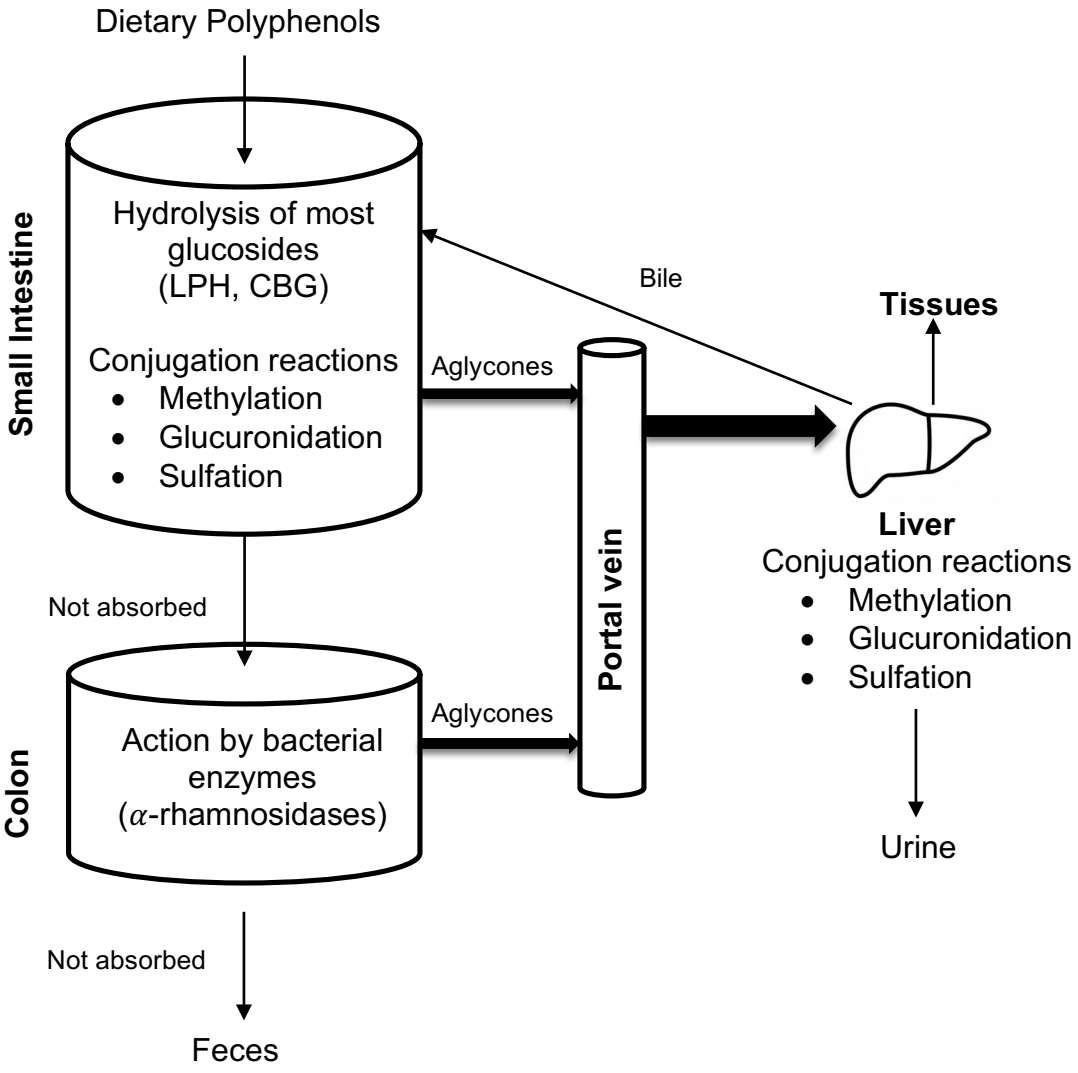
Solution A potassium phosphate 0.2M

Solution B dipotassium phosphate 0.2M

Calculations for HOMA-IR and HOMA-%B:

$$\text{HOMA-IR} = \frac{\text{FPI} \times \text{FPG}}{22.5 \times 18}$$

$$\text{HOMA-\%B} = \frac{\left(20 \times \text{FPI}\right)}{\frac{\text{FPG}}{18} - 3.5}$$



(Martin et al., 2015)

Figure A1: Absorption and metabolism routes for dietary polyphenols and their derivatives in humans

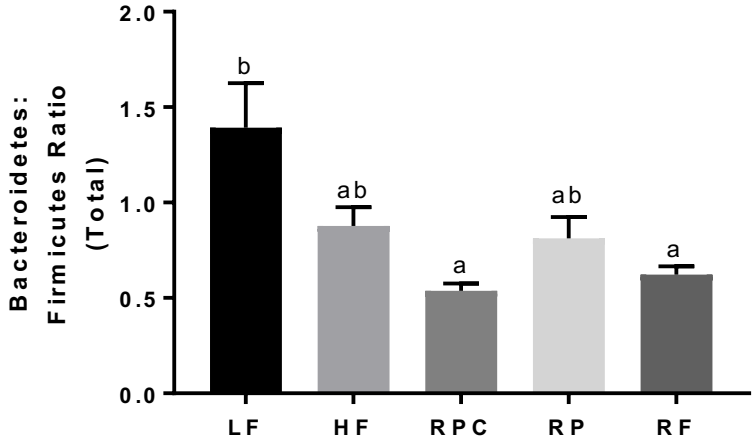


Figure A2: Bacteroidetes to firmicutes ratio (total)

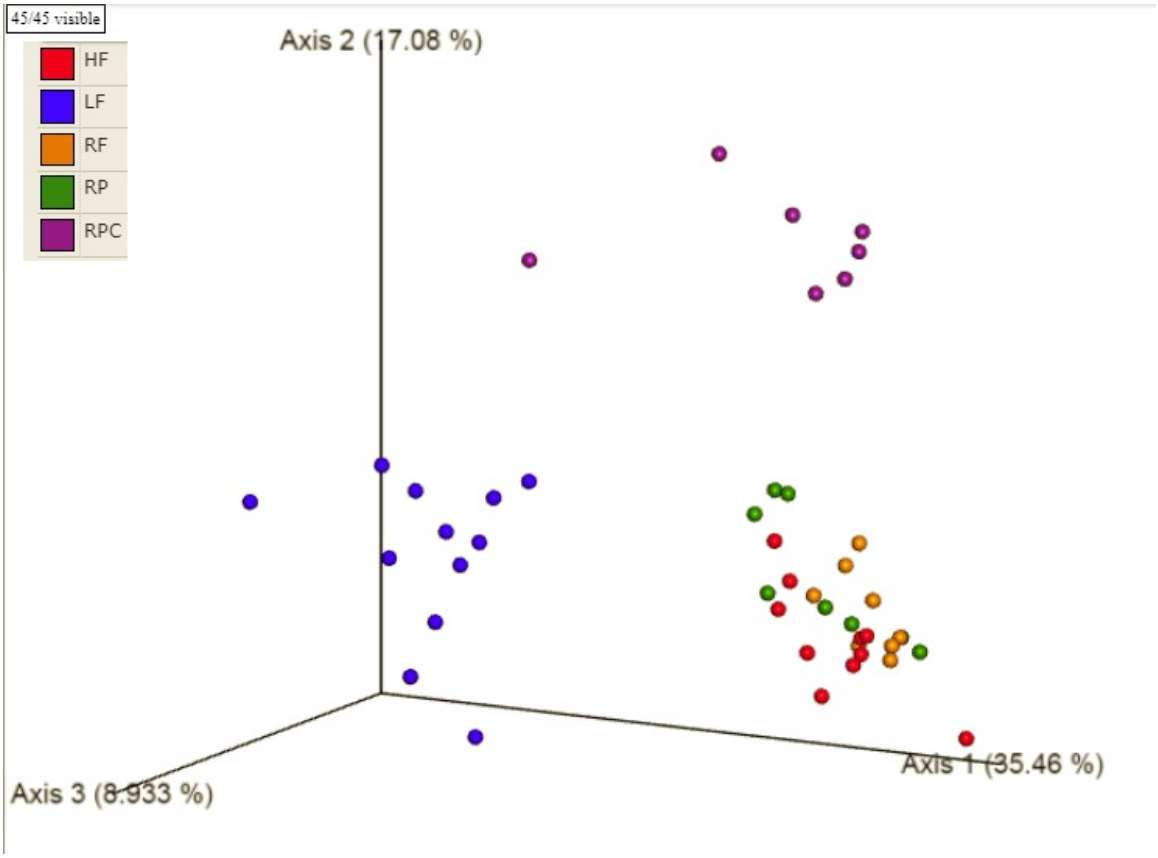


Figure A3: Bray-Curtis PCA plot in C57 male mice fed a low fat (LF), a high fat (HF) diet alone, or HF plus red raspberry puree concentrate (RPC), enriched red raspberry polyphenol (RP), or enriched red raspberry fiber (RF).

Calculations for fiber content of HF, RPC, RF and RP:

HF: 50g cellulose / 866.6 g total dry wt. = 5.8% fiber (dry)

RPC: (42.5 cellulose) + (375g wet RPC x 0.03 % fiber) =53.8 g fiber in RPC

53.8g fiber / 1145.15g wet RPC diet = 4.7% fiber wet w/w

53.8g fiber / 869.9g dry RPC diet = 6.2% fiber dry w/w

RP: 50g cellulose / 880.8g RP dry = 5.6% fiber from cellulose (dry)

50g cellulose / 894.95 g RP wet 5.7% fiber from cellulose (wet)

RF: 50g cellulose / 925.65 dry RF diet = 5.4% fiber dry

6.93% fiber (by assay) – 5.4% fiber (diet w/o RP) = 1.53% fiber RF

14.2 g raspberry fiber / 28.3g RF used = 50.2% fiber in product

Table A1: Fiber content of the diets

Measurement	HF	RPC	RP	RF
% fiber (dry bases)	5.8	6.2*	5.6	6.93

*Both wet and dry fiber percentages were reported for the RPC diet because this diet was not completely dried down. The fiber content would more accurately be represented by a number falling between these two values.

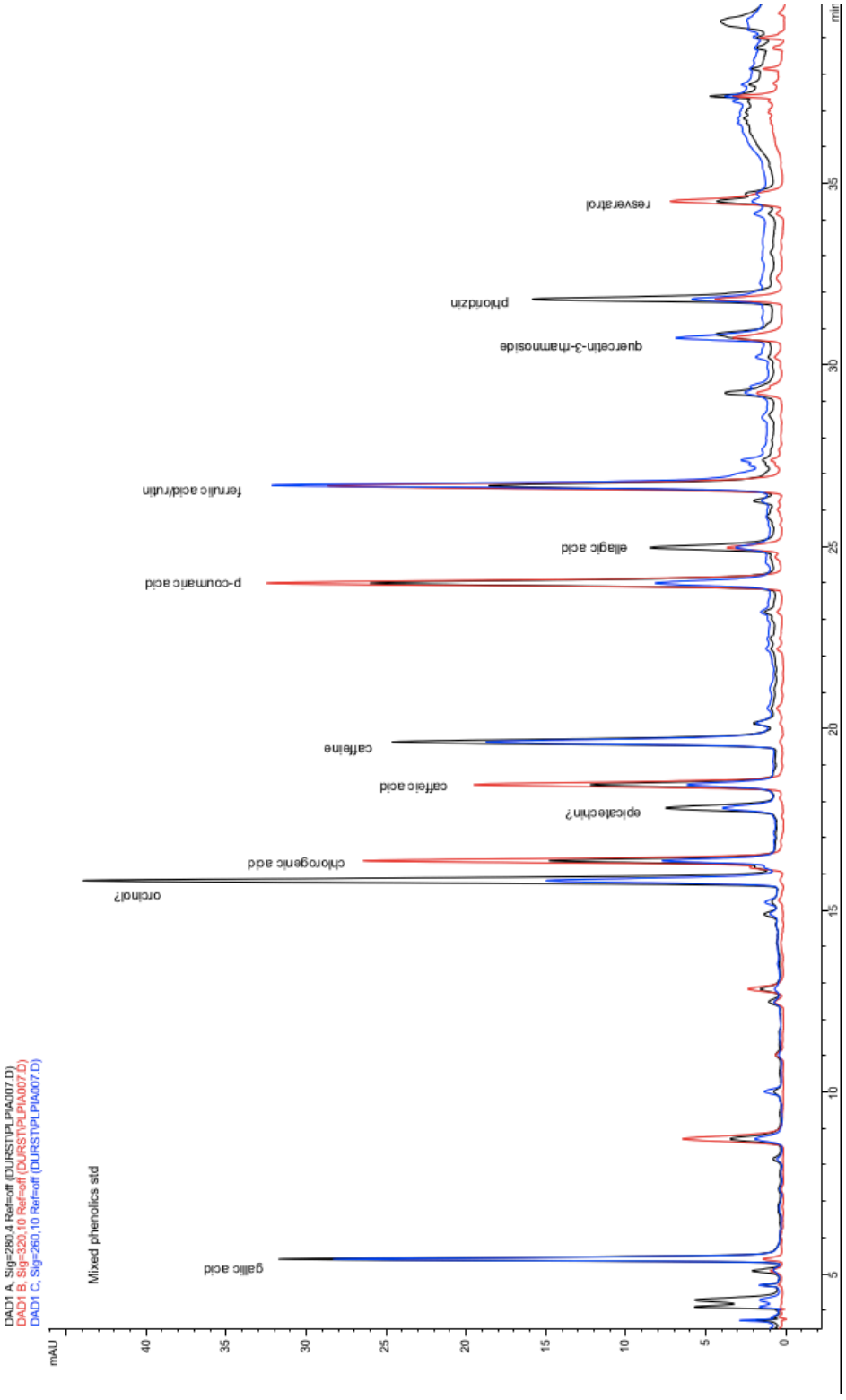


Figure A4: HPLC phenol standards

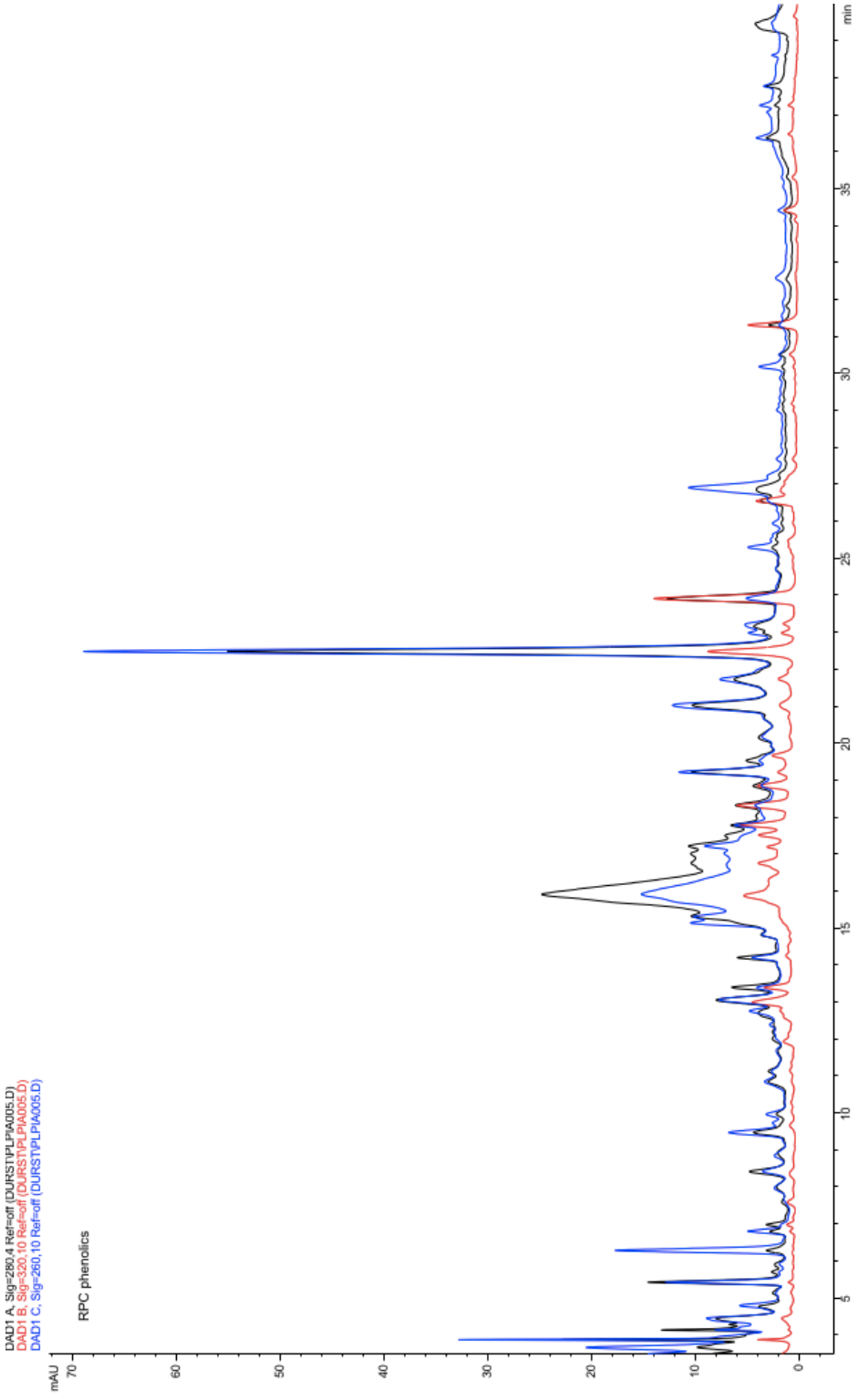


Figure A5: RPC HPLC phenol analysis

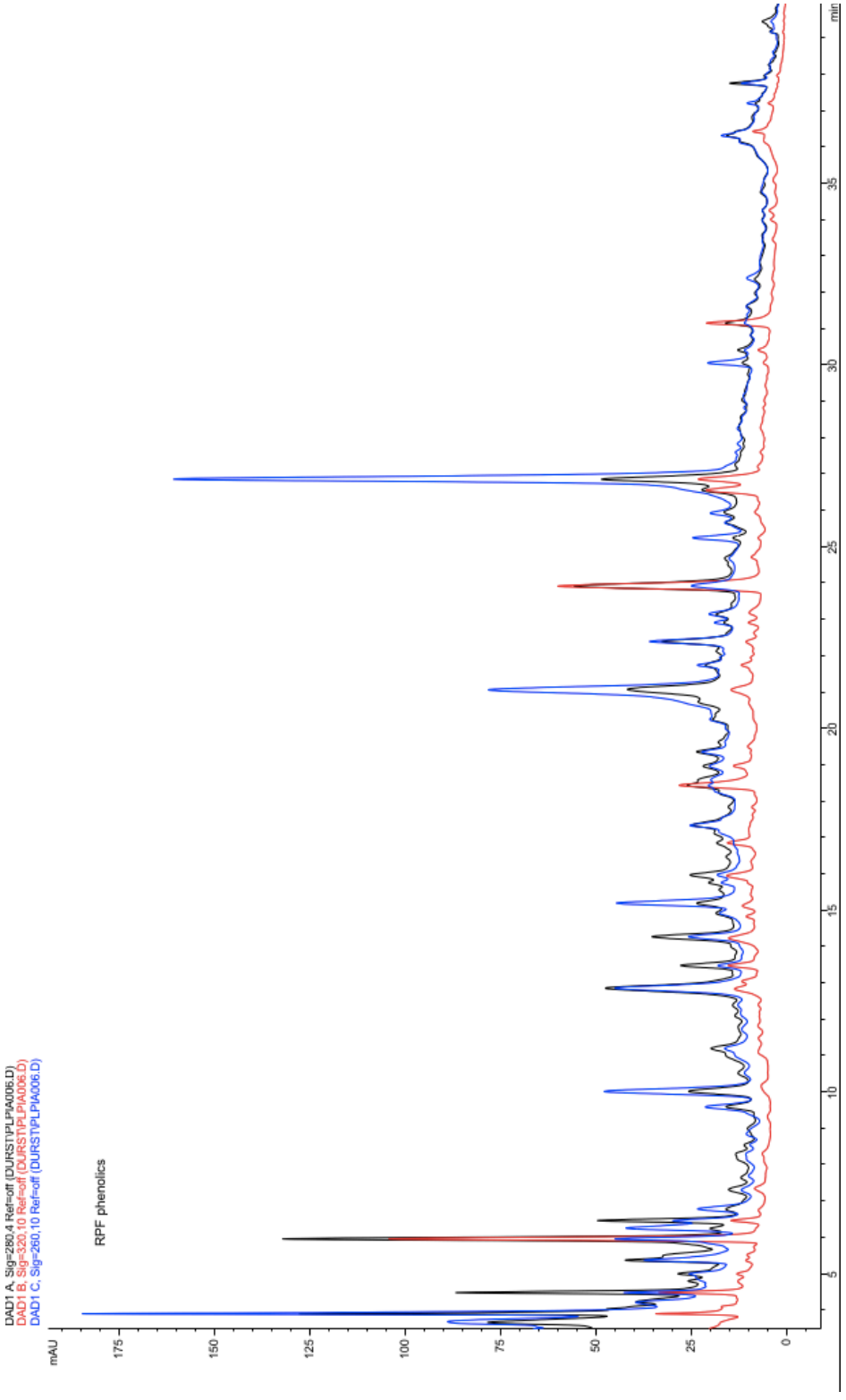


Figure A6: RP HPLC phenol analysis

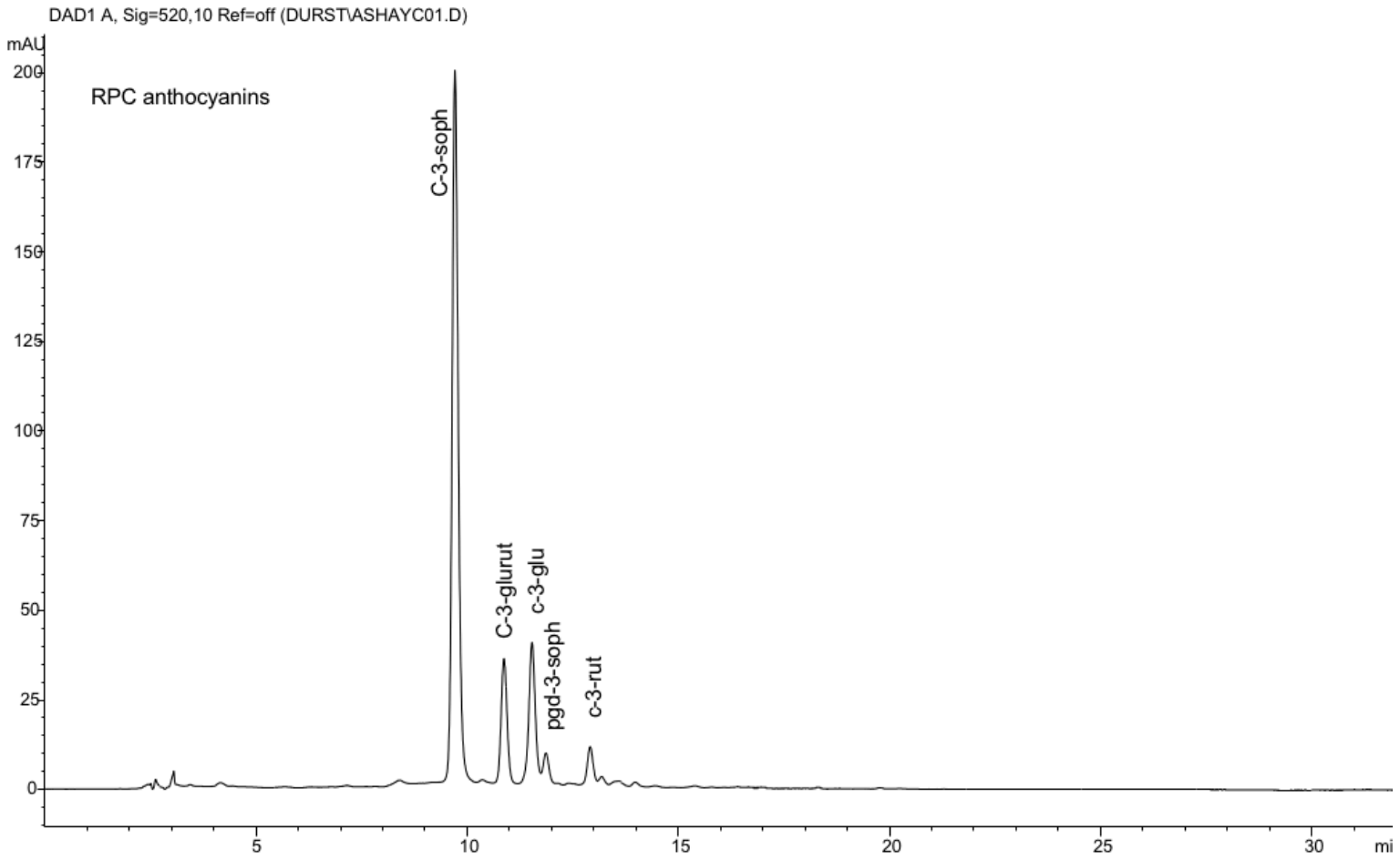


Figure A7: RPC HPLC anthocyanin analysis

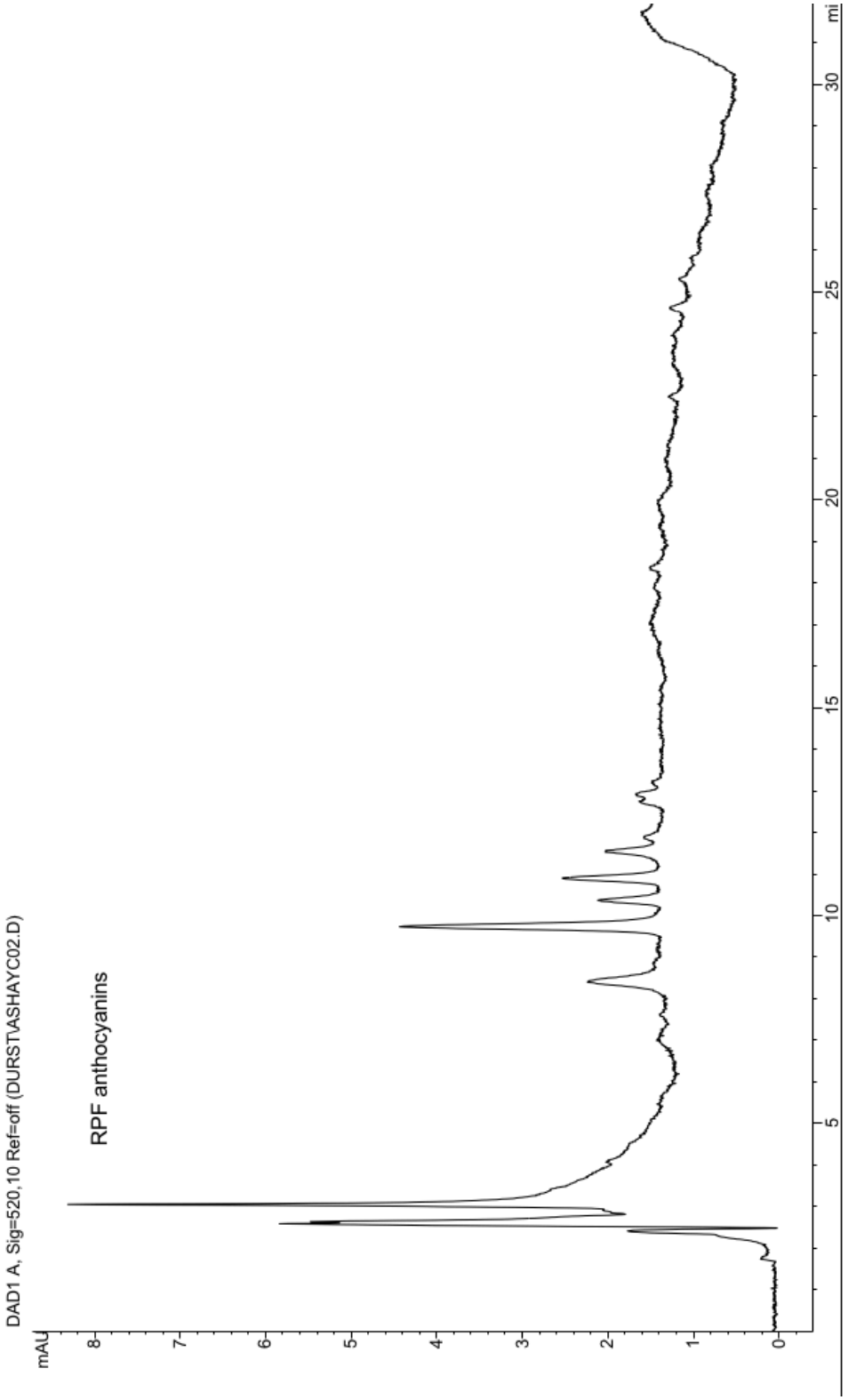


Figure A8: RP HPLC anthocyanin analysis

RED RASPBERRY PUREE CONCENTRATE

28 BRIX Nutritional Information

milnefruit.com

28 BRIX

Analysis Name	Results	Limit of Quantitation	Units
Moisture (Vacuum Only)	73.4	0.01	% by wt.
Total Carbohydrate	22.2	0.1	% by wt.
Estimated Caloric Value	106	2	Cal/100g
The Estimated Caloric Value has been calculated according to the deÀnition found in the nutrition labeling regulations printed on January 6, 1993 in CFR Part 101.9, where: Calories/100g = 4 (% protein) + 9 (% fat) + 4 (% carbs)			
Vitamin C	32	1	mg/100g
Total Dietary Fiber	2.0	0.1	% by wt.
Protein (modiÀed Dumas)	2.3	0.1	% by wt.
The % protein was calculated from % nitrogen using a factor of 6.25.			
Fat (Acid Hydrolysis)	0.9	0.1	% by wt.
Beta Carotene	80	20	IU/100g
Lycopene	0	0.5	mg/100g
Total Sugar	16	1	% by wt.
Fructose	8.0	0.1	% by wt.
Dextrose	7.7	0.1	% by wt.
Sucrose	0.2	0.2	% by wt.
Maltose	< 0.3	0.3	% by wt.
Lactose	< 0.5	0.5	% by wt.
The total sugar result does not include trisaccharides or tetrasaccharides.			
Ash	1.29	0.01	% by wt.
Calcium	38.3	0.5	mg/100g
Sodium	23.9	0.2	mg/100g

This nutritional information does not represent lot speciÀc testing and is not to be considered a speciÀcation.

Milne Est. 1956

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Updated 9.25.17

RED RASPBERRY PUREE CONCENTRATE

28 BRIX Technical Data Sheet

Product Summary

- Processed from freshly harvested and frozen red raspberries
- Contains no artificial ingredients or fillers
- This product complies with the Federal Juice HACCP Regulation 21 CFR Part 120
- This product contains lead and possibly other chemicals regulated by the California Proposition 65
- This product complies with the Federal Insecticide, Fungicide and Rodenticide Act (7 U.S.C. §136 et seq.)

Analytical & Sensory

Brix:	28° +/- 0.5 or as specified
pH:	2.9 – 3.8; varies with crop year
Acidity:	3.2 – 4.5% w/w as citric; varies with crop year
Appearance:	Typical for red raspberry puree concentrate; bright red color
Flavor/Aroma:	Typical of red raspberries; free from fermented, metallic or other objectionable flavors or odors

Microbiological

Total Plate Count:	<2000/g
Yeast & Mold:	<100/g
Coliform:	<10/g

Packaging & Storing

- Steel open head drums with two 4 mil poly liners, 466 lb fill
- Plastic pails, 47 lb fill
- Store at less than 0°F, shelf life at least 3 years from date of manufacture, in unopened containers
- Ship via refrigerated vans frozen

Each pail/drum is labeled with:

- Lot number
- Product ID
- Julian date of production
- Crop year
- Brix
- Essence designator
- Fill (gallons/net weight)
- Customer designator

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Updated 9.25.17

Product Specification

Alfa Aesar

J62162 Pancreatin, porcine pancreas

Product Number:	J62162
CAS number:	8049-47-6
MDL number:	MFCD00131789

Product Specification

Assay (unspecified):	Amylase activity: Minimum 25 USP u/mg
Assay (unspecified):	Lipase activity: Minimum 2.0 USP u/mg
Assay (unspecified):	Protease activity: Minimum 25 USP u/mg
Comment:	Source: Porcine pancreas

Date of Print:	April 7, 2017
Version:	1

Product Specifications are subject to amendment and may change over time.

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