The Influence of Dietary Flavanol Mean Degrees of Polymerization on Sensory Preference Trends and the Metabolic Syndrome

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ABSTRACT

According to the Centers for Disease Control, roughly 9.4% of the US population is diabetic, and at least 35% of the US population has metabolic syndrome. These diseases are associated with increased mortality risk, reduced quality of life, and altered taste perception of foods. With increased occurrence of these metabolic diseases, there is a greater need for research oriented towards using lifestyle modifications to combat illness. A relationship between flavanol consumption, health benefits, and taste perception has been well documented. Dietary flavanols are secondary plant metabolites that exist naturally in a wide array of polymerization states. The mechanisms behind the protective effects of flavanols are not entirely understood, particularly when considering how the mean degrees of polymerization (mDP), or average compound size, impacts the health benefits. Moreover, it is known that flavanol mDP influences the sensory attributes of flavanol-rich foods including bitterness and astringency. It is known that obesity and sensitivity to bitterness both influence perception of certain taste attributes such as sweetness and bitterness. The influence of these bitter and astringent sensations determined by flavanol mDP on consumer preferences for flavanol-rich products remains unknown. These influences on preference pose potential barriers to consumption, resulting in the loss of health benefits. The objectives of the research detailed here were i) to determine the effect of dietary consumption of small to medium-sized flavanols on markers of metabolic syndrome that were brought on by diet-induced obesity, ii) to determine how flavanol mDP influences the consumer perception and liking of flavanol-rich, wine-like products based on differences in consumer phenotype, and iii) to explore the potential to manipulate mDP of wine using traditional winemaking techniques. By way of an *in vivo* mouse model, it was observed that regardless of mDP, flavanols delivered at low dose, as part of a high-fat diet, reduced adipose-derived inflammatory cytokine production but did not prevent associated weight and fat gain. This suggests that small to medium sized flavanols may, at low dose, delay the onset of the pro-inflammatory state, which could ultimately protect against metabolic derangements associated with obesity and diabetes. Regarding the consumer acceptance of wine-like products made from flavanols of different mDP, and therefore different in bitterness and astringency intensity, it was observed in a consumer panel (n = 102) that when segmenting the panelists by body fat % and BMI classification, increased adiposity was associated with decreased ability to differentiate wine samples made with flavanols of different mDP. Moreover, differences in liking and ability to differentiate bitterness and astringency intensities were not as pronounced when segmenting the panelists based on bitterness sensitivity. This suggests that obesity may impact preference for flavanol-rich foods more so than sensitivity to flavor attributes associated with these products. Finally, in an exploratory effort to manipulate mDP of red and rosé wines using traditional winemaking techniques, no differences in mDP were observed in young wines, but significant differences in flavanol concentration were

detected. It is hypothesized that aging of these wines could lead to greater differences in mDP, especially for those that had a high flavanol concentration at baseline. Future work will continue to build off these studies so that flavanol-rich products such as red wine can be optimized for health benefits and consumer acceptability of dietary polyphenols.

The Influence of Dietary Flavanol Mean Degrees of Polymerization on Sensory Preference Trends and the Metabolic Syndrome

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GENERAL AUDIENCE ABSTRACT

According to the Centers for Disease Control, roughly 9.4% of the US population is diabetic, and at least 35% of the US population has metabolic syndrome. These diseases are associated with increased mortality risk, reduced quality of life, and altered taste perception for certain food types. With increased occurrence of these metabolic diseases, there is a greater need for research oriented towards using lifestyle modifications to combat illness. Dietary flavanols, which are potent antioxidants derived from plants, are being explored for their ability to mitigate chronic disease. They exist naturally in a wide variety of sizes and structures depending on plant of origin, growing conditions, and food processing conditions. It is believed that the size of these compounds impacts their health effects and influences their taste profile; smaller compounds are more bitter while larger compounds are more astringent. The purpose of this research was to determine the effect of flavanol supplementation on markers of the metabolic syndrome and how differences in taste due to differences in flavanol size influence consumer liking and perception of wine-like products. It was determined in this study that dietary flavanols, delivered at low dose in the context of a high-fat diet can slightly improve fasting blood glucose levels and prevent inflammation. When examining consumer preferences for wines made from dietary flavanols that are distinctly different in terms of bitterness and astringency, it was determined that overall, consumers liked wines that were less intense in terms of bitterness and astringency. However, when examining consumers classified as having a high body fat percentage or high BMI, their ability to differentiate the wines was decreased compared to lean counterparts. These findings suggest that dietary flavanol supplementation at a physiologically relevant dose may improve symptoms of diabetes and metabolic syndrome. Future work confirming these observations in humans is warranted, as are studies devoted to better understanding of the taste preferences of the obese population. This will allow for optimization of flavanol-rich foods that maximize health benefit while also being palatable to consumers.

Dedication

To my family. Thank you for your support and encouragement over the years.

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Metabolic syndrome; MetS, cardiovascular disease; CVD, diabetes mellitus; DM, diet induced obesity; DIO, low density lipoprotein; LDL, high density lipoprotein; HDL, small heterodimer partner; SHP, steroid response element binding protein 1c; SREBP1, degrees of polymerization; DP, mean degrees of polymerization; mDP, grape seed extract; GSE, pine bark extract; PBE, high-fat; HF, (±)-catechin; C, (−)- epicatechin; EC, (−)-epigallocatechin; EGC, (−)-epicatechin gallate; ECG, (−)-epigallocatetchin gallate; EGCG, procyanidins; PCs, methanol; MeOH, Folin-Ciocalteu; FC, 4-dimethylaminocinnamaldehyde; DMAC, oral glucose tolerance test; OGTT, intraperitoneal glucose tolerance test i.p.GTT, insulin tolerance test; ITT, malondialdehyde; MDA, interleukin-6; IL-6, tumor necrosis factor- α; TNF- α, area under the curve; AUC, maximum concentration; Cmax, propylthiouracil; PROP, phenylthiocarbamide; PTC, ultra-performance liquid chromatography tandem mass spectrometry; UPLC-MS/MS, labeled magnitude scale; LMS, American Council of Sports Medicine; ACSM, check-all-that-apply; CATA

Attributions

Several colleagues contributed to chapters 3-5 and Appendix E of this dissertation. A brief description of their contributions are included here.

Chapter 3: Flavanol Supplementation Protects Against Obesity-Associated Inflammation by an Obesity-Independent Pathway
Dane W. Fausnacht, PhD, a former doctoral student and current adjunct professor in the Department of Human, Nutrition, Foods, and Exercise at Virginia Tech assisted with data collection and interpretation of the glucose tolerance and insulin tolerance tests.

Jessica L. Tuzo, a former undergraduate student the Department of Human, Nutrition, Foods, and Exercise at Virginia Tech assisted with data collection and interpretation of the glucose tolerance and insulin tolerance tests, as well as the inflammatory cytokine assays.

Adele K. Addington, PhD, a research associate in the Metabolic Phenotyping Core at Virginia Tech assisted with data collection and interpretation of the glucose tolerance and insulin tolerance tests and the inflammatory cytokine assays.

Kathryn C. Racine, a current graduate student in the Department of Food Science and Technology at Virginia Tech assisted in the data collection and interpretation related to the inflammatory cytokine assays and insulin tolerance tests.

Haiyan Zhang, MS, the facility manager of the Metabolic Phenotyping Core at Virginia Tech assisted in the data collection and interpretation of the insulin tolerance tests.

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Richard S. Bruno, PhD, a current professor in the Department of Nutrition and Consumer Sciences at The Ohio State University assisted in the data interpretation of the serum and liver assays.

Sean F. O'Keefe, PhD, a current professor in the Department of Food Science and Technology at Virginia Tech assisted in the characterization of the flavanol extracts used in this study.

Andrew P. Neilson, PhD, a current professor in the Department of Food Science and Technology at Virginia Tech assisted with the study design, compilation and completion of the manuscript.

Amanda C. Stewart, PhD, a current professor in the Department of Food Science and Technology at Virginia Tech assisted with the study design, compilation and completion of the manuscript.

Chapter 4: Preference for and Sensitivity to Flavanol Mean Degree of Polymerization in Model Wines is Correlated with Body Composition

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Lindsey E. Miller, PhD, a current professor in the Elson S. Floyd College of Medicine at Washington State University assisted in the method development and interpretation of the panelist physiology tests.

Andrew P. Neilson, PhD, a current professor in the Department of Food Science and Technology at Virginia Tech assisted with the study design development and completion of the manuscript.

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Amanda C. Stewart, PhD, a current professor in the Department of Food Science and Technology at Virginia Tech assisted with the study design development and completion of the manuscript.

Chapter 5: Processing Strategies to Enhance the Health-Promoting Properties of Wine

Brianna Ewing, MS, a former graduate student in the Department of Food Science and Technology at Virginia Tech and current professor in the School of Food Science at Washington State University assisted in the method development and wine fermentation for this study.

Sean F. O'Keefe, PhD, a current professor in the Department of Food Science and Technology at Virginia Tech assisted in the characterization of the wine used in this study.

Tony K. Wolf, PhD, a current professor at the Alson H. Smith Jr. Agricultural Research and Extension Center assisted in the growth and management of the grapes used in this study.

Andrew P. Neilson, PhD, a current professor in the Department of Food Science and Technology at Virginia Tech assisted with the compilation and completion of the manuscript.

Amanda C. Stewart, PhD, a current professor in the Department of Food Science and Technology at Virginia Tech assisted with the method development and completion of the manuscript.

Appendix E: Alternations to Metabolically Active Bacteria in the Mucosa of the Small Intestine Predict Anti-Obesity and Anti-Diabetic Activities of Grape Seed Extract in Mice

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Ryan P. McMillan, PhD, a current research professor in the Department of Human Nutrition, Foods, and Exercise at Virginia Tech, assisted in data analysis and interpretation for the skeletal muscle flexibility assays.

Matthew W. Hulver, PhD, the current Department Head for the Department of Human Nutrition, Foods, and Exercise at Virginia Tech, assisted in the interpretation of the skeletal muscle assay data.

Monica A. Ponder, PhD, a current professor in the Department of Food Science and Technology at Virginia Tech assisted in results interpretation.

Andrew P. Neilson, PhD, a current professor in the Department of Food Science and Technology at Virginia Tech assisted with the study design compilation and completion of the manuscript.

CHAPTER I. INTRODUCTION AND JUSTIFICATION

The metabolic syndrome (MetS), otherwise known as syndrome X or dysmetabolic syndrome, encompasses multiple risk factors that increase the likelihood of developing cardiovascular disease (CVD).[1] Patients are diagnosed with the disease if they exhibit at least three of the characteristic symptoms (**Table 1.1**).[2] Abdominal obesity is the most common symptom exhibited by patients with MetS.[3] It is projected that people with MetS are 5-fold more likely to develop type 2 diabetes mellitus (DM) and 2 times more likely to develop coronary heart disease (CHD) compared to people without MetS.[1–3] Globally speaking, prevalence of the syndrome is higher in Americans and Canadians compared to Europeans and Asians, but overall incidence of the syndrome is increasing in children, adults, and older populations.[1,2]

Table 1.1 *Characteristic risk factors for Metabolic Syndrome*[2]

Risk Factor	Required Level
Blood Pressure	> 130/85 mmHg
Hypertriglyceridemia	> 150 mg/dL
HDL Cholesterol	< 40 mg/dL (males) <50 mg/dL (females)
Fasting Blood Glucose	$\geq$ 100 mg/dL
Abdominal Waist Circumference	$\geq$ 102 cm (males) $\geq$ 88 cm (females)

***** Taking medication for these conditions also counts as a risk for MetS

Given the increasing incidence of MetS and the inflation of medical expenses due to the variety of symptoms requiring treatment, MetS poses a huge threat to public health.[1,3,4] It has been observed that patients with MetS have 1.6 times greater health care costs compared to patients without, and total cost of healthcare increases by 24% with each additional risk factor contracted.[5] Given the staggering economic costs and reduced quality of life associated with MetS, there is great need for research on treatment and prevention measures. Lifestyle modifications, specifically dietary changes, may be one such way in which risk and symptoms of MetS can be reduced.[1] Flavanols in particular are one such dietary component that have been associated with prevention and reduction of metabolic diseases, though mechanisms of action are not completely understood.[6,7] Although it would appear that flavanol consumption may protect against metabolic derangements brought about by traditional Western diets and lifestyle, a secondary challenge is to produce flavanol-rich foods that consumers find acceptable. It has been observed that overweight and obese populations tend to prefer high-fat and high-sugar foods, which do not typically contain flavanols.[8,9] Furthermore, sensitivity to bitter compounds is highly variable among humans and could further affect selection and preference for flavanol-rich foods, as many of them are astringent and bitter.[10–13]

Flavanols exhibit structural complexity, and can be found in a variety of food sources including fruits, tea, cocoa, and wine.[6,7] Differences in flavanol structure can be attributed to variation in plant material, as well as processing and storage conditions.[14] Flavanols of varying size and structure (varying mean degrees of polymerization (mDP)) appear to exhibit different biological activity in relation to disease reduction. mDP effects appear to vary depending on the disease model and tissues under investigation.[7] Flavanol

mDP also dictates the bitterness and astringency intensity of wine and other products, as smaller compounds are bitter while larger compounds are astringent.[7,10,15]

The overall goal of this research is to determine how flavanol mDP impacts bioactivity using diet-induced obesity (DIO) models and how consumer sensory preferences for wines made with flavanols of different mDP changes due to differences in phenotype. The central hypothesis is that biological effects will be different based on flavanol mDP and preferences for flavanol-rich wines will differ based on weight status and bitterness sensitivity. In order to meet the overall goal and test the central hypothesis, the following objectives were proposed:

1) Conduct a DIO mouse-model study using flavanols extracts of variable mDP to determine the effect of mDP on markers of MetS including obesity and insulin resistance brought about by high-fat feeding. We hypothesize that bioactivity will increase with increasing mDP.
2) Determine how differences in phenotype relate to preference for wine made from flavanols with different mDP. We hypothesize overweight and obese individuals will have reduced preference and sensitivity for flavanol-rich wines, regardless of mDP.
3) Evaluate the extent to which manipulation of winemaking processing conditions can drive large differences in mDP in wine and wine-like products fit for human consumption. We hypothesize that manipulation of flavanol extraction through processing techniques will result in wines or wine-like products with large differences in mDP and flavanol concentration.

REFERENCES

(1) Galisteo, M.; Duarte, J.; Zarzuelo, A. Effects of Dietary Fibers on Disturbances Clustered in the Metabolic Syndrome. *J. Nutr. Biochem.* **2008**, *19* (2), 71–84.

(2) Olde, D.; Alpert, P.; Dalusung-Angosta, A. Metabolic Syndrome: Clinical Perspective for Best Practice. *J. Am. Assoc. Nurse Pract.* **2013**, *25* (12), 644–652.

(3) Scholze, J.; Alegria, E.; Ferri, C.; Langham, S.; Stevens, W.; Jeffries, D.; Uhl-Hochgraeber, K. Epidemiological and Economic Burden of Metabolic Syndrome and Its Consequences in Patients with Hypertension in Germany, Spain and Italy; a Prevalence-Based Model. *BMC Public Health* **2010**, *10*, 529.

(4) Nichols, G. A.; Moler, E. J. Metabolic Syndrome Components Are Associated with Future Medical Costs Independent of Cardiovascular Hospitalization and Incident Diabetes. *Metab. Syndr. Relat. Disord.* **2011**, *9* (2), 127–133.

(5) Boudreau, D. m.; Malone, D. c.; Raebel, M. a.; Fishman, P. a.; Nichols, G. a.; Feldstein, A. c.; Boscoe, A. n.; Ben-Joseph, R. h.; Magid, D. j.; Okamoto, L. j. Health Care Utilization and Costs by Metabolic Syndrome Risk Factors. *Metab. Syndr. Relat. Disord.* **2009**, *7* (4), 305–314.

(6) Xu, Y.; Simon, J. E.; Welch, C.; Wightman, J. D.; Ferruzzi, M. G.; Ho, L.; Passinetti, G. M.; Wu, Q. Survey of Polyphenol Constituents in Grapes and Grape-Derived Products. *J. Agric. Food Chem.* **2011**, *59* (19), 10586–10593.

(7) Neilson, A. P.; O'Keefe, S. F.; Bolling, B. W. High-Molecular-Weight Proanthocyanidins in Foods: Overcoming Analytical Challenges in Pursuit of Novel Dietary Bioactive Components. *Annu. Rev. Food Sci. Technol.* **2016**, *7* (1), 43–64.

(8) Drewnowski, A. Taste Preferences and Food Intake. *Annu. Rev. Nutr.* **1997**, *17* (1), 237–253.
(9) Drewnowski, A.; Brunzell, J. D.; Sande, K.; Iverius, P. H.; Greenwood, M. R. C. Sweet Tooth Reconsidered: Taste Responsiveness in Human Obesity. *Physiol. Behav.* **1985**, *35* (4), 617–622.
(10) Lesschaeve, I.; Noble, A. C. Polyphenols: Factors Influencing Their Sensory Properties and Their Effects on Food and Beverage Preferences. *Am. J. Clin. Nutr.* **2005**, *81* (1), 330S–335S.
(11) Pronin, A. N.; Xu, H.; Tang, H.; Zhang, L.; Li, Q.; Li, X. Specific Alleles of Bitter Receptor Genes Influence Human Sensitivity to the Bitterness of Aloin and Saccharin. *Curr. Biol.* **2007**, *17* (16), 1403–1408.
(12) Harwood, M. L.; Ziegler, G. R.; Hayes, J. E. Rejection Thresholds in Chocolate Milk: Evidence for Segmentation. *Food Qual. Prefer.* **2012**, *26* (1), 128–133.
(13) Cox, D. N.; Hendrie, G. A.; Carty, D. Sensitivity, Hedonics and Preferences for Basic Tastes and Fat amongst Adults and Children of Differing Weight Status: A Comprehensive Review. *Food Qual. Prefer.* **2016**, *48*, 359–367.
(14) Fang, F.; Li, J.-M.; Zhang, P.; Tang, K.; Wang, W.; Pan, Q.-H.; Huang, W.-D. Effects of Grape Variety, Harvest Date, Fermentation Vessel and Wine Ageing on Flavonoid Concentration in Red Wines. *Food Res. Int.* **2008**, *41* (1), 53–60.
(15) Busse-Valverde, N.; Gómez-Plaza, E.; López-Roca, J. M.; Gil-Muñoz, R.; Fernández-Fernández, J. I.; Bautista-Ortín, A. B. Effect of Different Enological Practices on Skin and Seed Proanthocyanidins in Three Varietal Wines. *J. Agric. Food Chem.* **2010**, *58* (21), 11333–11339.

CHAPTER 2. LITERATURE REVIEW

2.1.1 Polyphenols. Polyphenols are natural compounds that can be identified by the presence of hydroxylated phenyl moieties.[1,2] This particular structure allows polyphenols to quench free radicals, chelate metals, and precipitate proteins.[3] As such, these compounds are the most abundant natural dietary antioxidants.[4] These compounds are secondary metabolites of plants that are used in defense mechanisms. Polyphenols are the most diverse group of non-nutritive substances found in plants, of which, there are over 15 sub-classes consisting of small monomers to high molecular weight polymers.[5,6]

Polyphenols vary widely in terms of their structure and can be classified accordingly (**Figure 2.1**). Major groups are designated by the number of phenolic (benzene) rings and the functional groups attached to them.[3] Polyphenol classifications include phenolic acids, flavonoids, stilbenes, and lignins. To date, over 8,000 dietary polyphenols have been identified in edible plants. Predominant food sources include fruits, coffee, tea, wine, and cocoa.[4] Interest in polyphenols has increased in recent years due to the recognition that polyphenols have antioxidant properties and can possibly prevent the onset of certain diseases related to oxidative stress including cancer, heart disease, and stroke.[3,7]

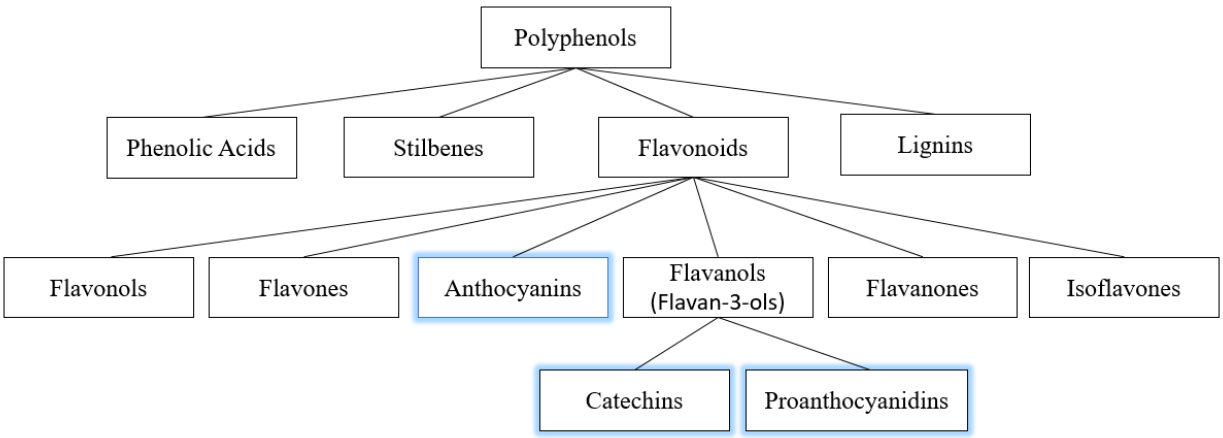

Figure 2.1 *Classification of polyphenols*[7,8]

2.1.2 Flavonoid Composition of Wine. Grapes and wine are rich in flavonoids, one of the predominant classes of polyphenols.[2,8] Flavonoids contain a common repeating structure of two aromatic rings bound together by a pyran carbon ring in a C6-C3-C6 configuration.[7,9,10] Flavonoids can be further divided into subclasses depending on the connection of the pyran ring to an aromatic ring, the oxidation state of the pyran ring, or the functional groups on the pyran ring.[11]

Grapes and wine are specifically rich in flavanols (including procyanidins (PCs) and catechin monomers) and anthocyanins, all of which belong to the flavonoid class (**Figure 2.2**).[2,7] These compounds are observed in monomer and polymerized forms. They can bind to each other, to sugars (forming glucosides), and to other non-flavonoids (forming acyl derivatives). Collectively, these polyphenol compounds typically comprise at least 85% of the total phenolic composition of red wines.[9,12]

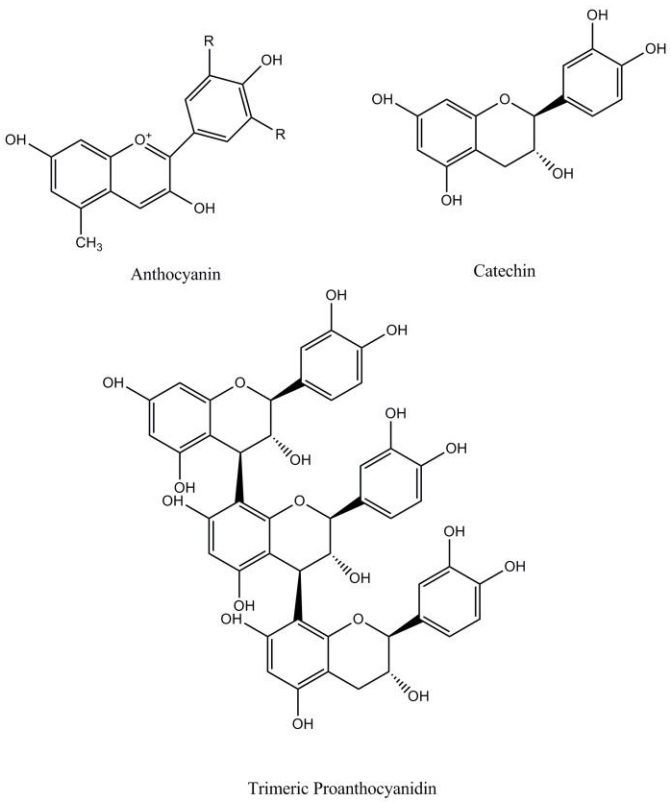

Figure 2.2 *Chemical structures of flavonoids found in grapes and wine*[7]

Anthocyanins concentrate in grape skins and are responsible for the color of red wine as well as many other red and purple plant tissues. Specifically in *V. vinifera* grapes, the primary anthocyanin is malvidin-3-O-glucoside (and acylated forms).[2] Anthocyanin content in wine is generally proportional to color and concentration typically ranges between 200-350 mg/L. As mentioned above, flavanols (specifically, flavan-3-ols) include catechin monomer units and PC polymer units.[7] Flavanol monomer units, including (+)-catechin (C), (−)- epicatechin (EC), and (−)-epicatechin-3-O gallate (ECG) have been found in red wine at concentrations up to 300 mg/L.[2,7] These monomers are most concentrated in the seeds and skins of grapes, as opposed to the pulp. PCs are polymerized forms of the flavanol monomer units and are commonly known as tannins.[2] Polymerization of monomers occurs during plant maturation and also during post-harvest processing of plant-based products during food production.[11] As such, a wide variety of molecular weights and levels of polymerization can be observed in grapes, wine, and other flavanol-rich products.[2] Typically, C and EC are the base units for polymerization of PCs, and the units are linked together to make dimers, trimers, oligomers, and large polymers.[7] PCs are extracted from skin, seeds, and stems of grapes.[2] Oftentimes, PCs are complexed with alkaloids, polysaccharides, or proteins.[8] Due to their abundance, PCs and anthocyanins are the compounds most studied in wine for their potential health benefits and impact on sensory characteristics.[1] However, there is great difficulty specifically in studying PCs due to their high complexity and variability. Variability within grapes and wine alone can be due to differences between grape species, cultivars within each species, environmental growing conditions, wine processing methods, and storage conditions.[2,13]

2.2 Flavanol Variation in Grapes and Wine. Many aspects of enology and viticulture impact the flavanol composition of grapes and wine.[13] As with all flavanol-rich substances, plant species and cultivar is a key factor determining flavanol composition of subsequent products. Grape cultivars can express different thicknesses in grape skin, can ripen differently, or respond differently to horticultural and environmental factors and variations in climate and growing season. Furthermore, flavanol variation can arise within the same cultivar and growing season if grapes are harvested at different maturities.[13]

Environmental factors play a key role in the profile and concentrations of plant flavanols, as soil composition, rainfall, climate, and sunlight all influence the development of polyphenols. It has been observed that excessive rainfall or waterlogging can reduce PC content, but water deficit does not impact PC content.[14] Anthocyanin production and stability is impacted by temperature. For ideal anthocyanin development, temperatures should rise to 20-25ºC during the day and fall to 10-15ºC at night. This is optimal for the production of sugar in berries, which stimulates anthocyanin synthesis.[9] Anthocyanin synthesis is also impacted by soil composition and nutrient availability. While high plant nitrogen content promotes amino acid synthesis, it also inhibits anthocyanin synthesis, as does high potassium content.[3,9,14] Vine vigor may also impact flavanol composition. Grape skins from low-vigor vines generally have higher levels of PCs.[14] Environmental stressors may also stimulate flavanol development.[6] Plants that have been exposed to infection or damage may contain higher concentrations of polyphenols compared to plants that have not been stressed.[7] Exposure to UV light or insect attack may also induce a stress response and lead to increased production of certain flavanols.[3,6]

The ripening process is also critical to the development of certain polyphenols. Anthocyanins develop during the ripening stage, with the amount increasing as the sugar content of the berry increases. Overcropping can stunt anthocyanin development in grapes since it decreases berry yield, and delays maturation and sugar accumulation. As a result, anthocyanin development is diminished.[9] Seed flavanols, on the other hand, develop early during ripening and do not change during berry maturation. Larger PCs from the skins peak in concentration at veraison and decrease as the grapes continue to ripen.[12,15]

In addition to environmental and horticultural factors, wine production and aging techniques also influence the flavanol profile of wines.[16] The basic process of winemaking is outlined in **Figure 2.3**.[9,12] Grapes are destemmed, macerated (red wine), and pressed either before (white/rosé wine) or after fermentation (red wine).[9] Flavanols are transferred from the skin, seed, and stem material of grapes into wine during the crushing, maceration, and fermentation processes. Extraction of flavanols is limited through white wine production techniques, and encouraged through red wine production techniques.[17] Using a maceration period during the red wine production process increases phenolic extraction due to lengthened seed and skin contact with grape must.[18] However, the impact of maceration procedures on wine flavanol concentration and composition will vary depending on maceration duration and conditions. Extraction of seed flavanols is slow compared to extraction of anthocyanins and skin PCs.[10] Flavanol monomers from grape seeds are found at higher concentrations in must and wine that is made with longer maceration times, heating at the end of maceration, and higher final alcohol concentrations.[1,18,19] In contrast, grape skin PCs and anthocyanins are extracted relatively early during the maceration period, and heating after the maceration period reduces their

presence in must.[19] The effect of maceration conditions and duration on red wine flavanol content remains a topic of current research.[18]

Aging is another factor that influences the final polyphenol profile of wines. Fermentation vessel, temperature, and contact time (red wine) are factors known to in turn influence PC polymerization during aging.[20] Fermentation vessel type can also influence the extent of flavanol extraction, as can vessel size.[13,20] After fermentation, wines can be aged in a variety of vessels including stainless steel tanks, wooden barrels and glass bottles. In terms of aging, anthocyanins in particular are unstable and degrade or undergo many reactions with other PCs.[10,21] This affects the polyphenolic profile as well as the color of red wine.[1,10] In addition to reaction with anthocyanins, flavanols increase in size during aging through polymerization and oxidation reactions.[10,21] Wine pH, proportion of anthocyanins to flavanols, and oxygen exposure all play a role in the development of the final polyphenol profile during wine aging.[21]

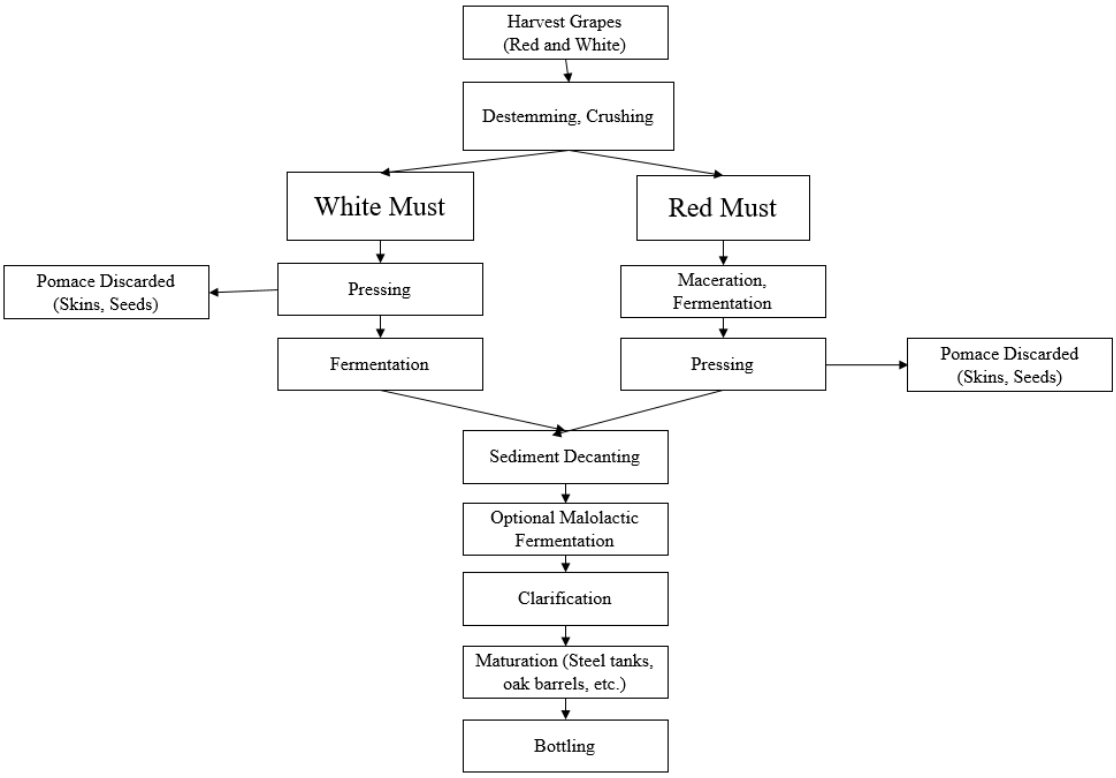

Figure 2.3 *Overview of winemaking practices*[9,12]

2.3 Flavanol Mean Degrees of Polymerization, Bioactivity and Bioavailability. Flavanol PCs exist in a wide variety of molecular weights in wine, due to the changes incurred during development and processing described above. As such, flavanols can be classified by their degree of polymerization (DP) (**Figure 2.4**). Monomers have a DP equal to 1, dimers have a DP equal to 2, oligomers have DP of 3-7, and polymers consist of DP greater than 7. Flavanol-rich foods, including wine and cocoa, can be characterized by their mean degrees of polymerization (mDP), or the average flavanol size in a substance.[22] mDP of a substance can be estimated by examining the relative abundance of flavanols based on

DP, or calculated directly using a thiolytic reaction and UPLC/MS-MS quantification methods.[23] It has also been reported that PCs extracted from grape skins have a higher mDP than PCs from grape seeds.[10,18] Grape seeds have DPs ranging from 2 to17, while grape skins have DPs ranging from 3 to 83.[24] The contributions of each of these materials to the total wine polyphenol composition determine mDP, along with other environmental, processing and aging conditions.[10]

Flavanol mDP can play a role in both the sensory characteristics imparted by flavanols, and the health benefits of flavanol consumption.[22,23,25,26] Flavanols are naturally bitter and astringent, the extent of each attribute being attributed to the DP of the flavanols consumed.[26,27] Bitterness is a basic taste detected on the tongue, while astringency is a mouth-drying sensation detected throughout the oral cavity.[27] It has been observed that flavanols of smaller DP are more bitter, while larger DP flavanols are more astringent; smaller compounds can enter the taste buds to cause a bitter sensation, while larger compounds are sterically inhibited and instead interact with salivary proteins, causing precipitation and perceived astringency. Thus, wine flavor is impacted by the mDP of flavanol compounds present.[1,28]

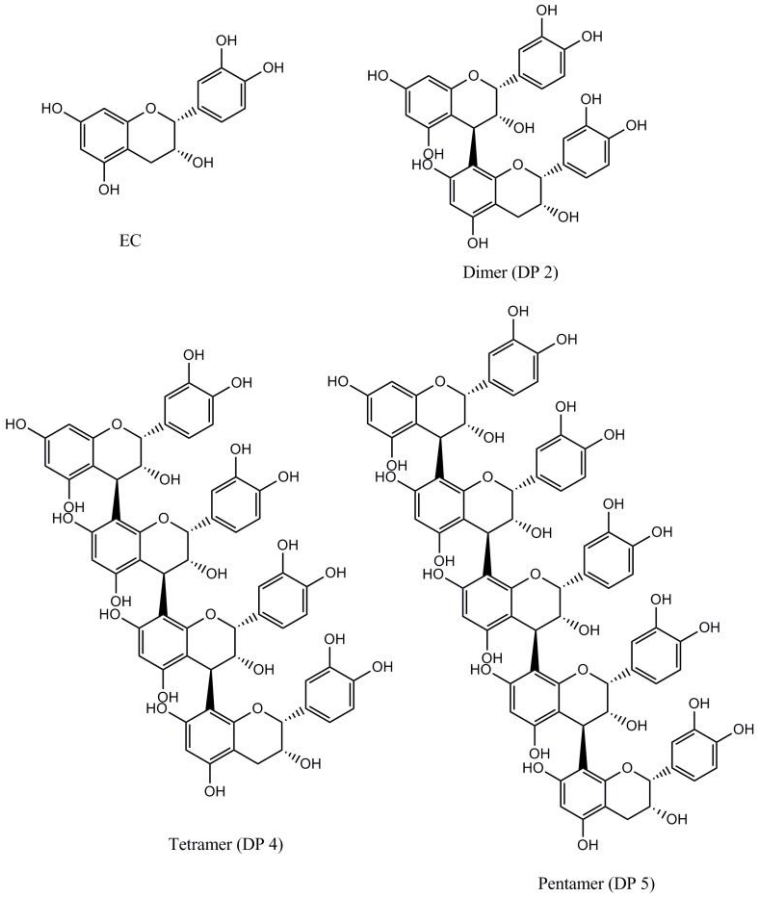

Figure 2.4 *Degrees of polymerization of flavanol compounds*[23]

In addition to influencing sensory characteristics, flavanol mDP has also been demonstrated to impact human health benefits imparted by dietary consumption of flavanol-rich foods. *In vitro* models evaluating effects of dietary interventions with PCs

extracted and fractionated from cocoa have shown that polymeric PCs have the greatest anti-inflammatory function in colonic tissue, while oligomeric and polymeric PCs stimulate glycogen synthesis and glucose uptake in muscle cells.[22,25] Other studies have indicated that oligomeric PCs were more effective than monomers or polymers at reducing hepatic and renal inflammation in diabetic rats.[29] When looking at liver and prostate cancer cells, a polymeric PC fraction had greater antiproliferative and cytotoxic effects than fractions with a smaller mDP. When studying human melanoma cells, oligomer PCs appeared to have greater antiproliferative effects compared to monomers.[30] Additionally, an *in vivo* mouse study suggested that oligomeric PCs from cocoa provide the most protection against weight gain, fat gain, insulin insensitivity and glucose intolerance.[23] When looking at gut enzyme inhibition, again polymer PCs from persimmons and cocoa were more effective at reducing pancreatic enzymes than the smaller PCs. Overall these findings indicate that flavanol mDP influences bioactivity, but there does not appear to be a specific correlation between size and function; in some instances, mDP and effect are positively correlated and in others they are negatively correlated. These observations do suggest, however, that grapes and wine, which can have variable mDPs, may exert different biological effects.[30]

Although flavanols are some of the most prominent sources of polyphenols in the diet and mDP plays a role in their protective health effects, it is worth noting that abundance does not necessarily dictate bioactivity.[7] It has been reported that bioavailability of flavanols is relatively low, particularly for oligomeric and polymeric PC compounds.[30,31] Monomers and dimers can be detected in the bloodstream within half an hour of consumption following simple deconjugation and deglycosylation reactions.[32,33] Animal models indicate that PCs up to pentamers (DP $\leq$ 5) can be absorbed in the small intestine.[30] Larger PCs, however, exhibit poor epithelial permeability and transport is limited. It is suspected that larger DP compounds exert their effects locally in the gut or systemically after conversion to microbial metabolites in the colon, which are more bioavailable than parent compounds.[4,7,30,34] Despite apparent poor bioavailability, larger PCs still appear to exert significant bioactivity in some model systems, as described above.[22,23,25,30,35]

The notion that flavanol mDP relates to bioactivity is relatively novel. Moreover, the effects of flavanols on sensory attributes and how they are related to preferences for flavanol-rich products such as red wine have not fully been explored. A review of current knowledge regarding grape-flavanol sensory properties and bioactivity in relation to mDP is outlined below.[7,17,31]

2.4 Epidemiological Evidence of Grape and Wine Health Effects. Both epidemiological and experimental data indicate that grape and wine polyphenols protect against the onset of certain diseases including cardiovascular disease (CVD), neurodegenerative diseases, metabolic syndrome (MetS), and some forms of cancer.[2,9] Research in this area began to surface shortly after the French Paradox was observed in 1992 but much of the early focus was on epidemiological studies.[36]

There have been numerous reports of the beneficial effects of wine consumption on CVD.[37] When looking at a cohort of 8,000 men from the Health Professionals follow up study, the cohort of 27,000 patients from the INTER-HEART study, and a cohort of 11,000 hypertensive men, light to moderate alcohol consumption has repeatedly demonstrated the ability to reduce the risk of myocardial infarction, stroke, and dementia in men and

women.[38] A cross-cultural correlation study found an inverse association between flavonoid intake and CVD mortality across 16 cohorts from 7 countries after 25 years of follow up.[39] Another study of 13,000 men and women aged 30 to 79 involved the analysis of CVD mortality risk and alcoholic beverage type. It was observed that relative CVD mortality risk decreased from 1.00 in non-drinkers to 0.4 in moderate consumers of red wine, while moderate beer consumption only reduced risk to 0.72.[40] A similar study was designed to look at stroke risk and alcohol type. Again, red wine consumers had slightly reduced risk of stroke compared to other types of alcohol consumption.[40] Women have not been studied as extensively as men, but the Nurses' Health Study, one of the largest and longest epidemiological studies devoted to investigating risk factors for major chronic diseases in women, indicated that a correlation existed between women's health and wine consumption. It was observed that cardiovascular mortality and all-cause mortality rates were reduced in women who classified themselves as moderate drinkers.[41]

More recently, there has been a shift from the previously somewhat singular focus on the cardiovascular benefits of wine consumption to the possible reduction of risk of the MetS. MetS is a series of metabolic disturbances that ultimately increase the risk for type 2 DM and CVD. Obesity, elevated triglyceride levels, reduced high-density lipoprotein (HDL) cholesterol levels, elevated blood pressure, elevated fasting blood glucose, and insulin resistance are all symptoms of MetS.[42,43] Wine, as part of a plant based diet, appeared to increase insulin sensitivity compared to traditional Western diets when 980 subjects aged 40-69 years were analyzed in a cross-sectional study.[44] Even more promising results were obtained from a study designed to investigate the effects of alcohol type on MetS, DM, and CVD in another cross-sectional analysis of 4,153 Greek adults. Moderate alcohol consumption, regardless of type, was associated with reduced levels of MetS, DM, and CVD. However, wine consumers exhibited lower levels of CVD compared to consumers of beer and spirits.[45] Similarly, in a cross-sectional study of 4,232 men and women 60 years of age, overall, MetS was most common in non-drinkers, while moderate wine drinkers had the lowest levels of MetS compared to other groups. Wine drinkers also exhibited lower levels of fasting insulin, elevated levels of HDL-cholesterol, and lower fibrinogen levels. Moreover, it was observed that women specifically had reduced incidence of MetS when they consumed red wine moderately.[42]

It is worth noting that while these epidemiological studies captured information from thousands of patients, the methods of data collection were mostly via self-reported questionnaires.[41] Moreover, it would appear that socioeconomic status may be a confounding variable.[41,42] Wine drinkers tend to be of higher socioeconomic status and are more health conscious than non-drinkers or drinkers of other sources of alcohol.[41] Additionally, given the chemically complex nature of wine, it is impossible to determine if the health effects reported by these epidemiological studies were due to flavanols, anthocyanins, ethanol, or a combination.[9] Therefore, these epidemiological observations alone are not enough to prove that wine consumption reduced the risk of disease.

Limited experimental human and animal studies have been performed to evaluate factors underlying the epidemiological findings linking wine to prevention of disease. Several notable animal studies have been conducted. In one such study, 48 rabbits were given a high cholesterol diet supplemented with water, red wine, white wine, beer, whiskey, or ethanol. With the exception of beer, all treatment groups had reduced levels of atherosclerotic lesions after 3 months compared to control, but the red wine group had the

most dramatic reduction.[46] Similarly, in rats fed a high cholesterol diet, total and LDL cholesterol levels were reduced by administration monomeric or polymeric flavanols. It was also noted in this instance that polymer PC compounds (exact mDPs not provided) were more effective than monomer compounds.[47] Finally, in anesthetized dogs mechanically manipulated to induce cyclic flow reductions in coronary blood flow, it was observed that the groups treated with 4 mL/kg bw red wine and 2.0 mL/kg grape juice completely eliminated the cyclic flow reductions. White wine was unable to produce the same results.[48]

The human intervention studies that have been performed mostly focus on consumption of red wine compared to white wine, or consumption of PC extracts, not specifically red wine.[9,31] In studies comparing the effects of red and white wine consumption, it was observed in 20 healthy males that 400 mL/d red wine consumption increased HDL cholesterol levels in 2 weeks, but had no effect on LDL cholesterol. These effects were not observed in the white wine consumption group.[49] However, in hypercholesterolemic patients who drank red or white wine over a 3-month period, it was observed that both treatments were effective at reducing LDL cholesterol levels and thrombin-initiated platelet aggregation.[50] Yet another study examining wine consumption of 17 healthy males over a 2 week period indicated that 400 mL/d Cabernet Sauvignon wine reduced LDL lipid peroxidation while 400 mL/d white wine increased lipid peroxidation.[51]

Considering studies that go beyond comparing white and red wine, some interesting results have been observed. One such double-blind placebo-controlled study concluded that supplementation of 400 mg/d grape seed extract (GSE) along with a chromium supplement for two months improved total cholesterol and low-density lipoprotein (LDL) cholesterol levels in 40 hypercholesterolemic patients. However, HDL cholesterol and triglyceride levels were not significantly different from control.[52] In a different double-blind crossover study, 24 health male heavy smokers were given 150 mg/d GSE supplements. Smoking was intended as a model for oxidative stress, and it was observed that oxidative stress was reduced, but total, HDL, and LDL cholesterol levels were unaffected.[53]

It is clear from epidemiological and limited clinical evidence that wine consumption may convey certain health benefits.[9,36,38,39,41,42,44–51,54] However, much of this evidence is not recent and largely observational. More recently, attention has been directed towards understanding the mechanisms behind the protective effects of red wine and more specifically the polyphenols that are abundant in red wine.[8] These findings are discussed in the following section.

2.5.1 Mechanisms of Health Effects. Despite the fact that numerous studies have concluded that grape and wine polyphenols protect against certain diseases, the mechanisms of action are not completely understood. Epidemiological and observational studies merely suggest an association of flavanol consumption with health effects, but do not directly link consumption with specific health biomarkers or pathways of disease.[31] It is in fact believed that many biological pathways are affected by grape and wine consumption. Effects observed through experimental studies relate to the inhibition of lipid peroxidation, scavenging of free-radicals, inhibition of blood platelet aggregation,

reduction of blood pressure and vasorelaxing activity, anti-inflammatory activity, improved endothelial function, improved insulin sensitivity and anticancer activity.[37]

2.5.2 Atherosclerosis Inhibition. Atherosclerosis, the inflammatory disease characterized by lipid accumulation in arteries, is one of the main factors contributing to the development of CVD. **Figure 2.5** depicts some of the aspects of CVD that may be modulated by red wine consumption. The first step in the development of atherosclerosis occurs when elevated levels of oxidized LDL cholesterol begin to accumulate in the arteries, ultimately generating atherosclerotic lesions and fibrous caps, also known as plaque. When thick enough, plaque buildup can rupture, leading to thrombosis and myocardial infarction (MI).[31]

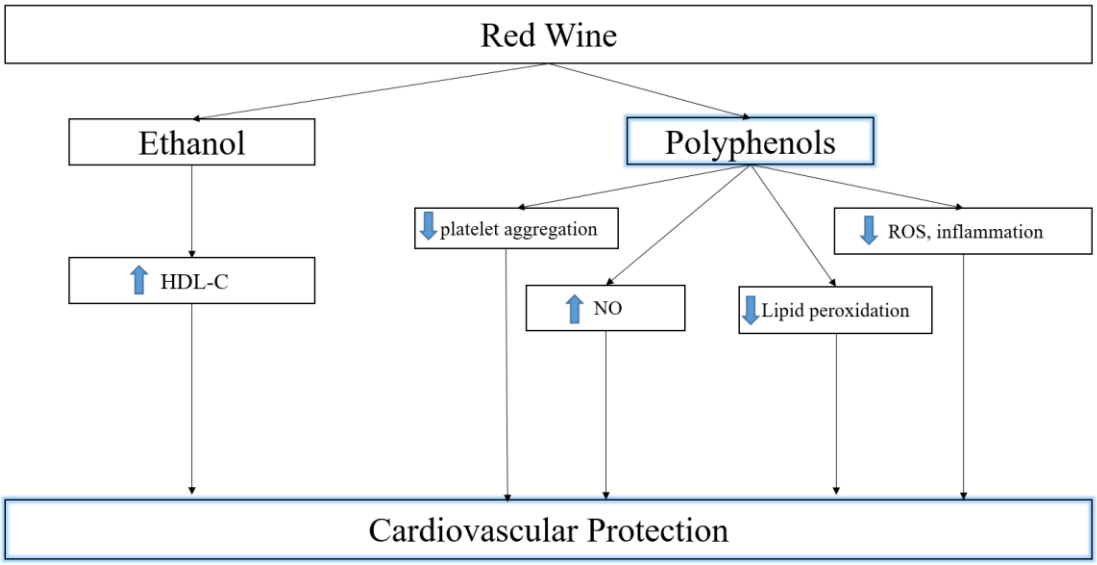

Figure 2.5 *Effects of red wine on CVD*[55]

Lipid peroxidation is primarily caused by oxidation via reactive oxygen species including hydroperoxyl radicals, nitrogenoxide radicals, singlet oxygen, and superoxide anion radicals, all of which are naturally generated by the body. Flavanols are thought to scavenge these reactive oxygen species, reducing the risk of CVD.[31] The characteristic substituted benzene ring structure of flavanols allows for stability through resonance, thus allowing them to scavenge free radicals.[1,2] In a study looking at the effect of red wine flavanols on LDL cholesterol oxidation, it was observed in apo E deficient mice that 2 months of red wine supplementation decreased LDL cholesterol oxidation by 40% compared to control.[56] Additionally, it has been speculated that flavanols reduce the onset of CVD through of anti-inflammatory mechanisms. Several studies have indicated that red wine flavanols reduce the expression of inflammatory biomarkers and adhesion molecules. These effects have been observed with GSE in *in vitro* models, in a human study comparing Merlot wine and gin consumption (320 mL wine or 100 mL gin/d for 1 month), and a human study involving GSE supplementation (100mg/d for 1 month) in atherosclerotic patients, among others.[31,57–59] In terms of mDP, it has been observed in multiple *in vitro* studies that oligomer compounds have the greatest protection against lipid peroxidation. While these studies were not performed using PCs from grapes or wine, it was noted that

increasing chain length was associated with reduced incidence of LDL cholesterol oxidation in the size range of DP 3 up to DP 9.[60–63] However, as mentioned previously, these compounds may not remain intact after entering the human digestive tract and the effects could be due to gut metabolites of PCs with DP 3 to DP 6, rather than intact large PCs.[64]

In addition to decreasing levels of oxidized LDL cholesterol, it is worth mentioning that wine has also demonstrated the ability to dramatically increase high density lipoprotein (HDL) cholesterol levels. This is one of the hallmark mechanisms behind the protective role of wine consumption on CVD. However, it has been established that this effect is due to ethanol, not flavanols.[65] A dose-dependent positive correlation between ethanol consumption and elevated HDL cholesterol has been observed.[66]

Hypertriglyceridemia is another independent risk factor for CVD development. GSE flavanols specifically appear to be able to acutely reduce blood triglyceride levels in both normolipidemic and hyperlipidemic rats at a dose of 250 mg/kg bw.[67] Studies suggest that the lipid-lowering effect of flavanols could be due to decreased triglyceride absorption in the gut or reduced chylomicron secretion. In fact, oligomer flavanols have demonstrated their potency in terms of acutely reducing triglyceride absorption (600 mg dose during triglyceride tolerance test).[68,69] It has also been observed that GSE flavanols reduce triglyceride levels by mimicking the effects of bile acids on lipid homeostasis; GSE is able to upregulate the bile acid receptor FXR much like bile salts. This causes a chain reaction causing an upregulation of small heterodimer partner (SHP), downregulation of steroid response element binding protein 1c (SREBP1), and ultimately suppressing hepatic fatty acid synthesis and increasing triglyceride catabolism.[67,70]

Endothelial dysfunction from vasoconstriction, or high blood pressure, is another marker of CVD risk. The vasodilating effects of red wine have been extensively studied.[31,37,71] There is evidence that red wine and grape flavanols promote the synthesis of nitric oxide (NO) by upregulation of NO synthesis promoter activity.[71] Increased circulation of NO has a vasodilating effect which reduces the risk of endothelial dysfunction and CVD.[31,37,72,73] *In vivo* rat studies indicate that red wine flavanol administration could reduce myocardial fibrosis and prevent aortic thickening.[72–74] In addition to upregulation of NO synthesis, *in vivo* animal models have suggested that even short term administration of red wine flavanols (of the Cabernet Sauvignon and Concord grape variety) reduce systolic blood pressure.[73,75] Most interestingly, it has been observed using *in vitro* studies involving rat aorta that monomer wine PCs demonstrate no vasodilating effect, while oligomer and polymer PCs from Cabernet Sauvignon grapes had higher vasodilating activity.[76,77] Despite these reports of the positive effect of wine flavanols on blood pressure and vasodilation, there have been other reports, especially in human clinical trials, of no effect of wine consumption on hypertension. This is still an area of ambiguity.[37]

Platelet aggregation, a symptom of CVD related to endothelial dysfunction, produces a pro-inflammatory response in the body, which ultimately leads to atherosclerosis and CVD.[31] Normally, blood platelets do not adhere to arteries due to an inhibitory mechanism initiated by NO. However, when NO is deficient, platelet aggregation can pose a threat to cardiovascular health.[78] Moderate wine consumption has been shown to reduce collagen-induced platelet aggregation in multiple studies.[37] Red grape juice, wine, and GSE have reportedly decreased platelet aggregation in various cell,

animal, and human studies in as little as a week. The effect was not observed in response to other types of beverages including orange juice, grapefruit juice, ethanol, or white wine. These data indicate that red wine decreases platelet aggregation, and again, that flavanols are partially responsible for the cardiovascular benefits of wine consumption.[9,31,37,78] It is also worth noting, however, that the dosage and also the type of wine used may be related to the perceived health effects and studies that further examine these specific variables is warranted.[79]

2.5.3 Metabolic Syndrome Inhibition. As mentioned in Chapter 1, MetS consists of a series of metabolic disturbances that ultimately lead to the onset of DM and CVD (**Figure 2.6**). With the onset of these diseases, morbidity and mortality rates increase, while quality of life decreases.[80] Some symptoms of MetS overlap with the symptoms of atherosclerosis, and include abdominal obesity, hyperglycemia, insulin resistance, hypertension, and dyslipidemia.[42,43] It has been established that diet plays a pivotal role in the onset of MetS and that red wine is one dietary component that can act as a preventative measure.[43,65]

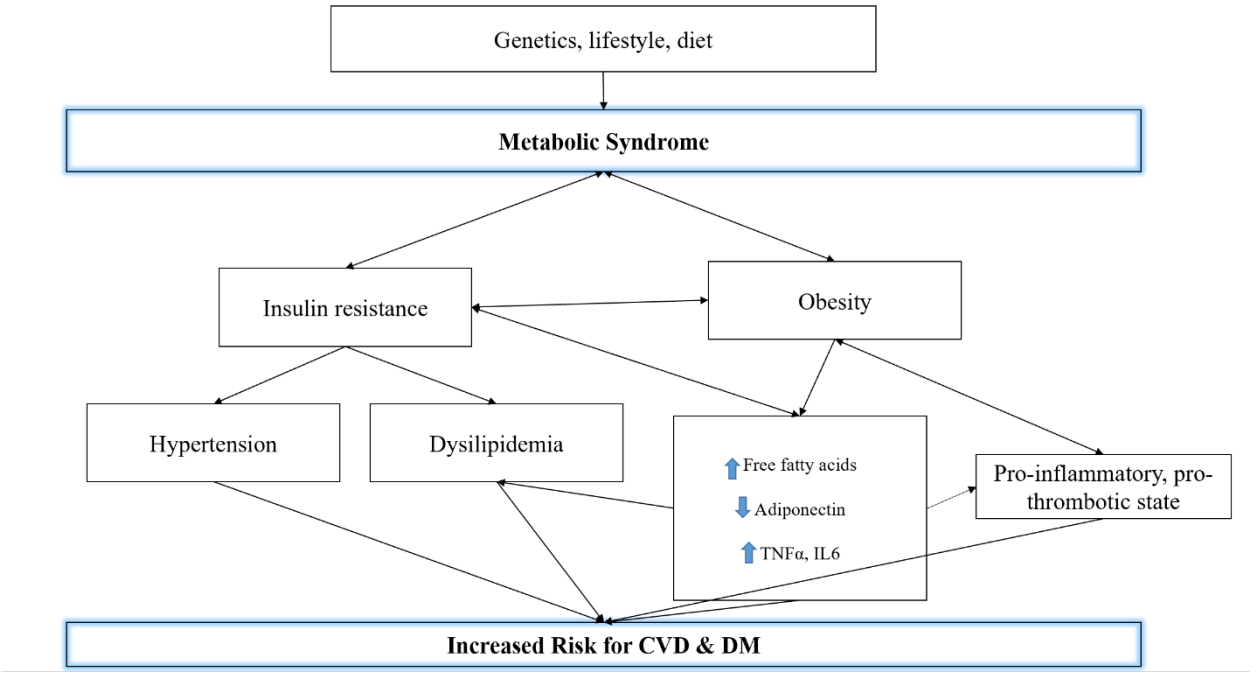

Figure 2.6 *Risk factors associated with MetS*[81]

Long term positive energy balance results in obesity, which is a risk factor for CVD and insulin resistance.[82,83] While somewhat controversial due to the caloric density of ethanol, there is some indication that moderate wine consumption prevents the onset of obesity.[84] It has been observed that red wine flavanols may actually reduce adiposity by increasing cAMP levels and genetic expression of hormone sensitive lipase, the rate limiting enzyme in lipolysis. An *in vitro* adipocyte study demonstrated the ability for oligomer and polymer grape PCs, but not monomers, to acutely stimulate lipolysis and inhibit lipogenesis in 3T3-L1 cells at a dose of 150 µM, thus promoting the breakdown of stored lipids.[84] In an animal study examining the anti-obesity effects of young Priorat wine

consumption in male Zucker rats, *ad libitum* wine supplementation prevented excessive weight gain. Additionally, the rats consumed less food. Given the decreased food intake of the wine consuming group, these results suggest that red wine consumption may trigger satiety mechanisms or interfere with fat absorption.[85] Reduced fat absorption capacity has been confirmed in animal studies involving cocoa flavanols.[86,87] A similarly designed, long-term study using muscadine grapes and muscadine wine extract instead of wine produced similar results; mice on high fat diets supplemented with muscadine grape or wine extract (at 4% of diet formulation for 15 weeks) had lower body weights, reduced circulating free fatty acid, triglyceride, and cholesterol levels, reduced inflammation, and increased insulin sensitivity compared to mice on high fat diets alone, but not compared to mice on standard diets. This study suggests that the flavanols in grapes and wine are responsible for preventing weight gain, rather than the ethanol.[88] Even though it would seem from these studies that red wine consumption may reduce the risk of obesity, it is worth mentioning, that in a meta-analysis of epidemiological studies about obesity risk and alcohol consumption, it was observed that risk reduction was not different between lean and obese study participants.[89]

Epidemiological data indicate that moderate alcohol consumption has a positive effect on insulin sensitivity and other markers of DM and MetS.[90] Controlled studies have also reported a positive effect of moderate wine consumption on insulin sensitivity, fasting glucose levels, dyslipidemia, and reduced incidence of DM.[37,91,92] One of the first clinical analyses of the effect of wine consumption on DM markers was performed on postmenopausal women in a controlled crossover trial. It was observed that consumption of 30 g/d of alcohol as part of a clinically controlled diet significantly reduced fasting insulin levels, reduced fasting triglyceride levels, and increased insulin sensitivity compared to 0 g/d alcohol consumption. While these effects were observed independently of BMI, the source of alcohol consumed was not specified. Thus, the effects cannot be solely attributed to red wine based on this study.[90] Another study on long-term GSE supplementation in mice fed a high-fructose diet showed similar results of improved insulin sensitivity, and also improved functionality of hepatic superoxide dismutase and catalase, when GSE was administered moderately at 1% of the diet for 8 weeks.[80] GSE supplementation in mice fed a high-fat diet has also indicated that supplementation increases oral glucose tolerance as well as fasting glucose concentration. Together, these studies indicate that wine flavanols could indeed be responsible for the effects of wine consumption on insulin resistance.[93] However, it must be noted that several studies on resveratrol, a stilbene that is concentrated in red wine, have also indicated protective effects, which are unrelated to dietary flavanol effects. Studies on resveratrol supplementation have indicated that resveratrol was responsible for reducing fasting glucose and triglyceride concentrations. These effects were observed within 2 weeks when fed to DM-induced rats. Furthermore, it was observed that glucose uptake by skeletal muscle, hepatic tissue, and adipocytes was also stimulated by resveratrol supplementation (60 mg/kg bw/d).[94]

Wine has also demonstrated the ability to influence circulating glucose levels in addition to increasing insulin sensitivity. It has been postulated that the acidity of wine slows down gastric emptying and certain components may inhibit key digestive enzymes related to starch digestion, including α-amylase. In a study comparing the postprandial levels of glucose after consumption of red wine, beer, or gin, it was observed that all three

alcohol types reduced postprandial glucose response, but red wine reduced it to the greatest extent. This postprandial glucose concentration lowering effect was observed when alcohol was consumed with or before a carbohydrate-rich meal.[91] Another suggested mechanism of glucose control is through the moderation of adiponectin.[95] Adiponectin is an adipose tissue-derived hormone that assists in the regulation of glucose and lipid metabolism. It has been observed in randomized controlled trials that moderate alcohol consumption (not specifically flavanols) increases adiponectin levels compared to control groups when consumed daily for 2 weeks.[96] It has also been observed in postmenopausal women that white wine consumption (25 g alcohol/d for 6 weeks) upregulated adiponectin gene expression, which in turn decreased fasting triglyceride and LDL cholesterol levels compared to a grape juice control. These observations suggest that ethanol, not just flavanols, may be responsible for the regulation of adiponectin.[92] Still, other studies indicate that GSE supplementation in rats as part of a high-fructose diet (0.5-1% GSE) improves insulin sensitivity by upregulating genetic expression of insulin signaling pathway proteins, adiponectin, and glycogen synthase.[95]

Metabolic flexibility refers to the ability to metabolize different substrates (lipids, glucose) depending on available fuel sources. An insulin resistant state is typically associated with reduced metabolic flexibility which contributes to reduced glucose oxidation rates in an insulin-stimulated state and reduced activation of lipid catabolism in a fasted or glucagon-stimulated state.[97] Short term (3 weeks) GSE supplementation (25 mg/kg bw/d) appears to have a positive impact on skeletal muscle beta-oxidation, mitochondrial function, and oxidative capacity in male Wistar rats in addition to reducing insulin resistance.[98] Short term and long term resveratrol supplementation has also demonstrated the ability to increase skeletal muscle mitochondrial function at variable dosages ranging from 5 mg – 1500 mg daily.[99] While it appears promising that skeletal muscle metabolism derangements might be reduced by grape and wine flavanols, no other notable studies have been performed on this topic.

As mentioned previously, it has been established that red wine flavanols improve symptoms of dyslipidemia, which is characterized by high circulating concentrations of triglycerides, total and LDL cholesterol, and low circulating concentrations of HDL cholesterol. Animal and human studies, have demonstrated that red wine flavanols are able to reduce plasma lipid concentrations, reduce cholesterol levels, and improve the ratio of LDL cholesterol to HDL cholesterol.[65,83,88,90,94,100] It has been suggested that the mechanism of action is by the interaction and subsequent blocking of cholesterol carriers and brush border membrane transporters.[83] It has also been observed that GSE can inhibit pancreatic lipase and cholesterol esterase, interfere with cholesterol micelle formation, downregulate SREBP1 expression which reduces hepatic lipogenesis, and bind bile acid.[67,70,100]

It is clear that grape and wine flavanols have the potential to prevent the development of MetS through various mechanisms. However, the effects of grape and wine flavanol mDP on health outcomes related to MetS, as well as dose effect, remain unclear. Given that other flavanol-rich substances of variable mDP appear to differ in the extent of health effects conferred depending on DP and dosage, it seems likely that the extent of perceived health effects due to grape and wine consumption would also be related to flavanol mDP.[23,30] This is an area where more research is needed.

2.5.4 Cancer Inhibition. Cancer is the uncontrolled growth and spreading of cells. Environment, genetics, and lifestyle contribute to the risk of cancer development and to cancer prevention.[78] It is believed that wine flavanols can inhibit carcinogenesis.[8,30] Administration of GSE and Cabernet Sauvignon wine has resulted in cytotoxicity towards human lung, breast, prostate, and gastric adenocarcinoma cells. GSE also appears to stimulate normal functioning of human gastric mucosal cells at different dosages.[9,63,101–103] Resveratrol has also demonstrated the ability to induce apoptosis in human breast cancer cells and ability to down-regulate key enzymes involved in colon cancer cell growth.[78,104] It has also been speculated that the cancer-inhibiting properties of red wine polyphenols are due to their ability to interact with steroid receptors and exert antioxidant and anti-inflammatory properties on harmful ROS. Collectively, these mechanisms may be related to the reduced cancer risk associated with wine consumption.[101,103]

When looking specifically at the effect of grape PC fractions on human colon carcinoma cells, it has been observed that PCs with greater mDP and percent galloylation were most effective at inhibiting cellular proliferation, inducing cell cycle arrest, and inducing cell apoptosis.[105] Oligomeric and polymeric fractions of GSE have demonstrated the ability to prevent tumor growth in mouse skin epidermal tissue.[106] It has also been observed that monomeric and dimeric PCs are not able to prevent lymphocyte DNA damage.[78] This trend of greater activity with increasing mDP has been observed in other types of cancer cells treated with other PC-rich foods, but once more, it is unclear what role microbial metabolism plays, when considering the impact of flavanols on cancer.[30,64]

2.6.1 Wine Flavanols and Sensory Attributes. Bitterness and astringency are sensory attributes characteristic of fruits, vegetables, and beverages.[26] They are often perceived together given that the majority of astringent compounds are also bitter.[107] These attributes can be differentiated by the fact that bitterness is one of the five basic tastes, while astringency is a tactile sensation, or feeling factor.[28]

Bitterness is detected in taste buds located on papillae on the tongue.[26] The *TASTE 2 Receptor* (T2R) family, a specific set of taste receptors, is responsible for the detection of bitterness in humans. These receptors are fairly limited in number (approximately 30 receptors in this family have been identified), and it is not well understood how so few receptors can detect the thousands of bitter tasting compounds that naturally exist.[108,109] It is interesting to note that the T2R family of receptors is coded by a highly variable gene, which gives rise to variation in human sensitivity to bitterness.[110] It is believed that overall human bitterness sensitivity is correlated with sensitivity to certain compounds, such as 6-*n*-propyl-thiouracil (PROP) and phenylthiocarbamide (PTC). Methods developed for classification of bitterness sensitivity based on PROP or PTC rating can segment individuals into non-taster, taster, and supertaster categories.[111,112] Extremely PTC/PROP sensitive "supertasters" appear to have more taste buds sensitive to bitter compounds than nontasters.[5]

Astringency is a mouth drying, or puckering, tactile sensation perceived in the epithelium of the oral cavity.[26,27,113] The sensation is caused by the binding of certain compounds to salivary proteins, which causes them to precipitate and reduce oral lubrication.[10,114] Astringency is detected slowly and lingers in the mouth and throat.[115] Astringent sensations increase with repeated stimulation rather than decreasing due to adaptation. In contrast, sensations captured on taste buds (i.e. bitterness) decrease due to

adaptation with repeated stimulus.[107] Like bitterness, astringency perception can also vary in humans due to salivary flow rate, viscosity, and protein composition of the oral cavity.[24]

Bitterness and astringency are two of the dominant attributes of red wine flavor.[26,27] These attributes are directly linked to the DP of flavanols found in the wine.[26] Flavanol monomers and low DP compounds are more bitter while polymer compounds are more astringent.[1,28] Thus, grape seed flavanols are generally more bitter while grape skin flavanols are more astringent.[1,24] This phenomenon is likely due to the fact that larger molecules are not able to enter the taste buds and elicit a bitter response, but instead have greater protein-binding capabilities, which can lead to precipitation and astringency.[10]

There are several key factors related to bitterness and astringency perception and intensity. Concentration is one such factor. Scalar sensory techniques have indicated that higher concentrations of flavanols increase both astringency and bitterness and associated aftertastes of each attribute.[27] Examination of seed-dominant flavanols using time-intensity sensory procedures has indicated that epicatechin has a more intense and long-lasting bitter flavor and astringency perception compared to its chiral isomer, (+/−) catechin.[26,107] Polymerization of monomers to DP 2 and DP 3 reduces bitterness intensity duration, while also increasing astringency intensity and duration.[10] Specific linkages between monomers during polymerization also have an effect on perceived astringency.[26,115] Analytical and traditional sensory methods indicate that differences in protein binding due to structural variation of flavanols alter the intensity of astringent sensations.[114,116] Larger PCs with reportedly greater flexibility appear to be more able to bind proteins, particularly when the PCs contain more catechin subunits relative to epicatechin or epigallocatechin subunits. This directly increases perceived astringency. There does appear to be an upper limit to this trend, however, because increasing PC size decreases solubility, which in turn limits protein-binding capacity and perceived astringency.[24] Astringency also appears to increase with increasing galloylation of the PCs regardless of DP, which is due to the fact that galloylation increases protein interactions. [10,28,114,116] Additionally, polymers that are linked through a C4-C8 linkage have a more linear structure than C6-C8 linkages and therefore are more able to bind proteins and produce astringent sensations.[24]

Astringency and bitterness can also be influenced by other properties of wine in addition to flavanols. It has been observed that bitterness intensity increases proportionally with increasing alcohol concentration, but astringency does not.[26,113] In contrast, reducing pH by the addition of acids, regardless of the acid type, has been shown to increase astringency, but has no effect on bitterness.[26,114] Increasing the viscosity of wine decreases the intensity of both bitterness and astringency, as does the consumption of high-fat foods (like chocolate) with wine simultaneously.[24,26] Higher concentrations of sucrose and anthocyanins in wine have been shown to reduce astringency. This is likely due to the binding of PCs to these compounds, which prevents them from binding with salivary proteins.[24,114] Aging of wine can also influence astringency and bitterness. Polymerization of monomers to oligomers has been shown to increase astringency, but specific ethyl-linked catechin oligomers also have increased bitterness compared to other oligomers. Additionally, large polymer PCs oftentimes undergo cleavage reactions during aging, thus reducing astringency of aged wines. This observation explains why in some cases aged wine has reduced astringency despite the polymerization reactions which produce PC polymers that should increase astringency.[10] Finally, anthocyanins in wine have been shown to increase astringency more so than flavanols from skins or seeds alone.[28]

2.6.2 Bitterness, Astringency and Sensory Preferences. Astringency and bitterness of wine is attributed primarily to the flavanol profile of wine and is likewise related to the putative health benefits of wine consumption.[2,26] Despite these reported health benefits, however, it has been observed that humans tend to instinctively reject bitter tasting foods. Bitter tasting compounds are innately associated with being poisonous or toxic. As such, to increase consumer acceptance of food products, the food industry often endeavors to mask the flavors of flavanols and other health-promoting compounds in foods by adding sugar or other ingredients capable of masking bitterness, since taste is typically a bigger driver of liking, not healthfulness. Measures have [5,117] even been taken to selectively breed bitterants out of plant food material,[5] though interestingly, it has been observed that supplementing a food with flavanols may increase preference for an otherwise bitter food product.[118]

Trends in food selection and preference are complex and can be influenced by many factors including age, culture, ethnicity, gender, and exposure to certain foods.[119] It has been postulated that preference for bitter and astringent foods may be derived from association with a post-consumption desirable outcome or reward, such as increased nutritional benefit. In other words, preference towards the bitter and astringent taste of wine may be driven by inherent desire for the health-related outcomes associated with wine consumption.[120] Similarly, it has been observed that natural human preferences may be related to health status, as obese men and women tend to prefer high-fat foods compared to lean counterparts. It is, however, unknown whether chronic consumption of high-fat high sugar foods leads to the palate adapting to prefer these types of foods over time, or whether the obese phenotype is genetically predisposed to preferring the taste of these foods, thus contributing to obesity due to inherent preference. This suggests that either preference could fundamentally be altered by continuous selection of foods lacking nutrient density, or that inherent preference dictates health status by gravitating towards or away from bioactive compound containing foods.[121,122]

Regarding bitterness sensitivity and food selection, variable results have been reported. Some studies indicate decreased consumption of bitter foods (vegetables) with increased sensitivity to PROP, while others conclude no relation. Other reports indicate PROP tasters exhibit reduced liking for sweet foods, alcohol, and salad dressings.[123] This raises the question as whether bitterness sensitivity and health status are correlated. To date, only two studies have been performed on this topic, both of which indicated reduced bitterness sensitivity with increasing weight status. One of these studies indicated reduced bitterness sensitivity with increased weight status in children and adults, while the other indicated a correlation between liking of fatty foods and BMI. [124,125] Many questions remain, however, given that these studies were performed using survey methods, or using reference solutions for basic tastes (i.e. a sucrose solution for sweetness), rather than complex food systems with multiple taste and texture attributes. Moreover, the impact of flavanol mDP on consumer liking of flavanol-rich products has not been explored, nor has the impact of different consumer phenotypes on liking of a flavanol rich food-system.[119,123]

These observations raise the question as to how bitterness sensitivity, health status, and inherent preferences for bitter foods relate to preference of flavanol-rich wines with different mDPs. Studying these interactions would prove useful in the development of wine

and other flavanol-rich foods so that taste profiles related to flavanol mDP, bitterness, and astringency are optimized.

2.7 Conclusion. It is clear from this review of literature that the flavanols in wine impact sensory attributes and the health benefits of consumption. Wine constituents appear to exhibit a positive effect on various mechanisms related to MetS, DM, CVD, and cancer.[2,9,30,31,37,63,72,78,103] However, the role of flavanol mDP is unclear.[30] Furthermore, mDP influences the sensory characteristics of flavanol-rich foods, as smaller compounds are more bitter and larger compounds are more astringent.[1,24,28] The interaction between consumer phenotypes and mDP on food selection and preference is complex and not well understood.[119,123] The studies described in the following chapters were designed to examine how consumption of flavanols at different dosages and mDPs impacts the symptoms of MetS and whether sensory preference trends for wine are inherently driven by mDP, sensitivity and preference for astringent and bitter compounds, health status, or a combination of these factors. Flavanol extracts with distinctly different mDP were characterized and tested *in vivo* to determine biological activity related to symptoms of MetS at different dosages. Wine consumers were then given a wine-like solution made with the same flavanol extracts as part of a sensory preference test. Preference results were analyzed based on health status, preference for bitter foods, and PROP sensitivity. Finally, the ability to manipulate mDP in wine and wine-like products using different winemaking process approaches for the optimization of health benefits and sensory preference was explored.

REFERENCES

(1) Kennedy, J. A.; Saucier, C.; Glories, Y. Grape and Wine Phenolics: History and Perspective. *American Journal of Enology and Viticulture* **2006**, *57* (3), 239–248.

(2) Xu, Y.; Simon, J. E.; Welch, C.; Wightman, J. D.; Ferruzzi, M. G.; Ho, L.; Passinetti, G. M.; Wu, Q. Survey of Polyphenol Constituents in Grapes and Grape-Derived Products. *Journal of agricultural and food chemistry* **2011**, *59* (19), 10586–10593.

(3) Parr, A. J.; Bolwell, G. P. Phenols in the Plant and in Man. The Potential for Possible Nutritional Enhancement of the Diet by Modifying the Phenols Content or Profile. *Journal of the Science of Food and Agriculture* **2000**, *80* (7), 985–1012.

(4) Crozier, A.; Del Rio, D.; Clifford, M. N. Bioavailability of Dietary Flavonoids and Phenolic Compounds. *Molecular aspects of medicine* **2010**, *31* (6), 446–467.

(5) Drewnowski, A.; Gomez-Carneros, C. Bitter Taste, Phytonutrients, and the Consumer: A Review. *Am J Clin Nutr* **2000**, *72* (6), 1424–1435.

(6) Harnly, J. M.; Bhagwat, S.; Lin, L.-Z. Profiling Methods for the Determination of Phenolic Compounds in Foods and Dietary Supplements. *Analytical and bioanalytical chemistry* **2007**, *389* (1), 47–61.

(7) Manach, C.; Scalbert, A.; Morand, C.; Rémésy, C.; Jiménez, L. Polyphenols: Food Sources and Bioavailability. *The American journal of clinical nutrition* **2004**, *79* (5), 727–747.

(8) Han, X.; Shen, T.; Lou, H. Dietary Polyphenols and Their Biological Significance. *International Journal of Molecular Sciences* **2007**, *8* (9), 950–988.
(9) Soleas, G. J.; Diamandis, E. P.; Goldberg, D. M.; others. Wine as a Biological Fluid: History, Production, and Role in Disease Prevention. *Journal of clinical laboratory analysis* **1997**, *11* (5), 287–313.
(10) Cheynier, V.; Dueñas-Paton, M.; Salas, E.; Maury, C.; Souquet, J.-M.; Sarni-Manchado, P.; Fulcrand, H. Structure and Properties of Wine Pigments and Tannins. *American Journal of Enology and Viticulture* **2006**, *57* (3), 298–305.
(11) Beecher, G. R. Overview of Dietary Flavonoids: Nomenclature, Occurrence and Intake. *The Journal of nutrition* **2003**, *133* (10), 3248S–3254S.
(12) Boulton, R. B.; Singleton, V. L.; Bisson, L. F.; Kunkee, R. E. *Principles and Practices of Winemaking*; Springer Science & Business Media, 2013.
(13) Fang, F.; Li, J.-M.; Zhang, P.; Tang, K.; Wang, W.; Pan, Q.-H.; Huang, W.-D. Effects of Grape Variety, Harvest Date, Fermentation Vessel and Wine Ageing on Flavonoid Concentration in Red Wines. *Food Research International* **2008**, *41* (1), 53–60.
(14) Downey, M. O.; Dokoozlian, N. K.; Krstic, M. P. Cultural Practice and Environmental Impacts on the Flavonoid Composition of Grapes and Wine: A Review of Recent Research. *Am J Enol Vitic.* **2006**, *57* (3), 257–268.
(15) Jordão, A. M.; Ricardo-da-Silva, J. M.; Laureano, O. Evolution of Proanthocyanidins in Bunch Stems during Berry Development (Vitis Vinifera L.). *VITIS-Journal of Grapevine Research* **2001**, *40* (1), 17.
(16) Pérez-Magariño, S.; González-San José, M. L. Evolution of Flavanols, Anthocyanins, and Their Derivatives during the Aging of Red Wines Elaborated from Grapes Harvested at Different Stages of Ripening. *J. Agric. Food Chem.* **2004**, *52* (5), 1181–1189.
(17) Monagas, M.; Gómez-Cordovés, C.; Bartolomé, B.; Laureano, O.; Ricardo da Silva, J. M. Monomeric, Oligomeric, and Polymeric Flavan-3-Ol Composition of Wines and Grapes from Vitis Vinifera L. Cv. Graciano, Tempranillo, and Cabernet Sauvignon. *Journal of Agricultural and Food Chemistry* **2003**, *51* (22), 6475–6481.
(18) Federico Casassa, L.; Beaver, C. W.; Mireles, M. S.; Harbertson, J. F. Effect of Extended Maceration and Ethanol Concentration on the Extraction and Evolution of Phenolics, Colour Components and Sensory Attributes of Merlot Wines. *Australian Journal of Grape and Wine Research* **2013**, *19* (1), 25–39.
(19) Koyama, K.; Goto-Yamamoto, N.; Hashizume, K. Influence of Maceration Temperature in Red Wine Vinification on Extraction of Phenolics from Berry Skins and Seeds of Grape (Vitis Vinifera). *Bioscience, biotechnology, and biochemistry* **2007**, *71* (4), 958–965.
(20) Gay, M.; Apetrei, C.; Nevares, I.; del Alamo, M.; Zurro, J.; Prieto, N.; De Saja, J. A.; Rodríguez-Méndez, M. L. Application of an Electronic Tongue to Study the Effect of the Use of Pieces of Wood and Micro-Oxygenation in the Aging of Red Wine. *Electrochimica Acta* **2010**, *55* (22), 6782–6788.
(21) Fulcrand, H.; Dueñas, M.; Salas, E.; Cheynier, V. Phenolic Reactions during Winemaking and Aging. *American Journal of Enology and Viticulture* **2006**, *57* (3), 289–297.

(22) Bowser, S. M.; Moore, W. T.; McMillan, R. P.; Dorenkott, M. R.; Goodrich, K. M.; Ye, L.; O'Keefe, S. F.; Hulver, M. W.; Neilson, A. P. High Molecular Weight Cocoa Procyanidins Possess Enhanced Insulin-Enhancing and Insulin Mimetic Activities in Human Primary Skeletal Muscle Cells Compared to Smaller Procyanidins. *The Journal of Nutritional Biochemistry* **2016**.

(23) Dorenkott, M. R.; Griffin, L. E.; Goodrich, K. M.; Thompson-Witrick, K. A.; Fundaro, G.; Ye, L.; Stevens, J. R.; Ali, M.; O'Keefe, S. F.; Hulver, M. W.; et al. Oligomeric Cocoa Procyanidins Possess Enhanced Bioactivity Compared to Monomeric and Polymeric Cocoa Procyanidins for Preventing the Development of Obesity, Insulin Resistance, and Impaired Glucose Tolerance during High-Fat Feeding. *J. Agric. Food Chem.* **2014**, *62* (10), 2216–2227.

(24) McRae, J. M.; Kennedy, J. A. Wine and Grape Tannin Interactions with Salivary Proteins and Their Impact on Astringency: A Review of Current Research. *Molecules* **2011**, *16* (3), 2348–2364.

(25) Bitzer, Z. T.; Glisan, S. L.; Dorenkott, M. R.; Goodrich, K. M.; Ye, L.; O'Keefe, S. F.; Lambert, J. D.; Neilson, A. P. Cocoa Procyanidins with Different Degrees of Polymerization Possess Distinct Activities in Models of Colonic Inflammation. *The Journal of Nutritional Biochemistry* **2015**, *26* (8), 827–831.

(26) Lesschaeve, I.; Noble, A. C. Polyphenols: Factors Influencing Their Sensory Properties and Their Effects on Food and Beverage Preferences. *The American journal of clinical nutrition* **2005**, *81* (1), 330S–335S.

(27) Robichaud, J. L.; Noble, A. C. Astringency and Bitterness of Selected Phenolics in Wine. *Journal of the Science of Food and Agriculture* **1990**, *53* (3), 343–353.

(28) Brossaud, F.; Cheynier, V.; Noble, A. C. Bitterness and Astringency of Grape and Wine Polyphenols. *Australian Journal of grape and wine research* **2001**, *7* (1), 33–39.

(29) Lee, Y.; Kim, Y. J.; Eun, J. C.; Yokozawa, T. Ameliorative Effects of Proanthocyanidin on Oxidative Stress and Inflammation in Streptozotocin-Induced Diabetic Rats. *Journal of agricultural and food chemistry* **2007**, *55* (23), 9395–9400.

(30) Neilson, A. P.; O'Keefe, S. F.; Bolling, B. W. High-Molecular-Weight Proanthocyanidins in Foods: Overcoming Analytical Challenges in Pursuit of Novel Dietary Bioactive Components. *Annual Review of Food Science and Technology* **2016**, *7* (1), 43–64.

(31) Rasmussen, S. E.; Frederiksen, H.; Struntze Krogholm, K.; Poulsen, L. Dietary Proanthocyanidins: Occurrence, Dietary Intake, Bioavailability, and Protection against Cardiovascular Disease. *Molecular nutrition & food research* **2005**, *49* (2), 159–174.

(32) Holt, R. R.; Lazarus, S. A.; Sullards, M. C.; Zhu, Q. Y.; Schramm, D. D.; Hammerstone, J. F.; Fraga, C. G.; Schmitz, H. H.; Keen, C. L. Procyanidin Dimer B2 [Epicatechin-(4β-8)-Epicatechin] in Human Plasma after the Consumption of a Flavanol-Rich Cocoa. *Am J Clin Nutr* **2002**, *76* (4), 798–804.

(33) Cardona, F.; Andrés-Lacueva, C.; Tulipani, S.; Tinahones, F. J.; Queipo-Ortuño, M. I. Benefits of Polyphenols on Gut Microbiota and Implications in Human Health. *The Journal of Nutritional Biochemistry* **2013**, *24* (8), 1415–1422.

(34) Ozdal, T.; Sela, D. A.; Xiao, J.; Boyacioglu, D.; Chen, F.; Capanoglu, E.; Ozdal, T.; Sela, D. A.; Xiao, J.; Boyacioglu, D.; et al. The Reciprocal Interactions between Polyphenols and Gut Microbiota and Effects on Bioaccessibility. *Nutrients* **2016**, *8* (2), 78.

(35) Zhou, H.-C.; Lin, Y.-M.; Li, Y.-Y.; Li, M.; Wei, S.-D.; Chai, W.-M.; Tam, N. F. Antioxidant Properties of Polymeric Proanthocyanidins from Fruit Stones and Pericarps of Litchi Chinensis Sonn. *Food Research International* **2011**, *44* (2), 613–620.

(36) Renaud, S. Wine, Alcohol, Platelets, and the French Paradox for Coronary Heart Disease. *The Lancet* **1992**, *339* (8808), 1523–1526.

(37) Lippi, G.; Franchini, M.; Guidi, G. C. Red Wine and Cardiovascular Health: The "French Paradox" Revisited. *International Journal of Wine Research* **2010**, *2*, 1–7.

(38) O'Keefe, J. H.; Bybee, K. A.; Lavie, C. J. Alcohol and Cardiovascular Health: The Razor-Sharp Double-Edged Sword. *Journal of the American College of Cardiology* **2007**, *50* (11), 1009–1014.

(39) Hertog, M. G.; Kromhout, D.; Aravanis, C.; Blackburn, H.; Buzina, R.; Fidanza, F.; Giampaoli, S.; Jansen, A.; Menotti, A.; Nedeljkovic, S.; et al. Flavonoid Intake and Long-Term Risk of Coronary Heart Disease and Cancer in the Seven Countries Study. *Archives of internal medicine* **1995**, *155* (4), 381–386.

(40) Wollin, S. D.; Jones, P. J. H. Alcohol, Red Wine and Cardiovascular Disease. *J. Nutr.* **2001**, *131* (5), 1401–1404.

(41) Lindberg, M. L.; Amsterdam, E. A. Alcohol, Wine, and Cardiovascular Health. *Clin Cardiol* **2008**, *31* (8), 347–351.

(42) Rosell, M.; de Faire, U.; Hellénius, M.-L. Low Prevalence of the Metabolic Syndrome in Wine Drinkers—Is It the Alcohol Beverage or the Lifestyle? *Eur J Clin Nutr* **2003**, *57* (2), 227–234.

(43) Tulipani, S.; Llorach, R.; Jáuregui, O.; López-Uriarte, P.; Garcia-Aloy, M.; Bullo, M.; Salas-Salvadó, J.; Andrés-Lacueva, C. Metabolomics Unveils Urinary Changes in Subjects with Metabolic Syndrome Following 12-Week Nut Consumption. *Journal of proteome research* **2011**, *10* (11), 5047–5058.

(44) Fung, T. T.; Rimm, E. B.; Spiegelman, D.; Rifai, N.; Tofler, G. H.; Willett, W. C.; Hu, F. B. Association between Dietary Patterns and Plasma Biomarkers of Obesity and Cardiovascular Disease Risk. *Am J Clin Nutr* **2001**, *73* (1), 61–67.

(45) Athyros, V. G.; Liberopoulos, E. N.; Mikhailidis, D. P.; Papageorgiou, A. A.; Ganotakis, E. S.; Tziomalos, K.; Kakafika, A. I.; Karagiannis, A.; Lambropoulos, S.; Elisaf, M. Association of Drinking Pattern and Alcohol Beverage Type With the Prevalence of Metabolic Syndrome, Diabetes, Coronary Heart Disease, Stroke, and Peripheral Arterial Disease in a Mediterranean Cohort. *ANGIOLOGY* **2008**, *58* (6), 689–697.

(46) Klurfeld, D. M.; Kritchevsky, D. Differential Effects of Alcoholic Beverages on Experimental Atherosclerosis in Rabbits. *Experimental and molecular pathology* **1981**, *34* (1), 62–71.

(47) Tebib, K.; Bitri, L.; Besançon, P.; Rouanet, J.-M. Polymeric Grape Seed Tannins Prevent Plasma Cholesterol Changes in High-Cholesterol-Fed Rats. *Food Chemistry* **1994**, *49* (4), 403–406.

(48) Demrow, H. S.; Slane, P. R.; Folts, J. D. Administration of Wine and Grape Juice Inhibits in Vivo Platelet Activity and Thrombosis in Stenosed Canine Coronary Arteries. *Circulation* **1995**, *91* (4), 1182–1188.
(49) Lavy, A.; Fuhrman, B.; Markel, A.; Dankner, G.; Ben-Amotz, A.; Presser, D.; Aviram, M. Effect of Dietary Supplementation of Red or White Wine on Human Blood Chemistry, Hematology and Coagulation: Favorable Effect of Red Wine on Plasma High-Density Lipoprotein. *Annals of Nutrition and Metabolism* **1994**, *38* (5), 287–294.
(50) Struck, M.; Watkins, T.; Tomeo, A.; Halley, J.; Bierenbaum, M. Effect of Red and White Wine on Serum Lipids, Platelet Aggregation, Oxidation Products and Antioxidants: A Preliminary Report. *Nutrition Research* **1994**, *14* (12), 1811–1819.
(51) Fuhrman, B.; Lavy, A.; Aviram, M. Consumption of Red Wine with Meals Reduces the Susceptibility of Human Plasma and Low-Density Lipoprotein to Lipid Peroxidation. *The American journal of clinical nutrition* **1995**, *61* (3), 549–554.
(52) Preuss, H. G.; Wallerstedt, D.; Talpur, N.; Tutuncuoglu, S. O.; Echard, B.; Myers, A.; Bui, M.; Bagchi, D. Effects of Niacin-Bound Chromium and Grape Seed Proanthocyanidin Extract on the Lipid Profile of Hypercholesterolemic Subjects: A Pilot Study. *J Med* **2000**, *31* (5–6), 227–246.
(53) Vigna, G. B.; Costantini, F.; Aldini, G.; Carini, M.; Catapano, A.; Schena, F.; Tangerini, A.; Zanca, R.; Bombardelli, E.; Morazzoni, P.; et al. Effect of a Standardized Grape Seed Extract on Low-Density Lipoprotein Susceptibility to Oxidation in Heavy Smokers. *Metabolism* **2003**, *52* (10), 1250–1257.
(54) Yugarani, T.; Tan, B. K. H.; Teh, M.; Das, N. P. Effects of Polyphenolic Natural Products on the Lipid Profiles of Rats Fed High Fat Diets. *Lipids* **1992**, *27* (3), 181–186.
(55) Li, H.; Förstermann, U. Red Wine and Cardiovascular Health. *Circulation Research* **2012**, *111* (8), 959–961.
(56) Aviram, M.; Fuhrman, B. Wine Flavonoids Protect against LDL Oxidation and Atherosclerosis. *Annals of the New York Academy of Sciences* **2002**, *957* (1), 146–161.
(57) Sen, C. K.; Bagchi, D. Regulation of Inducible Adhesion Molecule Expression in Human Endothelial Cells by Grape Seed Proanthocyanidin Extract. *Molecular and cellular biochemistry* **2001**, *216* (1–2), 1–7.
(58) Estruch, R.; Sacanella, E.; Badia, E.; Antúnez, E.; Nicolás, J. M.; Fernández-Solá, J.; Rotilio, D.; de Gaetano, G.; Rubin, E.; Urbano-Márquez, A. Different Effects of Red Wine and Gin Consumption on Inflammatory Biomarkers of Atherosclerosis: A Prospective Randomized Crossover Trial: Effects of Wine on Inflammatory Markers. *Atherosclerosis* **2004**, *175* (1), 117–123.
(59) Kalfin, R.; Righi, A.; Del Rosso, A.; Bagchi, D.; Generini, S.; Guiducci, S.; Matucci Cerinic, M.; Das, D. K. Activin, a Grape Seed-Derived Proanthocyanidin Extract, Reduces Plasma Levels of Oxidative Stress and Adhesion Molecules (ICAM-1, VCAM-1 and E-Selectin) in Systemic Sclerosis. *Free radical research* **2002**, *36* (8), 819–825.

(60) Verstraeten, S. V.; Keen, C. L.; Schmitz, H. H.; Fraga, C. G.; Oteiza, P. I. Flavan-3-Ols and Procyanidins Protect Liposomes against Lipid Oxidation and Disruption of the Bilayer Structure. *Free Radical Biology and Medicine* **2003**, *34* (1), 84–92.

(61) Porter, M. L.; Krueger, C. G.; Wiebe, D. A.; Cunningham, D. G.; Reed, J. D. Cranberry Proanthocyanidins Associate with Low-Density Lipoprotein and Inhibit in Vitro Cu2+-Induced Oxidation. *Journal of the Science of Food and Agriculture* **2001**, *81* (14), 1306–1313.

(62) da Silva Porto, P. A. L.; Laranjinha, J. A. N.; de Freitas, V. A. P. Antioxidant Protection of Low Density Lipoprotein by Procyanidins: Structure/Activity Relationships. *Biochemical Pharmacology* **2003**, *66* (6), 947–954.

(63) Bagchi, D.; Bagchi, M.; Stohs, S. J.; Das, D. K.; Ray, S. D.; Kuszynski, C. A.; Joshi, S. S.; Pruess, H. G. Free Radicals and Grape Seed Proanthocyanidin Extract: Importance in Human Health and Disease Prevention. *Toxicology* **2000**, *148* (2–3), 187–197.

(64) Strat, K. M.; Rowley IV, T. J.; Smithson, A. T.; Tessem, J. S.; Hulver, M. W.; Liu, D.; Davy, B. M.; Davy, K. P.; Neilson, A. P. Mechanisms by Which Cocoa Flavanols Improve Metabolic Syndrome and Related Disorders. *The Journal of nutritional biochemistry* **2016**, *35*, 1–21.

(65) Leighton, F.; Miranda-Rottmann, S.; Urquiaga, I. A Central Role of ENOS in the Protective Effect of Wine against Metabolic Syndrome. *Cell biochemistry and function* **2006**, *24* (4), 291–298.

(66) Rimm, E. B.; Williams, P.; Fosher, K.; Criqui, M.; Stampfer, M. J. Moderate Alcohol Intake and Lower Risk of Coronary Heart Disease: Meta-Analysis of Effects on Lipids and Haemostatic Factors. *Bmj* **1999**, *319* (7224), 1523–1528.

(67) Bas, J. M. D.; Ricketts, M.-L.; Vaqué, M.; Sala, E.; Quesada, H.; Ardevol, A.; Salvadó, M. J.; Blay, M.; Arola, L.; Moore, D. D.; et al. DIETARY PROCYANIDINS ENHANCE TRANSCRIPTIONAL ACTIVITY OF BILE ACID-ACTIVATED FXR IN VITRO AND REDUCE TRIGLYCERIDEMIA IN VIVO IN A FXR-DEPENDENT MANNER. *Mol Nutr Food Res* **2009**, *53* (7), 805–814.

(68) Vidal, R.; Hernandez-Vallejo, S.; Pauquai, T.; Texier, O.; Rousset, M.; Chambaz, J.; Demignot, S.; Lacorte, J.-M. Apple Procyanidins Decrease Cholesterol Esterification and Lipoprotein Secretion in Caco-2/TC7 Enterocytes. *Journal of lipid research* **2005**, *46* (2), 258–268.

(69) Sugiyama, H.; Akazome, Y.; Shoji, T.; Yamaguchi, A.; Yasue, M.; Kanda, T.; Ohtake, Y. Oligomeric Procyanidins in Apple Polyphenol Are Main Active Components for Inhibition of Pancreatic Lipase and Triglyceride Absorption. *Journal of agricultural and food chemistry* **2007**, *55* (11), 4604–4609.

(70) Bas, J. M. D.; Ricketts, M. L.; Baiges, I.; Quesada, H.; Ardevol, A.; Salvadó, M. J.; Pujadas, G.; Blay, M.; Arola, L.; Bladé, C.; et al. Dietary Procyanidins Lower Triglyceride Levels Signaling through the Nuclear Receptor Small Heterodimer Partner. *Molecular Nutrition & Food Research* **2008**, *52* (10), 1172–1181.

(71) Dell'Agli, M.; Buscialà, A.; Bosisio, E. Vascular Effects of Wine Polyphenols. *Cardiovascular Research* **2004**, *63* (4), 593–602.

(72) Pechánová, O.; Bernátová, I.; Babál, P.; Martínez, M. C.; Kyselá, S.; Stvrtina, S.; Andriantsitohaina, R. Red Wine Polyphenols Prevent Cardiovascular Alterations in L-NAME-Induced Hypertension. *Journal of hypertension* **2004**, *22* (8), 1551–1559.

(73) Bernátová, I.; Pechánová, O.; Babál, P.; Kyselá, S.; Stvrtina, S.; Andriantsitohaina, R. Wine Polyphenols Improve Cardiovascular Remodeling and Vascular Function in NO-Deficient Hypertension. *American Journal of Physiology-Heart and Circulatory Physiology* **2002**, *282* (3), H942–H948.

(74) Al-Awwadi, N. A.; Bornet, A.; Azay, J.; Araiz, C.; Delbosc, S.; Cristol, J.-P.; Linck, N.; Cros, G.; Teissedre, P.-L. Red Wine Polyphenols Alone or in Association with Ethanol Prevent Hypertension, Cardiac Hypertrophy, and Production of Reactive Oxygen Species in the Insulin-Resistant Fructose-Fed Rat. *Journal of agricultural and food chemistry* **2004**, *52* (18), 5593–5597.

(75) Diebolt, M.; Bucher, B.; Andriantsitohaina, R. Wine Polyphenols Decrease Blood Pressure, Improve NO Vasodilatation, and Induce Gene Expression. *Hypertension* **2001**, *38* (2), 159–165.

(76) Fitzpatrick, D. F.; Bing, B.; Maggi, D. A.; Fleming, R. C.; O'MALLEY, R. M. Vasodilating Procyanidins Derived from Grape Seeds. *Annals of the New York Academy of Sciences* **2002**, *957* (1), 78–89.

(77) Andriambeloson, E.; Magnier, C.; Haan-Archipoff, G.; Lobstein, A.; Anton, R.; Beretz, A.; Stoclet, J. C.; Andriantsitohaina, R. Natural Dietary Polyphenolic Compounds Cause Endothelium-Dependent Vasorelaxation in Rat Thoracic Aorta. *The Journal of nutrition* **1998**, *128* (12), 2324–2333.

(78) Yilmaz, Y.; Toledo, R. Health Aspects of Functional Grape Seed Constituents. *Trends in Food Science & Technology 15* (9), 422–433.

(79) Ho, L.; Chen, L. H.; Wang, J.; Zhao, W.; Talcott, S. T.; Ono, K.; Teplow, D.; Humala, N.; Cheng, A.; Percival, S. S. Heterogeneity in Red Wine Polyphenolic Contents Differentially Influences Alzheimer's Disease-Type Neuropathology and Cognitive Deterioration. *Journal of Alzheimer's Disease* **2009**, *16* (1), 59–72.

(80) Suwannaphet, W.; Meeprom, A.; Yibchok-Anun, S.; Adisakwattana, S. Preventive Effect of Grape Seed Extract against High-Fructose Diet-Induced Insulin Resistance and Oxidative Stress in Rats. *Food and Chemical Toxicology* **2010**, *48* (7), 1853–1857.

(81) Galisteo, M.; Duarte, J.; Zarzuelo, A. Effects of Dietary Fibers on Disturbances Clustered in the Metabolic Syndrome. *J. Nutr. Biochem.* **2008**, *19* (2), 71–84.

(82) Pajuelo, D.; Quesada, H.; Díaz, S.; Fernández-Iglesias, A.; Arola-Arnal, A.; Bladé, C.; Salvadó, J.; Arola, L. Chronic Dietary Supplementation of Proanthocyanidins Corrects the Mitochondrial Dysfunction of Brown Adipose Tissue Caused by Diet-Induced Obesity in Wistar Rats. *British Journal of Nutrition* **2012**, *107* (02), 170–178.

(83) Agouni, A.; Lagrue-Lak-Hal, A.-H.; Mostefai, H. A.; Tesse, A.; Mulder, P.; Rouet, P.; Desmoulin, F.; Heymes, C.; Martínez, M. C.; Andriantsitohaina, R. Red Wine Polyphenols Prevent Metabolic and Cardiovascular Alterations Associated with Obesity in Zucker Fatty Rats (Fa/Fa). *PloS one* **2009**, *4* (5), e5557.

(84) Ardevol, A.; Blade, C.; Salvado, M. J.; Arola, L.; others. Changes in Lipolysis and Hormone-Sensitive Lipase Expression Caused by Procyanidins in 3T3-L1 Adipocytes. *International journal of obesity* **2000**, *24* (3), 319–324.
(85) Bargalló, M. V.; Grau, A. A.; de Dios Fernández-Larrea, J.; Anguiano, G. P.; Segarra, M. C. B.; Rovira, M. J. S.; Ferré, L. A.; Olivé, M. B. Moderate Red-Wine Consumption Partially Prevents Body Weight Gain in Rats Fed a Hyperlipidic Diet. *The Journal of nutritional biochemistry* **2006**, *17* (2), 139–142.
(86) Gu, Y.; Lambert, J. D. Modulation of Metabolic Syndrome-Related Inflammation by Cocoa. *Molecular nutrition & food research* **2013**, *57* (6), 948–961.
(87) Gu, Y.; Yu, S.; Lambert, J. D. Dietary Cocoa Ameliorates Obesity-Related Inflammation in High Fat-Fed Mice. *European journal of nutrition* **2014**, *53* (1), 149–158.
(88) Gourineni, V.; Shay, N. F.; Chung, S.; Sandhu, A. K.; Gu, L. Muscadine Grape (Vitis Rotundifolia) and Wine Phytochemicals Prevented Obesity-Associated Metabolic Complications in C57BL/6J Mice. *Journal of agricultural and food chemistry* **2012**, *60* (31), 7674–7681.
(89) Koppes, L. L.; Dekker, J. M.; Hendriks, H. F.; Bouter, L. M.; Heine, R. J. Moderate Alcohol Consumption Lowers the Risk of Type 2 Diabetes A Meta-Analysis of Prospective Observational Studies. *Diabetes care* **2005**, *28* (3), 719–725.
(90) Davies, M. J.; Baer, D. J.; Judd, J. T.; Brown, E. D.; Campbell, W. S.; Taylor, P. R. Effects of Moderate Alcohol Intake on Fasting Insulin and Glucose Concentrations and Insulin Sensitivity in Postmenopausal Women: A Randomized Controlled Trial. *Jama* **2002**, *287* (19), 2559–2562.
(91) Brand-Miller, J. C.; Fatima, K.; Middlemiss, C.; Bare, M.; Liu, V.; Atkinson, F.; Petocz, P. Effect of Alcoholic Beverages on Postprandial Glycemia and Insulinemia in Lean, Young, Healthy Adults. *The American journal of clinical nutrition* **2007**, *85* (6), 1545–1551.
(92) Joosten, M. M.; Beulens, J. W. J.; Kersten, S.; Hendriks, H. F. J. Moderate Alcohol Consumption Increases Insulin Sensitivity and ADIPOQ Expression in Postmenopausal Women: A Randomised, Crossover Trial. *Diabetologia* **2008**, *51* (8), 1375–1381.
(93) Griffin, L. E.; Dorenkott, M. R.; Goodrich, K. M.; Fundaro, G.; McMillan, R.; Hulver, M. W.; Ponder, M. A.; Neilson, A. P. Dietary Grape Seed Extract Inhibits Glucose Intolerance and Loss of Metabolic Flexibility Induced by High-Fat Diet. *FASEB J* **2013**, *27* (1 Supplement), 861.14-861.14.
(94) Su, H.-C.; Hung, L.-M.; Chen, J.-K. Resveratrol, a Red Wine Antioxidant, Possesses an Insulin-like Effect in Streptozotocin-Induced Diabetic Rats. *American Journal of Physiology-Endocrinology and Metabolism* **2006**, *290* (6), E1339–E1346.
(95) Meeprom, A.; Sompong, W.; Suwannaphet, W.; Yibchok-anun, S.; Adisakwattana, S. Grape Seed Extract Supplementation Prevents High-Fructose Diet-Induced Insulin Resistance in Rats by Improving Insulin and Adiponectin Signalling Pathways. *British journal of nutrition* **2011**, *106* (08), 1173–1181.
(96) Sierksma, A.; Patel, H.; Ouchi, N.; Kihara, S.; Funahashi, T.; Heine, R. J.; Grobbee, D. E.; Kluft, C.; Hendriks, H. F. Effect of Moderate Alcohol

Consumption on Adiponectin, Tumor Necrosis Factor-α, and Insulin Sensitivity. *Diabetes Care* **2004**, *27* (1), 184–189.

(97) Serrano, J. C. E.; Cassanye, A.; Martín-Gari, M.; Granado-Serrano, A. B.; Portero-Otín, M. Effect of Dietary Bioactive Compounds on Mitochondrial and Metabolic Flexibility. *Diseases* **2016**, *4* (1), 14.

(98) Casanova, E.; Baselga-Escudero, L.; Ribas-Latre, A.; Cedó, L.; Arola-Arnal, A.; Pinent, M.; Bladé, C.; Arola, L.; Salvadó, M. J. Chronic Intake of Proanthocyanidins and Docosahexaenoic Acid Improves Skeletal Muscle Oxidative Capacity in Diet-Obese Rats. *The Journal of Nutritional Biochemistry* **2014**, *25* (10), 1003–1010.

(99) Timmers, S.; Hesselink, M. K. C.; Schrauwen, P. Therapeutic Potential of Resveratrol in Obesity and Type 2 Diabetes: New Avenues for Health Benefits? *Annals of the New York Academy of Sciences* **2013**, *1290* (1), 83–89.

(100) Adisakwattana, S.; Moonrat, J.; Srichairat, S.; Chanasit, C.; Tirapongporn, H.; Chanathong, B.; Ngamukote, S.; Mauml, K.; Sapwarobol, S.; others. Lipid-Lowering Mechanisms of Grape Seed Extract (Vitis Vinifera L) and Its Antihyperlidemic Activity. *Journal of Medicinal Plants Research* **2010**, *4* (20), 2113–2120.

(101) Damianaki, A.; Bakogeorgou, E.; Kampa, M.; Notas, G.; Hatzoglou, A.; Panagiotou, S.; Gemetzi, C.; Kouroumalis, E.; Martin, P.-M.; Castanas, E. Potent Inhibitory Action of Red Wine Polyphenols on Human Breast Cancer Cells. *Journal of cellular biochemistry* **2000**, *78* (3), 429–441.

(102) Kampa, M.; Hatzoglou, A.; Notas, G.; Damianaki, A.; Bakogeorgou, E.; Gemetzi, C.; Kouroumalis, E.; Martin, P.-M.; Castanas, E. Wine Antioxidant Polyphenols Inhibit the Proliferation of Human Prostate Cancer Cell Lines. *Nutrition and cancer* **2000**, *37* (2), 223–233.

(103) Bianchini, F.; Vainio, H. Wine and Resveratrol: Mechanisms of Cancer Prevention? *European Journal of Cancer Prevention* **2003**, *12* (5), 417–425.

(104) Schneider, Y.; Vincent, F.; Duranton, B.; Badolo, L.; Gossé, F.; Bergmann, C.; Seiler, N.; Raul, F. Anti-Proliferative Effect of Resveratrol, a Natural Component of Grapes and Wine, on Human Colonic Cancer Cells. *Cancer letters* **2000**, *158* (1), 85–91.

(105) Lizarraga, D.; Lozano, C.; Briedé, J. J.; Van Delft, J. H.; Touriño, S.; Centelles, J. J.; Torres, J. L.; Cascante, M. The Importance of Polymerization and Galloylation for the Antiproliferative Properties of Procyanidin-Rich Natural Extracts. *FEBS journal* **2007**, *274* (18), 4802–4811.

(106) Bomser, J. A.; Singletary, K. W.; Wallig, M. A.; Smith, M. A. L. Inhibition of TPA-Induced Tumor Promotion in CD-1 Mouse Epidermis by a Polyphenolic Fraction from Grape Seeds. *Cancer letters* **1999**, *135* (2), 151–157.

(107) Kallithraka, S.; Bakker, J.; Clifford, M. N. Evaluation of Bitterness and Astringency of (+)-Catechin and (-)-Epicatechin in Red Wine and in Model Solution. *Journal of Sensory Studies* **1997**, *12* (1), 25–37.

(108) Meyerhof, W.; Batram, C.; Kuhn, C.; Brockhoff, A.; Chudoba, E.; Bufe, B.; Appendino, G.; Behrens, M. The Molecular Receptive Ranges of Human TAS2R Bitter Taste Receptors. *Chemical senses* **2010**, *35* (2), 157–170.

(109) Mueller, K. L.; Hoon, M. A.; Erlenbach, I.; Chandrashekar, J.; Zuker, C. S.; Ryba, N. J. P. The Receptors and Coding Logic for Bitter Taste. *Nature* **2005**, *434* (7030), 225–229.
(110) Pronin, A. N.; Xu, H.; Tang, H.; Zhang, L.; Li, Q.; Li, X. Specific Alleles of Bitter Receptor Genes Influence Human Sensitivity to the Bitterness of Aloin and Saccharin. *Current Biology* **2007**, *17* (16), 1403–1408.
(111) Prescott, J.; Tepper, B. J. *Genetic Variation in Taste Sensitivity*; CRC Press, 2004.
(112) Pickering, G. J.; Simunkova, K.; DiBattista, D. Intensity of Taste and Astringency Sensations Elicited by Red Wines Is Associated with Sensitivity to PROP (6-n-Propylthiouracil). *Food Quality and Preference* **2004**, *15* (2), 147–154.
(113) Fontoin, H.; Saucier, C.; Teissedre, P.-L.; Glories, Y. Effect of PH, Ethanol and Acidity on Astringency and Bitterness of Grape Seed Tannin Oligomers in Model Wine Solution. *Food Quality and Preference* **2008**, *19* (3), 286–291.
(114) Boulet, J.-C.; Trarieux, C.; Souquet, J.-M.; Ducasse, M.-A.; Caillé, S.; Samson, A.; Williams, P.; Doco, T.; Cheynier, V. Models Based on Ultraviolet Spectroscopy, Polyphenols, Oligosaccharides and Polysaccharides for Prediction of Wine Astringency. *Food Chemistry* **2016**, *190*, 357–363.
(115) Peleg, H.; Gacon, K.; Schlich, P.; Noble, A. C. Bitterness and Astringency of Flavan-3-Ol Monomers, Dimers and Trimers. *J. Sci. Food Agric.* **1999**, *79* (8), 1123–1128.
(116) Cheynier, V. Polyphenols in Foods Are More Complex than Often Thought. *The American journal of clinical nutrition* **2005**, *81* (1), 223S–229S.
(117) Roy, G. M. The Applications and Future Implications of Bitterness Reduction and Inhibition in Food Products. *Critical Reviews in Food Science & Nutrition* **1990**, *29* (2), 59–71.
(118) Kaushik, R.; Narayanan, P.; Vasudevan, V.; Muthukumaran, G.; Usha, A. Nutrient Composition of Cultivated Stevia Leaves and the Influence of Polyphenols and Plant Pigments on Sensory and Antioxidant Properties of Leaf Extracts. *Journal of food science and technology* **2010**, *47* (1), 27–33.
(119) Cox, D. N.; Hendrie, G. A.; Carty, D. Sensitivity, Hedonics and Preferences for Basic Tastes and Fat amongst Adults and Children of Differing Weight Status: A Comprehensive Review. *Food Quality and Preference* **2016**, *48*, 359–367.
(120) Drewnowski, A. Taste Preferences and Food Intake. *Annual Review of Nutrition* **1997**, *17* (1), 237–253.
(121) Drewnowski, A.; Brunzell, J. D.; Sande, K.; Iverius, P. H.; Greenwood, M. R. C. Sweet Tooth Reconsidered: Taste Responsiveness in Human Obesity. *Physiology & Behavior* **1985**, *35* (4), 617–622.
(122) Drewnowski, A.; Kurth, C.; Holden-Wiltse, J.; Saari, J. Food Preferences in Human Obesity: Carbohydrates versus Fats. *Appetite* **1992**, *18* (3), 207–221.
(123) Tepper, B. J.; White, E. A.; Koelliker, Y.; Lanzara, C.; D'Adamo, P.; Gasparini, P. Genetic Variation in Taste Sensitivity to 6-n-Propylthiouracil and Its Relationship to Taste Perception and Food Selection. *Annals of the New York Academy of Sciences* **2009**, *1170* (1), 126–139.

(124) Overberg, J.; Hummel, T.; Krude, H.; Wiegand, S. Differences in Taste Sensitivity between Obese and Non-Obese Children and Adolescents. *Archives of disease in childhood* **2012**, archdischild–2012.
(125) Simchen, U.; Koebnick, C.; Hoyer, S.; Issanchou, S.; Zunft, H.-J. Odour and Taste Sensitivity Is Associated with Body Weight and Extent of Misreporting of Body Weight. *European journal of clinical nutrition* **2006**, *60* (6), 698.

CHAPTER 3:
Flavanol Supplementation Protects Against Obesity-Associated Inflammation by an Obesity-Independent Pathway

Submitted to Nutrition Research

ABSTRACT

Weight gain and obesity are associated with increased inflammatory burden, including increased levels of pro-inflammatory cytokines. Studies have demonstrated the ability of dietary flavanols to reduce the severity of metabolic derangements associated with weight gain. Flavanol mean degrees of polymerization (mDP) appears to play a role in determining the extent of the protective effects. This study evaluated the preventative effects of grape seed and pine bark flavanol supplementation in the context of a high-fat (HF) diet. These extracts were significantly different in terms of flavanol mDP. For 13 weeks, mice were given 35 mg/kg bw*d grape seed or pine bark as part of a HFD and compared to mice fed a low-fat diet (LFD) and control HFD. All flavanol supplemented groups and the HF control incurred significantly higher weight gain compared to the lean control, and the grape seed group gained significantly more weight than the HF control. Increased weight gain of treatment groups was likely caused by hyperphagia. Despite lack of improvements to weight gain and glycemic control, it was observed that all flavanol treatment groups were able to significantly reduce IL-6 compared to HF control. The grape seed group, which gained the most weight overall also exhibited the lowest levels of IL-6 compared to other treatments. Overall, low-dose flavanol extract supplementation, regardless of mDP, blunted cytokine production despite increased weight gain. This obesity-independent effect suggests flavanols may be used as complementary interventions to ameliorate increased inflammatory tone in the contexts of obesity and diabetes.

3.1 INTRODUCTION

The metabolic syndrome, a cluster of metabolic derangements including obesity, hyperglycemia, hypertension, hypercholesterolemia, and elevated blood triglycerides, presently affects at least 35% of the US population [1]. Chronic low-grade inflammation is a prominent characteristic of those with the disease. Moreover, elevated cytokine production and inflammatory tone has also been linked to insulin resistance [2]. Many of the abnormalities related to the metabolic syndrome are associated with modifiable lifestyle factors. As such, it is pertinent to study modifiable risk factors, such as diet, in order to reduce the deleterious effects of this syndrome [1]. Supplementation of dietary flavanols is one such potential approach [3].

Flavanols are a subclass of flavonoids, and are found widely in foods such as red wine, tea, and chocolate [4]. Flavanols consist of monomers such as (+)-catechin and (−)-epicatechin, as well as oligomers and polymers of these monomers, known as procyanidins (PCs) [5,6]. Individual flavanols can be characterized by their degree of polymerization (DP). Foods containing a variety of flavanols can be characterized by their mean degree of polymerization (mDP), or the average DP of all constituent flavanols [7]. Flavanols comprise ~85% of the total phenolic composition of red wines [8,9]. However, the flavanol profile of an individual wine and its associated mDP can vary greatly due to differences in grape genotype, viticulture practices, environmental factors, and winemaking practices [10,11]. Differences in flavanol profile and mDP can also exist in commercially available dietary supplements [12].

Interest in flavanols has increased due to their potential role in the prevention of cardiovascular disease, type 2 diabetes, and cancer [4,5,12,13]. Studies from our group have indicated a protective effect of low-dose flavanol-rich grape seed extract (GSE) on gut health and diabetes [14,15]. Furthermore, there is a correlation between flavanol DP and bioactivity [7,16,17]. Polymeric flavanols (DP > 7) appear to improve glucose tolerance by stimulating glucose uptake in skeletal muscle [7] and slowing intestinal absorption of glucose [18]. Oligomeric flavanols (DP 2-6) also prevent excessive weight and fat gain in mouse fed a high-fat diet [16] while reducing blood glucose levels in diabetic rats [19]. Compounds of various DP have demonstrated protection against inflammation through mechanisms involving reduced NF-κB activation and enhanced IL-4 levels [20–22]. Overall, the metabolic benefits of differently sized flavanols depends on the source, disease and target tissue for activity [3].

The objective of this study was to determine whether foods containing flavanols of distinct mDP exert distinct benefits on markers of metabolic syndrome in the context of high-fat (HF) feeding. Two plant extracts having different mDP profiles [GSE and pine bark extract (PBE)] were provided at physiologically relevant doses to determine their effects in mice fed a high-fat (HF) diet.

3.2 MATERIALS AND METHODS

3.2.1 Chemical Standards and Reagents. Chemical standards included (±)-catechin (C), (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG), and (−)-epigallocatetchin gallate (EGCG) from Sigma, procyanidin (PC) dimer B_2 and trimer C_1 from ChromaDex, and PC dimer B_1, dimer B_2-gallate, dimer B_5, trimer T_2, tetramer A_2, and pentamers through decamers (DP5-DP10) from Planta Analytica. Solvents for sample preparation and normal phase HPLC were of ACS grade or better (Fisher, VWR). Solvents for UPLC-MS/MS were of LC-MS grade (VWR).

3.2.2 Polyphenol extracts. Vitaflavin, a white grape seed extract (GSE) (Lot 181476), and Oligopin, a maritime pine bark extract (PBE) (Lot 182938), that are commercially available for purchase and use, were obtained from DRT. These extracts were selected for use in this study based on manufacturer specifications (**Table A1**) indicating differences in mDP, which were confirmed in our lab prior to study initiation. Thus, GSE and PBE were specifically used in these studies to simulate consumption patterns of foods rich in small versus large flavanols without interference of other polyphenols, including anthocyanins. When not in use, the extracts were stored at −20°C.

3.2.3 Determination of mDP by Thiolysis. The mDP of GSE and PBE were determined by thiolysis as described previously [16] with minor modifications. Full details are found in Supplementary Information. **Table A2** details the elution gradient program for thiolysis and **Table A3** details the MRM parameters for the monomers and derivatives quantified.

3.2.4 Qualitative Analysis of PCs by Normal Phase HPLC. Qualitative polyphenol profiles of GSE and PBE were assessed by the method described previously [17]. Full details are found in Supplementary Information. **Table A4** details the binary elution gradient program used.

3.2.5 Quantitative Analysis of Individual PCs by Reverse Phase by UPLC. Individual monomeric flavanols and PCs were quantified by UPLC-MS/MS up to DP 10. GSE and PBE (n = 3) were diluted to 0.1 mg/mL with 0.1% formic acid in water/0.1% formic acid in acetonitrile (95:5). Samples were analyzed as described for thiolysis. **Table A5** details the MRM parameters for the monomers and PCs quantified. A complete list of quantified monomers and PCs for each of the dietary plant extracts can be found in **Table A6**.

3.2.6 Estimations of Total Polyphenols and Flavanols. The Folin-Ciocalteu (FC) assay was performed to estimate total polyphenols content of GSE and PBE based on the method described in Ainsworth & Gillespie with modification [23]. The 4-dimethylaminocinnamaldehyde (DMAC) assay was performed to estimate total flavanols in GSE and PBE. The method was adapted from Payne et al. [24]. Full details are found in Supplementary Information.

3.2.7 Animals. All animal experiments were approved by the Institutional Animal Care and Use Committee at Virginia Tech (protocol #17-085 FST) before beginning. Five-week old C57BL/6J male mice (n = 72, ~20 g) were obtained from Jackson Laboratory and acclimatized to the facility for 1 week before cage randomization to experimental diets. Mice were maintained in standard housing conditions (12 h light/dark cycle, 30-70% relative humidity, 20-26°C, 4 mice/cage) for the study duration. Cage bedding was changed twice weekly to minimize coprophagy. Mice were provided food and water *ad libitum* unless otherwise noted.

3.2.8 Dietary Treatments. Immediately following acclimatization, mice were assigned randomly to one of six diets (n = 12 mice/diet; **Table 3.1**). The control low-fat (LF; D12450J) and high-fat (HF; D12492) diets were purchased from Research Diets Inc. and contained 10% and 60% energy from fat, respectively, as detailed previously [16]. Both diets were matched for protein and sucrose content. Flavanols were incorporated into the HF diet to create experimental treatment diets to compare their potential mDP-dependent protective effects on metabolic syndrome. GSE was used to formulate a low mDP diet and PBE to provide a high mDP diet (**Figure 3.1A**). The extracts

were significantly different in terms of mDP, which we believe to be physiological relevant, despite small absolute differences between mDP (2.1 vs 2.7). Small but significant differences in mDP have yielded differences in obesity outcomes in our lab previously [16]. Experimental diets, detailed in **Table 3.2**, were formulated to provide ~35 mg extract/kg body wt/d (treatment G for GSE-weight basis and PW for PBE-weight basis). The results from the FC assay indicate that the PBE is not entirely comprised of flavanols (**Figure 3.1C**). As such, a third experimental treatment was formulated so that the dosage was matched to treatment G based on the total polyphenol content of PBE as determined by the Folin-Ciocalteu assay (treatment PF, PBE-Folin basis). GSE was comprised entirely of flavanols so an additional treatment adjusting for flavanol content was unnecessary. The exact diet formulations were made based on preliminary data estimating daily food consumption of 81.63 g HF food/kg body wt/d [14]. These low dosages were selected based on our preliminary data indicating that relatively lower flavanol dosage were more effective than higher doses, and they are also more physiologically relevant [15]. Humans would need, on average, to consume 2.8 mg extract/kg body mass (168 mg/60 kg) to achieve daily dosage equivalent to that given mice in this study based on body surface area [25].

Table 3.1 Description of Mouse Diets and Treatment Groups

Treatment	Abbreviation	Fat (% kcal)	Additive	Total Polyphenols (g GAE/g extract)[a]	Dose (mg extract/kg/d)[b]	Food Concentration (mg extract/kg diet)
Lean Control	LF	10	NA	NA	NA	NA
HF Control	HF	60	NA	NA	NA	NA
GSE by Weight	G	60	GSE	0.99	35	428.8
PBE by Weight	PW	60	PBE	0.68	35	428.8
PBE by Polyphenols	PF	60	PBE	0.68	51.3	628.1

[a]based on the Folin-Ciocalteu assay

[b]calculated based on pilot data, indicating that mice eat, on average, 81.63 g HF food/kg BW/d

Animals were maintained on experimental diets for 13 weeks. LF diet was replaced weekly and HF control and treatment diets were replaced twice weekly to prevent lipid oxidation and degradation of flavanols. Food intake on a per cage basis was calculated as the difference between food weight added and food weight remaining for each feeding period. When not in use, diets were stored at −20°C.

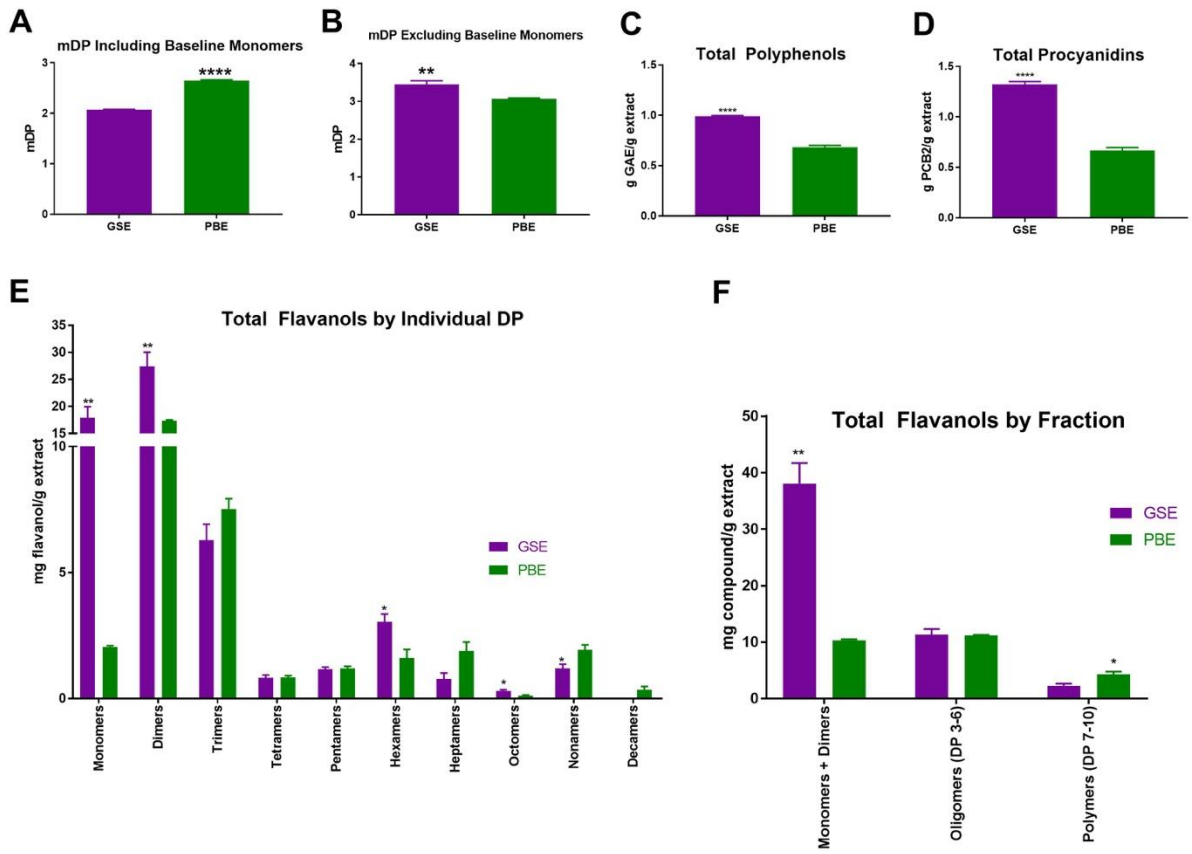

Figure 3.1 *Calculated mDP values for GSE and PBE when endogenous monomers (monomers present prior to thiolysis) are included in the calculation (A) and when these monomers are excluded from the calculation (B). mDP values are expressed as means (n = 5) ± SEM. Total polyphenols (Folin) in GSE and PBE expressed as g GAE/g extract (C) and total procyanidins (DMAC) in GSE and PBE expressed as g PCB2/g extract (D). Values are expressed as means (n = 7) ± SEM. LC-MS quantification of representative flavanols in GSE and PBE by individual DP (E) and grouped by monomer + dimer, oligomer, or polymer (F). Values are reported as means (n = 3) ± SEM. Significance for all analyses (between GSE and PBE for each measure) are denoted with asterisks, as calculated using unpaired Student's T tests: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. It must be noted for E and F that quantified compounds were those we had chemical standards for and do not include all PCs in each extract.*

Table 3.2 Composition of Mouse Diets from the Diet-Induced Obesity Series by Research Diets Inc.

Component	LF Diet (D12450J)		HF Diet (D12492)		GW Diet (17051705)		PW Diet (17051707)		PF Diet (17051708)	
	g %	kcal %	g %	kcal %	g %	kcal %	g %	kcal %	g %	kcal %
Protein	19.2	20	26.2	20	26.2	20	26.2	20	26.2	20
Carbohydrate	67.3	70	26.3	20.1	26.3	20.1	26.3	20.1	26.3	20.1
Fat	4.3	10	34.9	59.9	34.9	59.9	34.9	59.9	34.9	59.9
Total		100		100		100		100		100
kcal/g	3.9		5.24		5.24		5.24		5.24	
Ingredient	**g**	**kcal**	**g**	**kcal**	**g**	**kcal**	**g**	**kcal**	**g**	**kcal**
Casein, 80 Mesh	200	800	200	800	200	800	200	800	200	800
L-Cystine	3	12	3	12	3	12	3	12	3	12
Corn Starch	506.2	2024.8	0	0	0	0	0	0	0	0
Maltodextrin 10	125	500	125	500	125	500	125	500	125	500
Sucrose	68.8	275.2	68.8	275.2	68.8	275.2	68.8	275.2	68.8	275.2
Cellulose, BW200	50	0	50	0	50	0	50	0	50	0
Soybean Oil	25	225	25	225	25	225	25	225	25	225
Lard	20	180	245	2205	245	2205	245	2205	245	2205
Mineral Mix S10026	10	0	10	0	10	0	10	0	10	0
Dicalcium Phosphate	13	0	13	0	13	0	13	0	13	0
Calcium Carbonate	5.5	0	5.5	0	5.5	0	5.5	0	5.5	0
Potassium Citrate, 1 H2O	16.5	0	16.5	0	16.5	0	16.5	0	16.5	0
Vitamin Mix V10001	10	40	10	40	10	40	10	40	10	40
Choline Bitartrate	2	0	2	0	2	0	2	0	2	0
Grape Seed Extract	0	0	0	0	0.332	0	0	0	0	0
Pine Bark Extract	0	0	0	0	0	0	0.332	0	0.487	0
Total	1055.1	4057	773.85	4057	774.18	4057	774.18	4057	774.18	4057

3.2.9 Body Weight and Composition. Body mass was measured weekly. Body composition (fat, lean, and free fluid mass) was assessed at weeks 1, 6, 9, and 12 to using a Bruker LF90 NMR analyzer according to manufacturer specifications.

3.2.10 Glucose and Insulin Tolerance Tests. An oral glucose tolerance test (OGTT) was performed as described in Andrikopoulos et al. [26] at weeks 6 and 10 using a 20% dextrose solution and administered to provide 2 g glucose/kg body mass. Blood samples were collected at 0, 30, 60, 90, 120, and 180 min from the tail vein. In addition, an intraperitoneal (i.p.) GTT was performed in all mice at week 11 using a 15% dextrose solution that provided 1.5 g glucose/kg body mass. For the insulin tolerance test (ITT) performed in week 12, mice were given insulin i.p. at 0.65 U/kg body mass in saline. Full test details can be found in Supplementary Information. Area under the curve (AUC), Cmax (maximum concentration of blood glucose observed in the 2-hr period) and excursion (difference between minimum and maximum blood glucose values) were determined from the data collected.

3.2.11 Euthanasia and Necropsy. At study termination (week 13), mice were fasted 12 for hours (overnight) and euthanized by CO_2 followed by bilateral pneumothorax per AVMA guidelines. Liver and epididymal adipose tissues were excised, weighed, snap frozen in liquid nitrogen, and stored at −80°C until analysis. Blood was collected by cardiac puncture with 1 cc tuberculin syringes and 1/2" 26-gauge needles, clotted at room temperature for 30 min in BD Vacutainer® serum separation tubes, and centrifuged (15 min, 4°C, 1,500 x *g*). The resulting serum was collected and stored at −80°C.

3.2.12 Liver Analyses. Lipids were extracted from liver tissue. 200 mg of sample was weighed and placed in 1 mL of 25 µM HEPES (pH 7.4). Samples were individually transferred to a glass homogenizer tube, passed through a homogenizer 10 times (100 rpm), and the contents were poured back into the original tube. The homogenizer tube was then rinsed with 0.5 mL 25 µM HEPES, passed though the homogenizer 5 more times, and the residual was transferred to the sample tube. Once homogenized, 1 mL of each sample was aliquoted into a 15 mL plastic centrifuge tube with 2 mL of 3:2 heptane:isopropanol. Tubes were vortexed for 15 s and left on a rotator at room temperature overnight (60 rpm). A pea-sized amount of magnesium sulfate was added to each tube on the following day, samples were vortexed for 15 s, and centrifuged for 10 min at 1100 x *g*. The upper organic phase was transferred to a pre-weighed glass tube. The original sample material was washed with 3 mL heptane, vortexed, and centrifuged again for 10 min at 1100 x *g*. Again, the top layer was transferred to the pre-weighed glass tube. Solvents were dried down using a SpeedVap and residual oil was weighed. Hepatic lipid peroxidation was determined by measuring malondialdehyde (MDA) in liver homogenates by HPLC-FL as described previously [27]. MDA concentrations were normalized to hepatic protein, which was assessed using a Pierce BCA Protein Assay Kit purchased from Thermo Scientific.

3.2.13 Inflammatory Markers. ELISA kits for interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) were purchased from Cayman Chemical and utilized per manufacturer specifications for mouse adipose tissue. Adipose tissue was first prepared by weighing approximately 200 mg into a microcentrifuge tube with zirconium oxide beads (Next Advance) and 1 mL of RIPA buffer, beating samples with a Bullet Blender (Next Advance) for 5 min, ultrasonicating for 2 min on ice (Fisher Scientific), centrifuging (17,000 x *g*, 5 min, accuSpin Mircro 17, Fisher Scientific), and

collecting the bottom aqueous fraction. Analysis of TNF-α was performed per the manufacturer instructions with the 200 mg/mL adipose extract and the IL-6 analysis was performed per manufacturer instructions after further dilution of the extract to 10 mg/mL. Quantification of the protein in each of the adipose samples was determined in triplicate by BCA protein analysis (Pierce™ BCA Protein Assay Kit, Thermo Scientific). Analysis was performed according to manufacturer specifications after dilution of the samples to 40 mg/mL in RIPA buffer. Serum total cholesterol, glucose and triglycerides were measured using reagent kits purchased from Pointe Scientific.

3.2.14 Data and Statistical Analysis. Statistical analysis was performed by 1-way ANOVA with Fisher's LSD post-hoc test to assess pair-wise treatment differences using GraphPad Prism v.7. Dixon's Q-test was used beforehand to detect and remove outliers from treatment groups as needed. Significance was defined *a priori* as $p < 0.05$.

3.3 RESULTS

3.3.1 Flavanol Extracts. Total polyphenol (**Figure 3.1C**) and total PC (**Figure 3.1D**) concentrations were significantly higher in GSE compared to PBE. When comparing FC and DMAC values of each extract, it appeared that the lower values for PBE on a weight basis are due to the terpene content of PBE, as terpenes are abundant in pine [28]. The DMAC responses indicated a greater relative difference than FC values for the extracts, which can be explained by assay responses for PC standards (**Figure A1**). Monomer compounds exhibit approximately doubled response in DMAC relative to FC. Thiolysis was performed to estimate mDP of the constituent flavanols of GSE and PBE. mDP values were calculated two different ways: including and excluding the native monomers in the samples (**Figure 3.1A and B**). The flavanol extracts exhibited significantly different mDPs based on calculations including and excluding native monomers (equations shown in Supplementary Information). Including monomers (the best overall measure of mDP, including all flavanols), GSE and PBE exhibited mean mDPs of 2.1 ± 0.01 and 2.7 ± 0.01, respectively. When excluding monomers (i.e., the mDP of the oligomeric and polymeric flavanols, i.e. the procyanidins), GSE and PBE exhibited mean mDPs of 3.5 ± 0.1 and 3.1 ± 0.02, respectively. These data demonstrate that overall GSE has a lower mDP, which is primarily due to the influence of the large proportion of monomers in GSE (PBE has smaller oligomeric and polymeric flavanols, but more of them as a percentage of weight, and thus the weighted average results in a higher overall mDP for PBE).

While this analysis is somewhat qualitative and approximates relative differences between groups rather than true absolutes, these relative differences were significant and therefore likely to be relevant as a model of consumption of flavanol-rich foods with different mDPs in the monomer-oligomer range [16], consistent with our previous study in which larger mDPs were examined [16]. Other supporting analyses were performed to validate thiolysis findings. Qualitative analysis of the polyphenol profile of each extract by normal-phase HPLC indicated that similar DP ranges are present in both extracts (**Figure A2**). However, since fluorescence peak areas are not directly indicative of abundance due to decreasing response factors with increasing DP [29], individual flavanols were also quantified to confirm mDP results. Quantitative analysis of individual flavanols by UPLC-MS/MS indicated a significantly higher concentration of monomers in the GSE and a significantly higher concentration of polymers in the PBE, with roughly similar oligomer content, (**Figure 3.1E and F**), in agreement with the thiolysis findings. Given the

significant differences in mDP and flavanol profiles observed in the GSE and PBE, it was determined that they would be suitable to formulate rodent diets in the present study to test the hypothesis that HF-induced metabolic syndrome can be ameliorated in a mDP-dependent manner.

3.3.2 Weight and Body Composition. At study onset, there was no significant difference in body weight between treatment groups (**Figure A3**). Weight and fat gain over time are displayed in **Figure 3.2A and B**. The HF control and all experimental treatment groups progressively gained more weight and fat throughout the study compared to the LF control. Surprisingly, when examining total weight and adiposity at the end of the study (**Figure 3.2C and D**), the experimental flavanol groups did not significantly reduce weight gain compared to HF control. Rather, the G group exhibited significantly greater weight gain compared with the HF control and both PBE treated groups. Similarly, and again contrary to our hypothesis, total fat gain was not significantly improved due to flavanol treatments as no significant differences were observed between the HF group and the treatment groups. These results were much different than previously observed in similar HF feeding studies involving flavanol extracts dosed at similar levels [15,16]. In previous experiments from our lab, including one with the same GSE, beneficial whole-body treatment effects, such as reduced weight and fat gain, were observed [15,16]. It seemed possible that another variable was influencing the physiological changes observed in this study. Diet compositions were verified using formulation records obtained from the manufacturer to eliminate this as a factor.

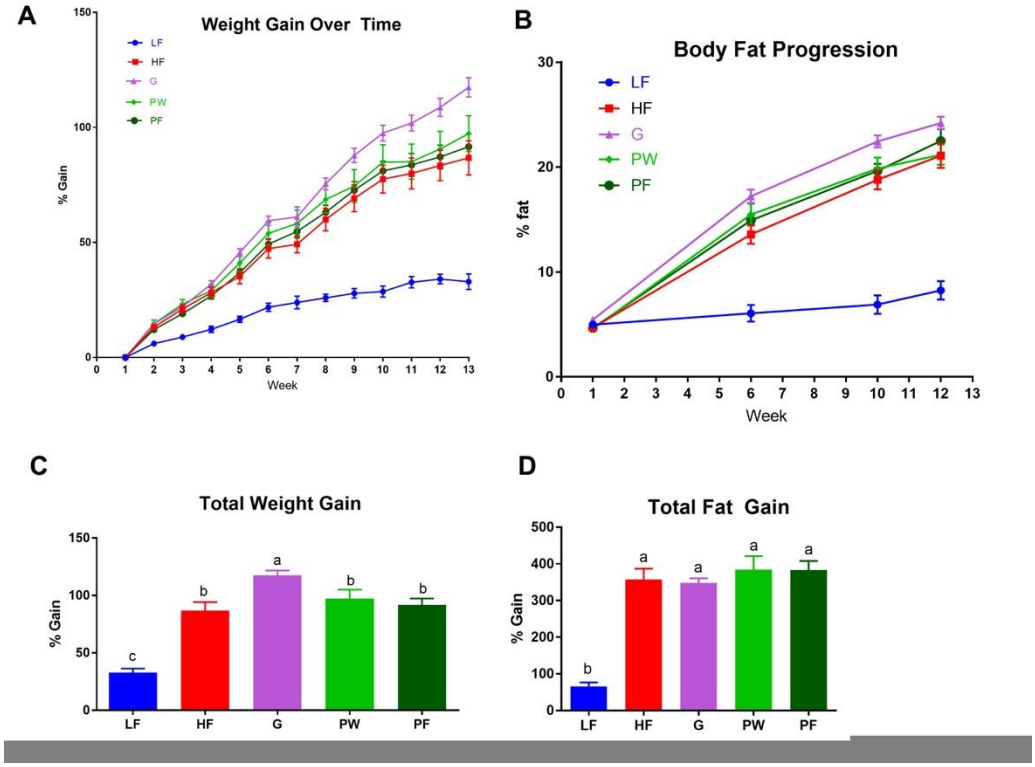

Figure 3.2 *A) Mouse weight gain over time based on percent of initial weight. B) Mouse body fat gain over time based on percent of initial fat mass. C) Total weight gain based on percent of initial weight. D) Total fat mass gain based on percent of initial fat mass. All values are expressed as means (n = 12/trt for HF, G and PF, n = 9 for LF, and n = 11 for PW) ± SEM. Significance of treatments was determined using 1-way ANOVA with Fisher's LSD post hoc test and was defined as p < 0.05 with different letters designating significantly different values for panels C and D.*

3.3.3 Food Intake. To investigate possible factors responsible for the unexpected observed results, we examined food and energy intake throughout the study. Weekly food intake data are shown in **Figure A4**. Trends in weekly food intake were generally consistent across diets. Cumulative weekly intake on a food weight basis was not significantly different across treatment groups (**Figure 3.3A**). As expected, cumulative calorie consumption was significantly higher for HF vs. LF controls, but unexpectedly, was significantly higher for the G group compared to the HF control (**Figure 3.3B**). Even though statistical differences were not observed between HF, PW, and PF regarding weekly calorie consumption, it should be noted that all three experimental groups (G, PW, PF) cumulatively consumed more calories compared to the HF control group. The HF group cumulatively consumed 939.2 kcals/cage, while the flavanol-supplemented groups cumulatively consumed 1000 to 1075 kcals/cage. This trend was not observed previously [15,16] and likely explains the lack of benefit of experimental treatments on weight and fat gain (and for G, increased weight gain) compared to the HF control. Despite the deviation in reported values compared to similar studies, we are confident that the data presented here are accurate. Pelleted diets were used so that accurate measurement of food consumption could be achieved, and minimal amounts of food waste were seen on the cage floors. This was more accurate than using a powdered diet delivered in feeding troughs. Twice-weekly weighing of food within all 4 HF groups facilitated accurate intake measurements and comparisons between these diets.

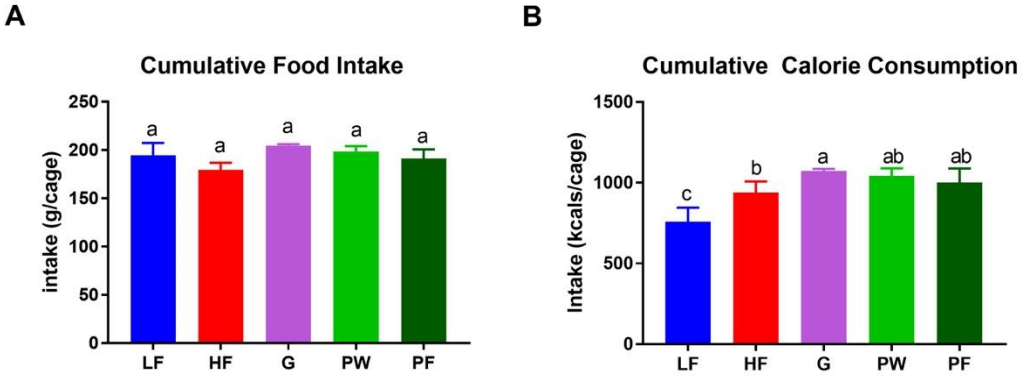

Figure 3.3 *Cumulative food intake (A), and average weekly calorie intake (B). Values are expressed as means (n =3 cages) ± SEM. Significance of treatments was determined using 1-way ANOVA with Fisher's LSD post hoc test and was defined as $p < 0.05$ with different letters designating significantly different values.*

3.3.4 Glucose and Insulin Tolerance. OGTTs were performed in weeks 6 (**Figure 3.4**) and 10 (**Figure 3.5**), ipGTTs were performed in week 11 (**Figure 3.6**), and ITTs were performed in week 12 (**Figure 3.7**). Week 6 OGTT results indicated significant difference in 12 h fasting blood glucose (**Figure 3.4B**), area under the curve (AUC) (**3.4C**), and maximum concentration (Cmax) (**3.4D**) between the LF group and all other treatments. Blood glucose excursion (**3.4E**) was not significantly different between any treatment groups. No significant differences were observed between the experimental groups and the HF control. At this point that the experimental model was producing the expected phenotypic changes, but it may have been too early to observe potential protective benefits from the experimental diets; however, G did prevent HF-induced excursions. During the week 10 OGTT, greater separation among the treatment groups was observed. The PF group exhibited reduced 12 hr fasting blood glucose measurements (**Figure 3.5B**) compared to the other flavanol treatment groups and was not significantly different to either LF or HF control. The experimental groups were significantly different from LF control and not significantly different from HF control for the other analyses including AUC, Cmax, and excursion

(**3.5C, D and E**). These findings suggest that PBE, when dosed based on 35 mg flavanols/kg bw/d, may help to partially prevent deterioration of fasting blood glucose control (possibly due to inhibited gluconeogenesis [30]) after 10 weeks on a high-fat diet, but not glucose clearance or insulin sensitivity when a glucose load was ingested orally. In the week 11 ipGTT, the G group surprisingly exhibited a 12 h fasting blood glucose level (**Figure 3.6B**) that was significantly higher than both controls. The PW and PF groups were not significantly different from the HF control, suggesting that PBE was able to blunt increases in fasting blood glucose control for longer than GSE, regardless of dosage. AUC was also significantly worse for the G group compared to both control groups (**3.6C).** Cmax and excursion (**3.6D and E**) values for experimental groups were not significantly different from HF control. Overall, no treatment groups were able to significantly prevent glucose insensitivity associated with high-fat feeding. Similar observations regarding the ITT were made. No differences were observed in 4 hr fasting blood glucose values (**Figure 3.7B**). The G group exhibited significantly higher AUC compared to both controls, while PW and PF AUCs did not differ from HF (**3.7C**).

Overall, it would appear that no flavanol treatment was able to effectively manage glucose tolerance after 6 weeks, but slight protection against HF-induced elevations in 12-hr fasting blood glucose were observed in only the PF group after 10 weeks. By 11 weeks, in terms of 12-hr fasting blood glucose, treatments were not significantly different from one another, and not significantly better than either control group. Glucose tolerance, regardless of glucose delivery (oral vs i.p. injection) was not significantly improved due to flavanol supplementation. Insulin sensitivity was not improved by any treatment group.

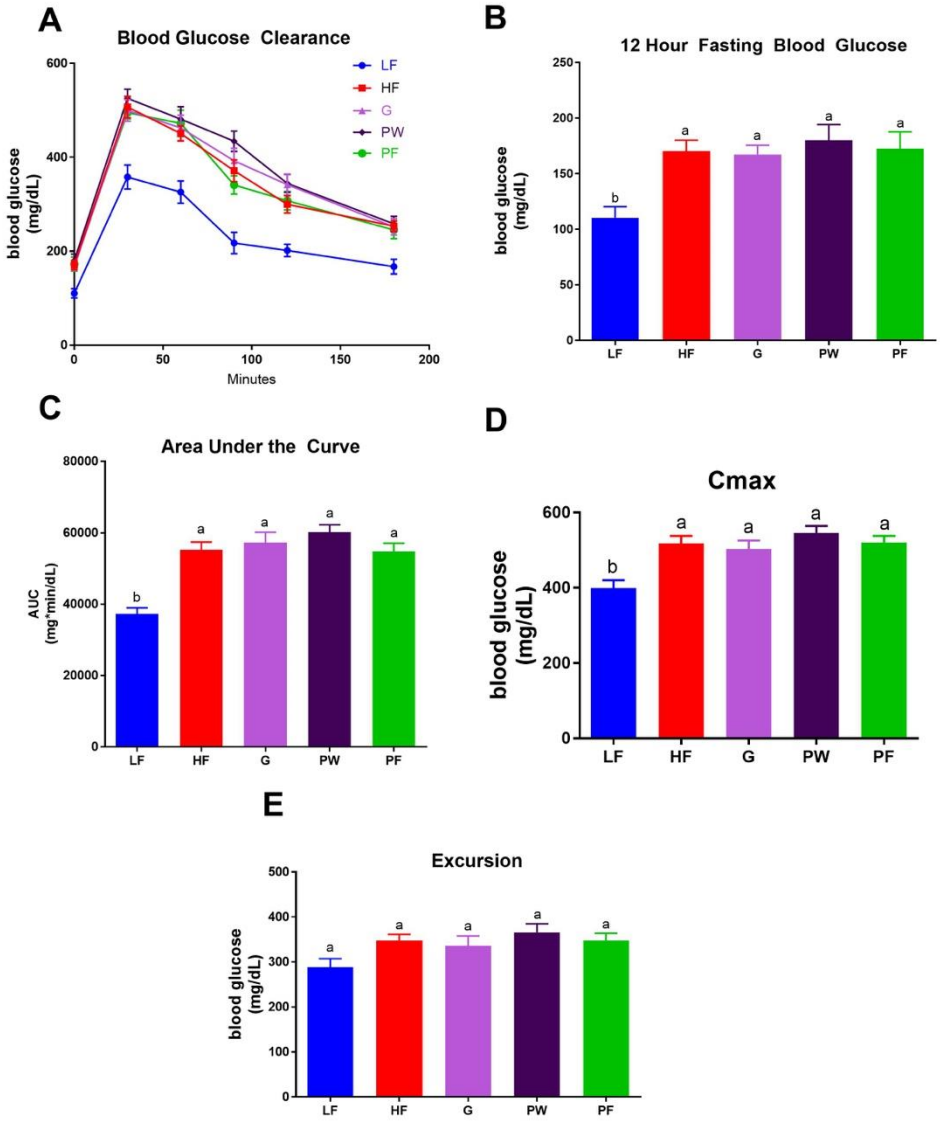

Figure 3.4 *Week 6 oral glucose tolerance test (OGTT): A) Blood glucose concentrations over time. B) 12-hr fasting blood glucose levels. C) Blood glucose area under the curve. D) Cmax (maximum blood glucose measurement obtained for OGTT). E) Excursion for OGTT (from baseline). Values are expressed as means (n = 12/trt for HF, G PW, and PF, n = 11 for LF) ± SEM. Significance of treatments was determined using 1-way ANOVA with Fisher's LSD post hoc test and was defined as p < 0.05 with different letters designating significantly different values for B-E.*

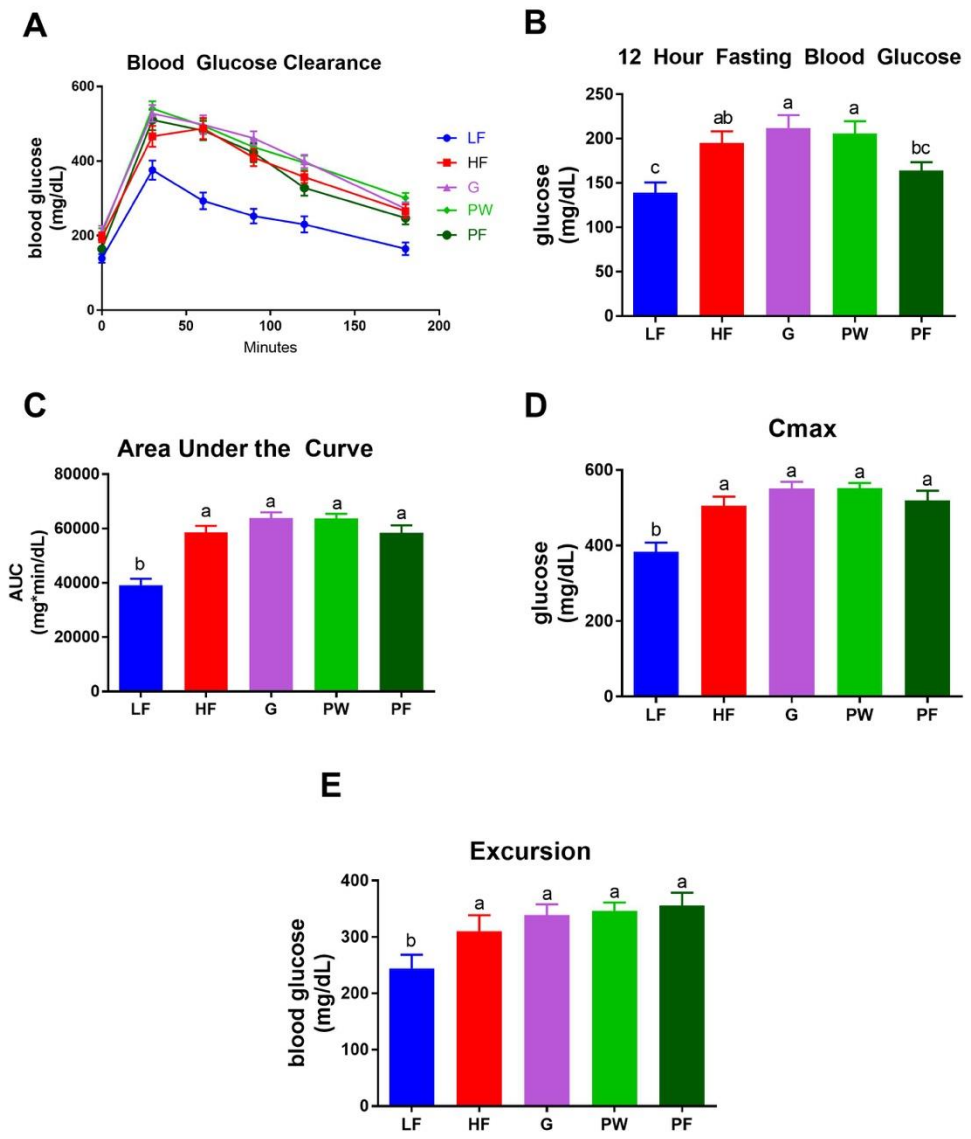

Figure 3.5 *Week 10 oral glucose tolerance test (OGTT): A) Blood glucose concentrations over time. B) 12-hr fasting blood glucose levels. C) Blood glucose area under the curve. D) Cmax (maximum blood glucose measurement obtained for OGTT). E) Excursion for OGTT (from baseline). Values are expressed as means (n = 12/trt for HF, G, PW, and PF, n = 11 for LF) ± SEM. Significance of treatments was determined using 1-way ANOVA with Fisher's LSD post hoc test and was defined as p < 0.05 with different letters designating significantly different values for B-E.*

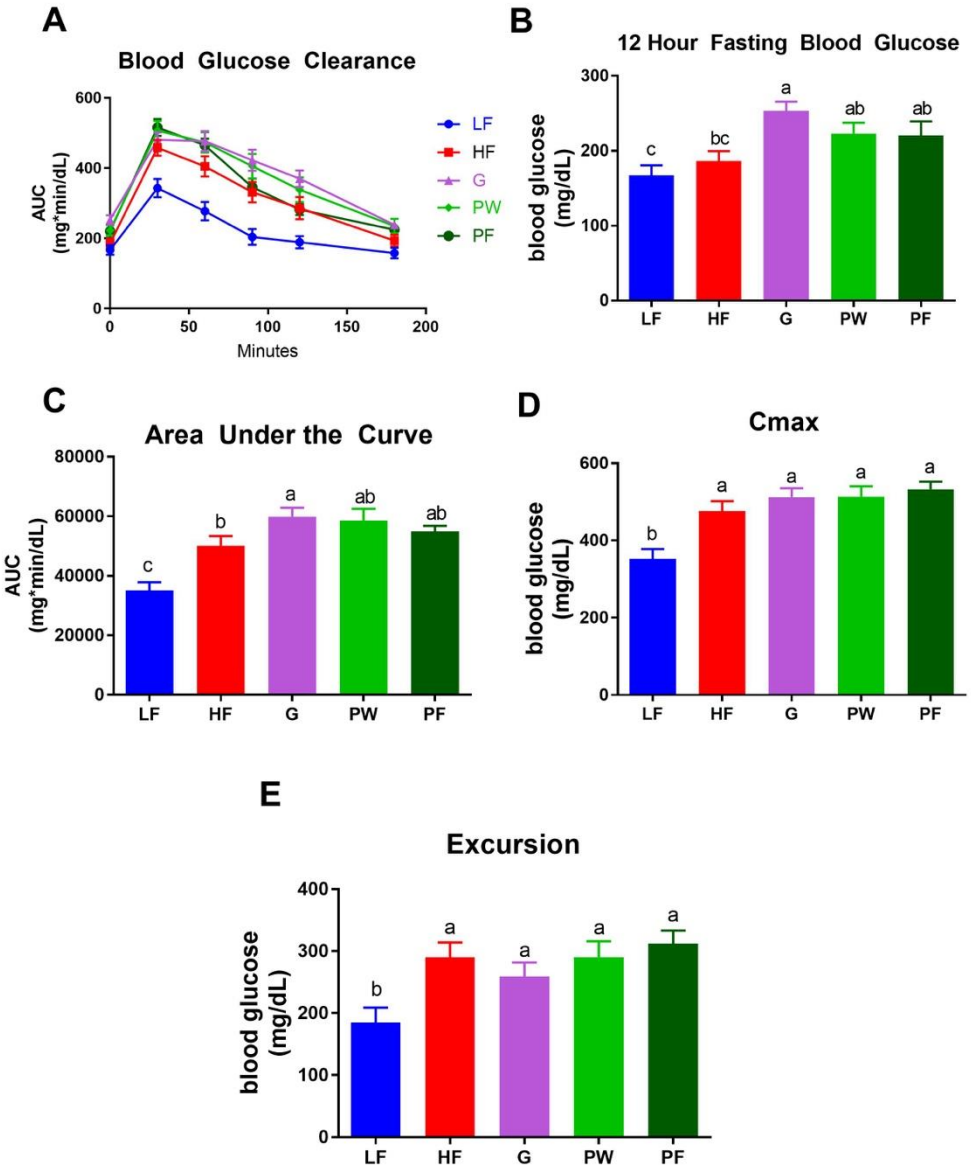

Figure 3.6 *Week 11 intraperitoneal glucose tolerance test (ipGTT). A) Blood glucose clearance over time. B) 12 hr-fasting blood glucose levels at. C) Blood glucose area under the curve. D) Cmax (maximum blood glucose measurement obtained for ipGTT). E) Excursion for ipGTT (from baseline). Values are expressed as means (n = 12/trt for HF, G, and PF, n = 10 for LF, n = 11 for PW) ± SEM. Significance of treatments was determined using 1-way ANOVA with Fisher's LSD post hoc test and was defined as p < 0.05 with different letters designating significantly different values for B-E.*

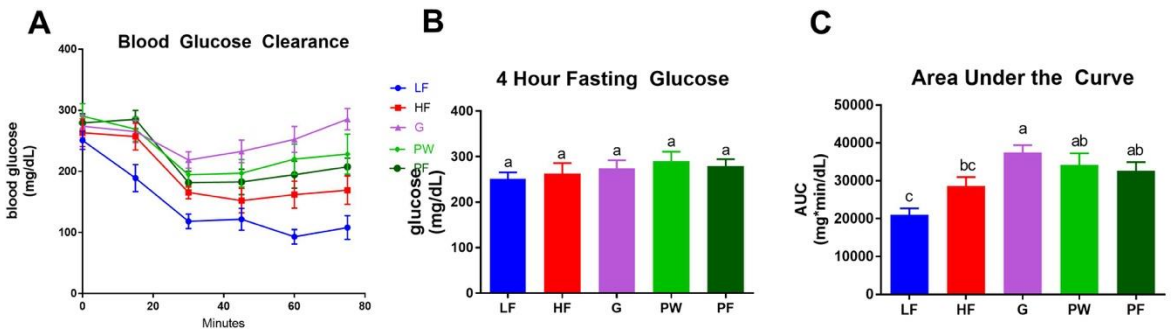

Figure 3.7 *Week 12 insulin tolerance test (ITT). A) Blood glucose clearance over time. B) 4 hr-fasting blood glucose levels. C) Blood glucose area under the curve. Values are expressed as means [n = 12/trt for HF, G, and PF, n = 6 for LF (4 mice exhibited signs of hypoglycemia and were removed from dataset), n = 11 for PW] ± SEM. Significance of treatments was determined using 1-way ANOVA with Fisher's LSD post hoc test and was defined as p < 0.05 with different letters designating significantly different values for B-E.*

3.3.5 Organ and Serum Analyses. Liver lipids, and liver and epididymal adipose tissue weights are shown in **Figure A5**. The LF group had a significantly higher liver weight (normalized to 25 g body wt) compared to all other groups except G. In contrast, HF and PW exhibited significantly lower liver weights compared to LF and G. High fat feeding is typically characterized by increased liver weight compared to lean counterparts [31,32], but in some cases liver weight is reduced. The results here are inconclusive. No experimental diet was able to significantly decrease epididymal adipose weight compared to HF control. Liver fat mass was not significantly different for any treatment group.

Serum glucose and liver MDA are shown in **Figure 3.8A-B**. Liver MDA concentrations (**Figure 3.8A**) were significantly elevated in HF compared to LF. PF exhibited marginal protection against HF-induced MDA increases, with levels that were not significantly different from LF or HF. G and PW were significantly higher than LF and not significantly different from HF. For serum glucose (**Figure 3.8B**), HF had significantly higher levels than LF, and G was significantly elevated compared to both. PW and PF had glucose levels significantly higher than LF but not significantly different from HF.

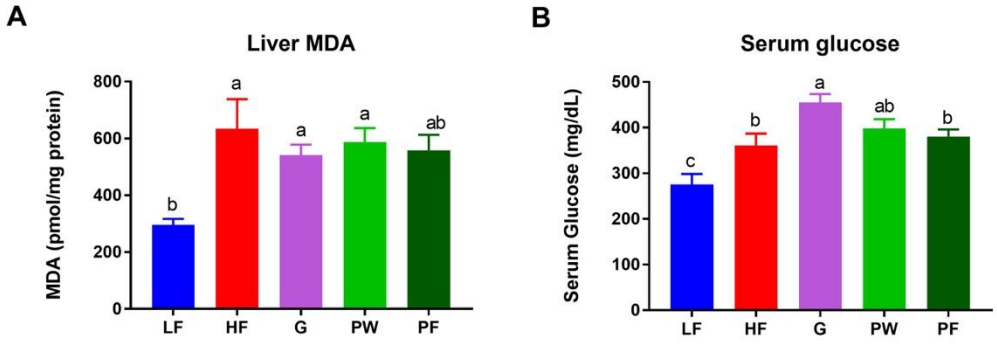

Figure 3.8 *Liver malondialdehyde (MDA) levels (A) and serum glucose levels (B). Values are expressed as means (n = 12/trt for HF, G, and PF, n = 9 for LF, and n = 11 for PW) ± SEM. Significance of treatments was determined using 1-way ANOVA with Fisher's LSD post hoc test and was defined as p < 0.05 with different letters designating significantly different values.*

3.3.6 Adipose Tissue Cytokines. Adipose IL-6 and TNF-α levels are shown in **Figure 3.9**. Notably significant differences in IL-6 concentration were detected (**3.9A**). As expected, the IL-6 in the HF control was significantly greater than the LF control group. While no statistically significant differences were observed between flavanol treatment groups, all 3 groups exhibited significantly lower IL-6 levels compared to HF but were also significantly higher than LF. Perhaps most interestingly, it must be noted that the G group, which gained the most weight and body fat mass, exhibited the lowest absolute IL-6 concentration among groups receiving the HF diet. In terms of TNF-α levels (**3.9B**), no significant differences were observed, but the treatment groups exhibited lower cytokine levels compared to control groups.

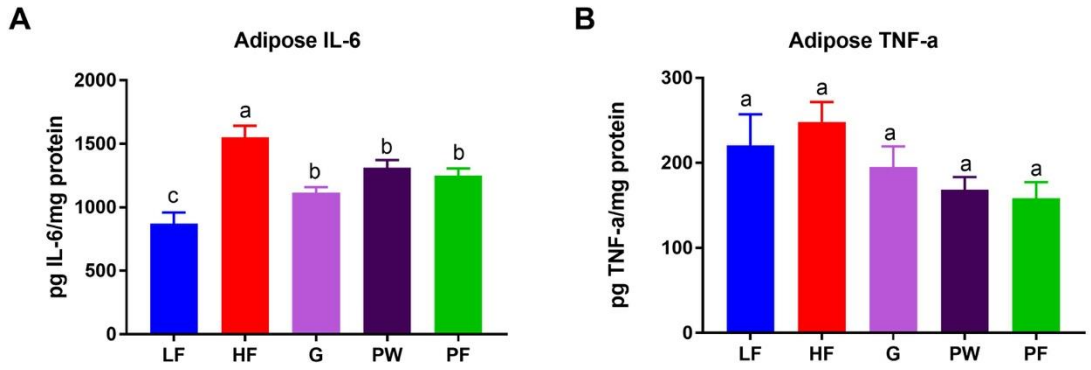

Figure 3.9 *Epididymal adipose IL-6 (A) and epididymal adipose TNF-α (B) across treatments expressed as means (n = 12/trt for HF, G, and PF, n = 9 for LF, and n = 11 for PW). Significantly different groups are designated by different letters ($p < 0.05$) as tested by 1-way ANOVA with Fisher's LSD post hoc test.*

3.4 DISCUSSION

Contrary to our hypothesis, the findings of this study, particularly regarding the increased weight gain and fasting blood glucose levels of the treatment groups compared to HF control, are not consistent with our expectations based on prior findings by our lab [15,16]. The C57Bl/6J mice were selected for this study as they reportedly model the human progression of metabolic syndrome, but only when exposed to a HF diet, and without genetic alteration [33]. It is recommended to keep these mice on HF diets for 8-16 weeks, which is a very wide range [34], and other studies have demonstrated that significant weight gain can happen as late as after 14 weeks on HF diet compared to LF controls [35]. Moreover, it has been reported that a large individual variation in weight gain of C57Bl/6J mice due to factors other than genetics (physical activity level, fat free mass, etc. prior to HF diet initiation) exists [36]. These factors could partially explain some of the observations regarding the increased weight gain of the experimental groups in this study, despite a lack of differences in body weight at the onset of the study (**Figure A3**). Additionally, treatment groups showed the greatest improvements in fasting blood glucose after 10 weeks on HF diets (**Figure 3.5**), that were not present during the final 12-hr fasting blood glucose measurement after 11 weeks (**Figure 3.6**), suggesting that overall results could have been very different if the diets had been administered for a shorter time. Alternative to this hypothesis is the possibility that flavanol supplementation, at the present dosages and study duration, was not able to prevent significant weight gain. While uncommon, there have been previously reported cases of no improvement of weight gain due to flavanol supplementation, notably when using

apple flavanols at low dosage, high dose cranberry extract, high dose cocoa extract, and high dose artic berry extract [37–40]. Similar to the trend observed in this study, a study involving GSE supplementation in male Wistar rats reported no significant reduction in weight gain, but significant anti-hypertrophic and hyperplasic effects in visceral white adipose tissue[41]. While it is possible that the size of the flavanol dose used in this study could be a reason for the lack of obesity improvements in this study, it represents a much more physiologically relevant dose as it would be physiologically achievable for a human to consume an equivalent amount. Moreover, we have shown improvement to weight gain in a similar study utilizing a lower dose than the one used here [15]. In either case, the DIO model with C57Bl/6J mice did not behave as expected based on previous experiments in our lab, but interesting findings related to inflammatory cytokines were still observed.

When examining the food intake of the experimental diet groups compared to the HF control, there was no statistically significant difference in overall intake based on weight, but G did consume significantly more kcals compared to HF control, while the other treatment groups consumed slightly, but not significantly more kcals (**Figure 3.3**). The observed trends in body weight, body fat and blood glucose data appear to be explained primarily by the food intake data. The only difference between the diets was the addition of the plant extracts which were, if anything, expected to cause the mice to eat less, as these plant extracts are astringent and bitter [42]. However, the dose was so low that bitterness or astringent sensations were likely below levels that would otherwise deter eating [15]. HF diets were refreshed twice weekly, so it is also unlikely that the HF control mice ate less due to fat oxidation and rancidity. All diets were sucrose-matched (**Table 3.2**). Even though the 3 flavanol experimental groups gained slightly more weight over time, it must be noted that the PBE groups did not gain significantly more weight than the HF control at the end of the study. This suggests that while the compounds did not prevent diet-induced weight gain compared to LF controls, perhaps they prevented the ability of excess calorie consumption to cause significantly higher weight gain compared to HF control in some groups.

It seems likely that the increased caloric consumption directly caused the increased weight gain and lack of treatment effects, however there are possible explanations that may have contributed to the observed results. It is well known that certain gut microbiome populations are linked with obesity and that environmental factors can influence an individual's microbiome [43]. There could have been alterations to the pathways associated with or functionality of metabolic hormones (i.e. leptin, ghrelin) which could have caused changes in appetite or energy expenditure in certain treatment groups [44].

Even though the added flavanols failed to significantly prevent weight gain or adiposity, they were remarkably able to prevent obesity-induced increases in adipose IL-6 and TNF-α levels. Most notably was the G group, which exhibited the greatest weight and % fat progression of all diets, and yet IL-6 levels not significantly different from the LF control (**Figures 3.2 and 3.9**). This was a very interesting finding, as it was clearly an obesity-independent effect (i.e., reductions in inflammation were mediated by a pathway unrelated to body fat amount). This finding overall suggests that flavanols with lower mDP may be more effective at reducing adipose IL-6 secretion in response to HF diet induced obesity. The PBE treatment groups were slightly, but not significantly, more effective at reducing TNF-α, but these groups did not gain as much weight as the G group, nor did they consume significantly more calories than the HF control. Still, it would appear that despite increased caloric intake and weight gain, the flavanol treatment groups, regardless of mDP, were able to protect against increased inflammatory cytokine production, which is one of the hallmark signs of metabolic derangement [2]. Obesity-independent effects such

as these may suggest that these compounds, even if not successful at preventing or inhibiting obesity, may still be used as adjuvants to reduce the deleterious side effects and consequences of obesity, such as increased inflammatory tone. It would be beneficial, as part of a future study, to examine the levels of other inflammatory cytokines in response to these compounds, particularly NF-κB, as it is a more upstream regulator of inflammatory pathways [45].

The purpose of this study was to determine how mDP impacts markers of metabolic syndrome when flavanols are consumed as part of a HF diet. Despite a lack of improvements in body weight and body composition, it was observed that flavanols, regardless of mDP significantly prevented increases in levels of inflammatory cytokines concurrent with obesity. It is possible that the smaller mDP treatment could be overall more effective than the larger mDP as reflected by the impressive improvements in cytokine levels, yet significantly worse body weight gain and no improvement to glucose homeostasis. The larger mDP treatment, however, exerted a greater effect on 12-hr fasting blood glucose levels, after 10 weeks, without adjusting for terpene content, suggesting that adjusting the dosage to account for terpene content of PBE did not change the effect. Very little research has previously been performed on terpenes. A brief discussion can be found in Supplementary Information. These findings overall once again indicate that flavanols function differently depending on the disease model in question and the target tissue, with mDP as well as structure playing determining roles [3,7,16,17,40]. It should also be mentioned that these effects were observed when very small, albeit significant, comparative differences in mDP between extracts were used. It is possible that larger differences in mDP would yield observable physiological differences in treatment groups or make the differences observed here more pronounced [16].

In conclusion, obesity and hyperglycemia were not significantly reduced by the flavanol-rich diets and there appeared to be a diet-induced hyperphagic effect in some treatment groups. This highlights the importance of measuring food intake in metabolic studies; without the food intake data, we would not have any ideas of the reasons for the observed weight and adiposity data. While these results are not in accordance with other published studies, we believe that this study demonstrates that results from DIO animal models may not be as consistent as often thought. It is pertinent to investigate factors related to the results of this study so that they can be properly controlled for in the future. Despite the lack of expected outcomes, we observed significantly reduced inflammation independent of obesity in treatment groups, which could indicate improved aspects of overall health status when considering other factors related to the metabolic syndrome. This improved inflammatory state in obese mice should be more closely examined in future studies, as this effect has now been observed when using flavanols from multiple plant sources [40].

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REFERENCES

[1] Galisteo M, Duarte J, Zarzuelo A. Effects of dietary fibers on disturbances clustered in the metabolic syndrome. J Nutr Biochem 2008;19:71–84.

[2] Lee Y-H, Pratley RE. The evolving role of inflammation in obesity and the metabolic syndrome. Curr Diab Rep 2005;5:70–5. doi:10.1007/s11892-005-0071-7.
[3] Neilson AP, O'Keefe SF, Bolling BW. High-Molecular-Weight Proanthocyanidins in Foods: Overcoming Analytical Challenges in Pursuit of Novel Dietary Bioactive Components. Annu Rev Food Sci Technol 2016;7:43–64. doi:10.1146/annurev-food-022814-015604.
[4] Han X, Shen T, Lou H. Dietary polyphenols and their biological significance. Int J Mol Sci 2007;8:950–988.
[5] Tsang C, Auger C, Mullen W, Bornet A, Rouanet J-M, Crozier A, et al. The absorption, metabolism and excretion of flavan-3-ols and procyanidins following the ingestion of a grape seed extract by rats. Br J Nutr 2005;94:170–181.
[6] Xu Y, Simon JE, Welch C, Wightman JD, Ferruzzi MG, Ho L, et al. Survey of polyphenol constituents in grapes and grape-derived products. J Agric Food Chem 2011;59:10586–10593.
[7] Bowser SM, Moore WT, McMillan RP, Dorenkott MR, Goodrich KM, Ye L, et al. High Molecular Weight Cocoa Procyanidins Possess Enhanced Insulin-Enhancing and Insulin Mimetic Activities in Human Primary Skeletal Muscle Cells Compared to Smaller Procyanidins. J Nutr Biochem 2016. doi:10.1016/j.jnutbio.2016.10.001.
[8] Soleas GJ, Diamandis EP, Goldberg DM, others. Wine as a biological fluid: history, production, and role in disease prevention. J Clin Lab Anal 1997;11:287–313.
[9] Boulton RB, Singleton VL, Bisson LF, Kunkee RE. Principles and Practices of Winemaking. Springer Science & Business Media; 2013.
[10] Manach C, Scalbert A, Morand C, Rémésy C, Jiménez L. Polyphenols: food sources and bioavailability. Am J Clin Nutr 2004;79:727–747.
[11] Monagas M, Gómez-Cordovés C, Bartolomé B, Laureano O, Ricardo da Silva JM. Monomeric, oligomeric, and polymeric flavan-3-ol composition of wines and grapes from Vitis vinifera L. Cv. Graciano, Tempranillo, and Cabernet Sauvignon. J Agric Food Chem 2003;51:6475–6481.
[12] Katalinić V, Možina SS, Skroza D, Generalić I, Abramovič H, Miloš M, et al. Polyphenolic profile, antioxidant properties and antimicrobial activity of grape skin extracts of 14 Vitis vinifera varieties grown in Dalmatia (Croatia). Food Chem 2010;119:715–723.
[13] Bagchi D, Bagchi M, Stohs SJ, Das DK, Ray SD, Kuszynski CA, et al. Free radicals and grape seed proanthocyanidin extract: importance in human health and disease prevention. Toxicology 2000;148:187–97. doi:10.1016/S0300-483X(00)00210-9.
[14] Goodrich KM, Fundaro G, Griffin LE, Hulver MW, Ponder MA, Neilson AP. Chronic administration of dietary grape seed extract increases colonic expression of gut tight junction protein occludin and reduces fecal calprotectin: a secondary analysis of healthy Wistar Furth rats. Nutr Res 2012;32:787–794.
[15] Griffin LE, Witrick KA, Klotz C, Dorenkott MR, Goodrich KM, Fundaro G, et al. Alterations to metabolically active bacteria in the mucosa of the small intestine predict anti-obesity and anti-diabetic activities of grape seed extract in mice. Food Funct 2017;8:3510–22. doi:10.1039/C7FO01236E.
[16] Dorenkott MR, Griffin LE, Goodrich KM, Thompson-Witrick KA, Fundaro G, Ye L, et al. Oligomeric Cocoa Procyanidins Possess Enhanced Bioactivity Compared to Monomeric and Polymeric Cocoa Procyanidins for Preventing the Development of Obesity, Insulin Resistance, and Impaired Glucose Tolerance during High-Fat Feeding. J Agric Food Chem 2014;62:2216–27. doi:10.1021/jf500333y.

[17] Bitzer ZT, Glisan SL, Dorenkott MR, Goodrich KM, Ye L, O'Keefe SF, et al. Cocoa procyanidins with different degrees of polymerization possess distinct activities in models of colonic inflammation. J Nutr Biochem 2015;26:827–31. doi:10.1016/j.jnutbio.2015.02.007.
[18] Yamashita Y, Okabe M, Natsume M, Ashida H. Comparison of anti-hyperglycemic activities between low- and high-degree of polymerization procyanidin fractions from cacao liquor extract. J Food Drug Anal 2012;20:283–7.
[19] Lee Y, Kim YJ, Eun JC, Yokozawa T. Ameliorative Effects of Proanthocyanidin on Oxidative Stress and Inflammation in Streptozotocin-Induced Diabetic Rats. J Agric Food Chem 2007;55:9395–400.
[20] Tatsuno T, Jinno M, Arima Y, Kawabata T, Hasegawa T, Yahagi N, et al. Anti-inflammatory and anti-melanogenic proanthocyanidin oligomers from peanut skin. Biol Pharaceutical Bull 2012;35:909–16.
[21] Gentile C, Allegra M, Angileri F, Pintaudi AM, Livrea MA, Tesoriere L. Polymeric proanthocyanidins from Sicilian pistachio (Pistacia vera L.) nut extract inhibit lipopolysaccharide-induced inflammatory response in RAW 264.7 cells. Eur J Nutr 2012;51:353–63.
[22] Mao TK, Powell JJ, van de Water J, Keen CL, Schmitz HH, Gershwin ME. Effect of Cocoa Procyanidins on the Secretion of Interleukin-4 in Peripheral Blood Mononuclear Cells. J Med Food 2004;3:107–14.
[23] Ainsworth EA, Gillespie KM. Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin-Ciocalteu reagent. Nat Protoc 2007;2:875–7. doi:10.1038/nprot.2007.102.
[24] Payne MJ, Hurst WJ, Stuart DA, Ou B, Fan E, Ji H, et al. Determination of total procyanidins in selected chocolate and confectionery products using DMAC. J AOAC Int 2010;93:89–96.
[25] Nair AB, Jacob S. A simple practice guide for dose conversion between animals and human. J Basic Clin Pharm 2016;7:27–31. doi:10.4103/0976-0105.177703.
[26] Andrikopoulos S, Blair AR, Deluca N, Fam BC, Proietto J. Evaluating the glucose tolerance test in mice. Am J Physiol-Endocrinol Metab 2008;295:E1323–32. doi:10.1152/ajpendo.90617.2008.
[27] Li J, Sapper TN, Mah E, Rudraiah S, Schill KE, Chitchumroonchokchai C, et al. Green tea extract provides extensive Nrf2-independent protection against lipid accumulation and NFκB pro-inflammatory responses during nonalcoholic steatohepatitis in mice fed a high-fat diet. Mol Nutr Food Res 2016;60:858–70. doi:10.1002/mnfr.201500814.
[28] Breitmaier E. Terpenes: Importance, General Structure, and Biosynthesis. Terpenes, Wiley-VCH Verlag GmbH & Co. KGaA; 2006, p. 1–9. doi:10.1002/9783527609949.ch1.
[29] Robbins RJ, Leonczak J, Li J, Johnson JC, Collins T, Kwik-Uribe C, et al. Determination of Flavanol and Procyanidin (by Degree of Polymerization 1–10) Content of Chocolate, Cocoa Liquors, Powder(s), and Cocoa Flavanol Extracts by Normal Phase High-Performance Liquid Chromatography: Collaborative Study. J AOAC Int 2012;95:1153–60. doi:10.5740/jaoacint.12-162.
[30] Rui L. Energy Metabolism in the Liver. Compr Physiol 2014;4:177–97. doi:10.1002/cphy.c130024.
[31] Milagro FI, Campión J, Martínez JA. Weight Gain Induced by High-Fat Feeding Involves Increased Liver Oxidative Stress. Obesity 2006;14:1118–23. doi:10.1038/oby.2006.128.

[32] Bose M, Lambert JD, Ju J, Reuhl KR, Shapses SA, Yang CS. The Major Green Tea Polyphenol, (-)-Epigallocatechin-3-Gallate, Inhibits Obesity, Metabolic Syndrome, and Fatty Liver Disease in High-Fat–Fed Mice. J Nutr 2008;138:1677–83.
[33] Srinivasan K, Poduri R. Animal models in type 2 diabetes research: An overview K. vol. 125. 2007.
[34] Reuter TY. Diet-induced models for obesity and type 2 diabetes. Drug Discov Today Dis Models 2007;4:3–8. doi:10.1016/j.ddmod.2007.09.004.
[35] Yang Y, Smith DL, Keating KD, Allison DB, Nagy TR. Variations in body weight, food intake and body composition after long-term high-fat diet feeding in C57BL/6J Mice. Obes Silver Spring Md 2014;22:2147–55. doi:10.1002/oby.20811.
[36] Zhang L-N, Morgan DG, Clapham JC, Speakman JR. Factors Predicting Nongenetic Variability in Body Weight Gain Induced by a High-Fat Diet in Inbred C57BL/6J Mice. Obesity 2012;20:1179–88. doi:10.1038/oby.2011.151.
[37] Anhê FF, Nachbar RT, Varin TV, Vilela V, Dudonné S, Pilon G, et al. A polyphenol-rich cranberry extract reverses insulin resistance and hepatic steatosis independently of body weight loss. Mol Metab 2017;6:1563–73. doi:10.1016/j.molmet.2017.10.003.
[38] Anhê FF, Varin TV, Le Barz M, Pilon G, Dudonné S, Trottier J, et al. Arctic berry extracts target the gut-liver axis to alleviate metabolic endotoxaemia, insulin resistance and hepatic steatosis in diet-induced obese mice. Diabetologia 2017. doi:10.1007/s00125-017-4520-z.
[39] Osada K, Suzuki T, Kawakami Y, Senda M, Kasai A, Sami M, et al. Dose-dependent hypocholesterolemic actions of dietary apple polyphenol in rats fed cholesterol. Lipids 2006;41:133–9. doi:10.1007/s11745-006-5081-y.
[40] Gu Y, Yu S, Lambert JD. Dietary cocoa ameliorates obesity-related inflammation in high fat-fed mice. Eur J Nutr 2014;53:149–158.
[41] Pascual-Serrano A, Arola-Arnal A, Suárez-García S, Bravo FI, Suárez M, Arola L, et al. Grape seed proanthocyanidin supplementation reduces adipocyte size and increases adipocyte number in obese rats. Int J Obes 2017;41:1246–55. doi:10.1038/ijo.2017.90.
[42] Peleg H, Gacon K, Schlich P, Noble AC. Bitterness and astringency of flavan-3-ol monomers, dimers and trimers. J Sci Food Agric 1999;79:1123–8. doi:10.1002/(SICI)1097-0010(199906)79:8<1123::AID-JSFA336>3.0.CO;2-D.
[43] Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature 2006;444:1027–31. doi:http://dx.doi.org.ezproxy.lib.vt.edu/10.1038/nature05414.
[44] Levin BE, Dunn-Meynell AA, Ricci MR, Cummings DE. Abnormalities of leptin and ghrelin regulation in obesity-prone juvenile rats. Am J Physiol-Endocrinol Metab 2003;285:E949–57. doi:10.1152/ajpendo.00186.2003.
[45] Lawrence T. The Nuclear Factor NF- B Pathway in Inflammation. Cold Spring Harb Perspect Biol 2009;1:a001651–a001651. doi:10.1101/cshperspect.a001651.

CHAPTER 4:

Preference for and Sensitivity to Flavanol Mean Degree of Polymerization in Model Wines is Correlated with Body Composition

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Submitted to Appetite

ABSTRACT

Bitterness and astringency are characteristic sensory attributes associated with flavanol-rich foods. It is known that the degree of polymerization (DP) of flavanol compounds influences their perceived bitter and astringent sensations. Smaller DP compounds can enter the papillae on the tongue and elicit a bitter response, while larger DP compounds are sterically inhibited from entering the papillae and instead interact with oral proteins, cause precipitation, and elicit astringent sensations. Previous research has indicated that preference for bitterness and astringency is related to health status and sensitivity to bitter compounds such as 6-*n*-propyl-thiouracil (PROP). The purpose of this study was to examine trends in liking, bitterness intensity, and astringency intensity of wine-like products with distinct flavanols of different DP in a consumer sensory panel when consumers were differentiated by phenotype. Recruited panelists (n=102) were segmented into different phenotypes based on body fat percentage (BF%), body mass index, PROP sensitivity, and stated bitter food preference. Differences in overall product liking, perceived bitterness intensity, and astringency intensity were observed between the three wine samples of varying flavanol mDP. Specifically, with increased mDP in the wine, overall liking and bitterness liking decreased, with concurrent increased perception of bitterness and astringency intensity. Based on panelist phenotype, greater differences between phenotypes were observed based on BF% and BMI classification, rather than PROP sensitivity classification. Reduced ability to detect differences in bitterness and astringency were noted in subjects of higher weight status. Overall, these data suggest that weight status is a greater predictor of liking of flavanol-rich foods than bitterness sensitivity, and that reduced perception of bitterness and astringency associated with weight gain may impact selection and preference for these foods.

4.1 INTRODUCTION

Flavanols are a sub-class of flavonoids naturally found in a variety of plant foods including cocoa, fruits, tea, and nuts (Neilson, O'Keefe, & Bolling, 2016). A wide variety of flavanol sizes and structures exist due to the polymerization of monomers (catechins) to oligomers and polymers (procyanidins) (**Figure 4.1A**). Many flavanols undergo polymerization reactions during plant development or during food processing, such as during the production of wine or chocolate (Beecher, 2003; Fulcrand, Dueñas, Salas, & Cheynier, 2006). Degree of polymerization (DP) refers to the size of an individual flavanol, and mean degree of polymerization (mDP) refers to the average size of all the flavanols in a given food (Neilson et al., 2016). Flavanol DP and food mDP appear to impact the sensory profile of flavanol-rich foods (Lesschaeve & Noble, 2005).

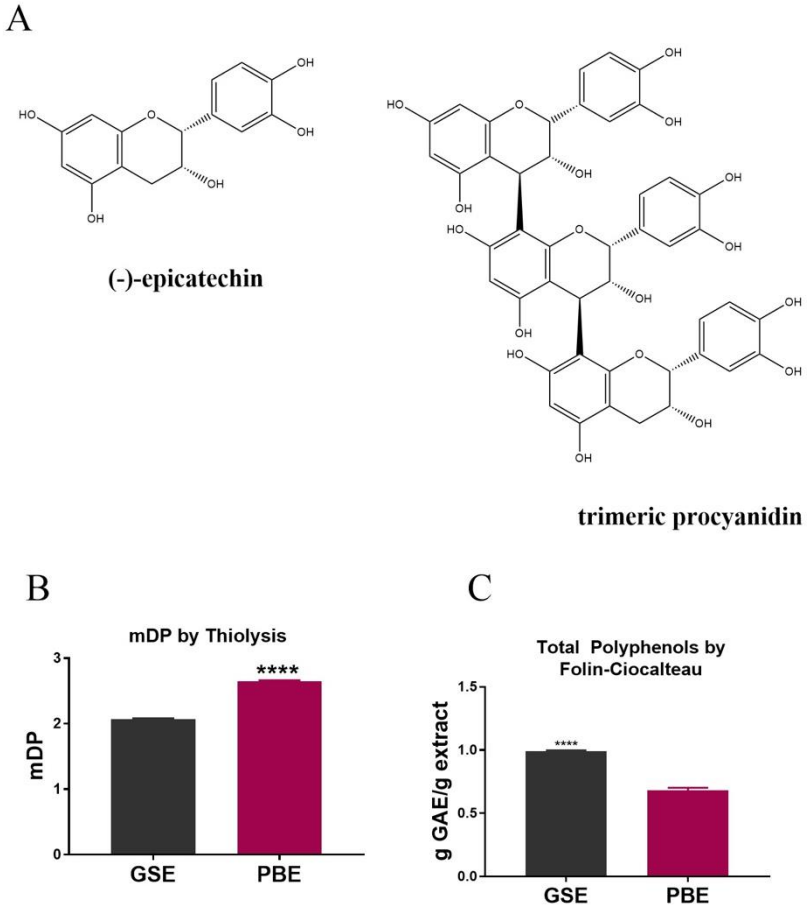

Figure 4.1. *Representative flavanols found in GSE and PBE with different DPs (A). Mean flavanol degree of polymerization for GSE and PBE expressed as means (n = 5) ± SEM (B) and total polyphenols for GSE and PBE as measured by the Folin-Ciocalteu assay expressed as means (n = 7) ± SEM (C). Significance is denoted by asterisks (****p < 0.0001) based on unpaired Student's T-tests.*

Bitterness and astringency are characteristic taste attributes of flavanol-rich foods (Lesschaeve & Noble, 2005). These attributes are often perceived together as many astringent compounds can also be bitter, but they can be differentiated by the fact that bitterness is specifically a taste perceived on the tongue papilla, while astringency is a tactile sensation perceived throughout the mouth (Brossaud, Cheynier, & Noble, 2001; Kallithraka, Bakker, & Clifford, 1997). Regarding

the flavanols that elicit these taste sensations, it has been observed that DP is directly related to the intensity of bitterness and astringency. Smaller flavanols, including monomers and dimers, are more bitter, while larger DP compounds are more astringent (Brossaud et al., 2001; Kennedy, Saucier, & Glories, 2006). The relationship between DP, bitterness, and astringency is likely due to the fact that smaller flavanols can enter the papillae and elicit bitter sensation, while larger compounds are sterically inhibited from entering the papillae and instead bind with oral proteins to cause protein precipitation and subsequent astringent sensations (Cheynier et al., 2006).

A vast number of natural compounds other than flavanols elicit bitter taste at very low dose in humans (Tepper et al., 2009). Despite the wide range of known bitter compounds, there are relatively few bitterness-specific taste receptors in humans. There are only approximately 30 known bitterness receptors, all of which belong to the *TASTE 2 Receptor* (TR2) family (Meyerhof et al., 2010; Mueller et al., 2005). This family of receptors is coded by a highly-variable gene, which subsequently allows for variation in individual bitterness detection and sensitivity in humans (Pronin et al., 2007). Current literature indicates that flavanols can directly interact with these receptors (Soares et al., 2018). It is believed that human bitterness perception can be described by sensitivity to certain compounds, such as 6-*n*-propyl-thiouracil (PROP) and phenylthiocarbamide (PTC). In fact, it has been previously shown that extremely sensitive PROP/PTC "supertasters" actually have greater expression of bitter taste receptors than tasters or non-tasters (Drewnowski & Gomez-Carneros, 2000). Moreover, people who are more sensitive to PROP/PTC are also more sensitive to caffeine, quinine, sweetness, capsaicin, alcohol, and oily textures (Tepper et al., 2009).

Food selection and preference in humans is influenced by a variety of factors including age, gender, culture, and ethnicity, as well as exposure to different foods and attitudes about nutrition (Cox, Hendrie, & Carty, 2016; Tepper et al., 2009). Studies examining food preferences based on bitterness sensitivity have produced variable results. Some studies report less vegetable consumption in PROP tasters compared to non-tasters, while others show no correlation. Other studies have indicated that in some cases, PROP tasters exhibit lower liking for sweet foods, salad dressings, and alcoholic beverages (Tepper et al., 2009). Other studies have examined the relationship between weight status, taste perception, and preferences. For example, it has been hypothesized that overweight and obese individuals are less sensitive to the texture and mouthfeel of lipids, thus requiring greater concentrations of fatty foods to detect mouthfeel and achieve satiation (J. E. Stewart & Keast, 2012; Jessica E. Stewart et al., 2010). Low sensitivity may lead to increased caloric intake and weight gain. Some studies have supported this hypothesis while others have rejected it (Cox et al., 2016). There are also reported cases of reduced bitter taste sensitivity with increased weight status in children and adults in response to standard basic taste solutions (Overberg, Hummel, Krude, & Wiegand, 2012; Simchen, Koebnick, Hoyer, Issanchou, & Zunft, 2006) and other studies indicating a correlation between liking of fatty foods and BMI (Cox et al., 2016). It is also worth noting that a study on food preferences before and after a low calorie diet with subsequent weight loss indicated decreased preference for high-fat and high-sugar foods after the intervention (Andriessen et al., 2018).

While there is evidence suggestive of correlations between weight status, bitterness sensitivity, and taste perceptions, much of the current research is based on qualitative analysis (i.e. surveys) and quantitative approaches utilizing reference solutions for basic tastes, rather than food systems displaying target characteristics of bitterness or astringency (Cox et al., 2016; Tepper et al., 2009). The purpose of the present study, therefore, was to determine how preferences for wine-like beverages made from flavanols of different DP (exhibiting different bitterness and astringency

intensities) differ among different phenotypes related to weight status, bitterness sensitivity, and bitterness preference. It was hypothesized that differences in liking of flavanols of different mDP and perceived intensities of bitterness and astringency would be correlated with differences in consumer phenotype due to previous observations of differences in food preference trends for lean and obese consumers (Tepper et al., 2009). By understanding the relationships between sensory preferences and phenotype, it might be possible to develop flavanol-rich products with optimized sensory characteristics for different consumer phenotypes.

4.2 MATERIALS AND METHODS

4.2.1 Chemical Standards and Reagents. Chemical standards included (±)-catechin (C), (−)-epicatechin (EC), (−)-epicatechin gallate (ECG), and (−)-catechin gallate (CG), and quinine sulfate were purchased from Sigma (St. Louis, MO, USA), and procyanidin (PC) dimer B_2 was purchased from ChromaDex (Irvine, CA, USA). Propylthiouracil (PROP) was purchased from Spectrum Chemical (New Brunswick, NJ, USA). Alum was purchased from McCormick (Baltimore, MD, USA) and sodium chloride was purchased from J.T. Baker Inc. (Phillipsburg, NJ, USA). Chemical standards for human consumption were USP grade or better. Solvents for UPLC MS/MS analysis were of LC-MS grade (VWR, Radnor, PA, USA). All other solvents were of ACS grade or better (Fisher, Pittsburgh, PA, USA).

4.2.2 Flavanol Extracts. Food grade grape seed extract (Vitaflavin, GSE) and pine bark extract (Oligopin, PBE) were purchased from DRT (Dax, France). Manufacturer specifications (**Table 4.1**) indicated differences in mDP, which were confirmed by subsequent analysis prior to usage in the study. PBE was selected instead of a grape skin extract due to the presence of anthocyanins in grape skin extract, which could confound result interpretation, as anthocyanins also impact astringency through flavanol interactions (Boulet et al., 2016; McRae & Kennedy, 2011; S. Vidal, Meudec, Cheynier, Skouroumounis, & Hayasaka, 2004). The use of these extracts would simulate the taste and consumption of dietary flavanols of different mDP present in wine without interference from other polyphenols. Extracts were stored in a food-grade −20°C freezer when not in use.

Table 4.1. Extract Specifications from Manufacturer

Parameter	Vitaflavin	Oligopin
Procyanidin Content (% w/w)	75.6	68.9
Monomers (% w/w)	24.4	31.1
Dimers (% w/w)	NA	16.8
Dimers + Trimers (% w/w)	43.6	NA
Timers + Tetramers (% w/w)	NA	52.1
Procyanidin-Porter content (% w/w)	66.0	66.0

4.2.3 Thiolysis. Flavanol mDP was assessed by thiolytic degradation followed by ultra-performance liquid chromatography with tandem mass spectrometry as described previously (Dorenkott et al., 2014). The analysis confirmed that the GSE and the PBE had significantly different mDP (**Figure 5.1B**) and were suitable for use in the study. Based on a weighted average calculation, the GSE and PBE mDPs were 2.1 ± 0.01 and 2.7 ± 0.01, respectively.

4.2.4 Folin-Ciocalteu Assay. The Folin-Ciocalteu assay was performed as described previously (Ryan et al., 2016) to approximate the concentration of flavanols in each extract (**Figure 4.1C**). The total polyphenol concentration of GSE was greater than PBE based on the assay, which was likely due to the presence of terpenes in the PBE (Breitmaier, 2006). This analysis allowed for consistent preparation of the wine samples based on equal total polyphenol content of each flavanol extract.

4.2.5 Wine Selection and Flavanol Dosage. Beringer Main & Vine Pinot Grigio (St. Helena, CA, USA) was selected as the control wine for the study. Full characterization of the wine was performed by the Virginia Tech Wine Lab (Blacksburg, VA, USA) (**Table B1**). Flavanol extracts were added to Beringer Main & Vine Pinot Grigio at the dosage of 1000 mg GAE/L for use in the study. This dosage fell within an acceptable commercial range for red wine, was palatable, and was devoid of anthocyanins. The FC assay was performed on the control wine and the wine samples fortified with extract to confirm that the experimental wines were matched in total polyphenol content (**Table B2)**. For full methodology of how potential control wines were screened and flavanol extract dosages selected, see Supplementary Information.

4.2.6 Wine Preparation. Wine samples were prepared 2 hours before the panel began. Three 1.5L bottles of Beringer Main & Vine were combined into a composite sample for each of the wines to be evaluated in the panel (n = 3). Flavanol extracts were added to respective wine samples and sonicated for 10 minutes to dissolve as outlined in **Table B2**. Wine solutions were mixed thoroughly and were poured into wine glasses labeled with randomly generated 3-digit codes in 30 mL aliquots using liquor pourers. Glasses were covered until samples were served. Wines were stored and served at room temperature (20°C).

4.2.7 Panelist Recruitment. Before beginning any sensory work, IRB approval was obtained at Washington State University (Protocol #16710-001). Sensory panelists were recruited from the Washington State University sensory database maintained using Compusense (Compusense Inc, Ontario, Canada). Panelists were screened for potential inclusion based on willingness to wear a nose clip while evaluating the wine samples, willingness to undergo body composition analysis, and willingness to participate in a 2-day sensory panel. Additionally, through a screening questionnaire, only panelists who scored at least a 6 (like slightly) on the hedonic scale (1-9, 1 = dislike extremely, 9 = like extremely) when asked how much they liked consuming red wine were selected for inclusion. If the panelists agreed to the conditions of the test and indicated that they liked to consume red wine, they were recruited for the sensory panel. Panelists were screened until at least 100 had been recruited and a total of 102 panelists completed the study. Subjects provided written informed consent before participating in any procedure.

4.2.8 Consumer Panel Overview. The consumer sensory assessment of the wine occurred over the course of two days. On the first day, panelists were asked to refrain from eating or drinking 1 hour prior to participating in the panel. Upon arrival, panelists participated in a brief orientation to familiarize them with bitterness and astringency perception, and how to differentiate the two sensations. Afterwards, they proceeded to the sensory booths to evaluate the three wine samples. To prevent fatigue, the remaining portions of the sensory test were performed the following day. On the second day, panelists were instructed to refrain from eating or drinking 3 hours before participating in the panel. Upon arrival, panelists' body composition assessments were made by

bioelectrical impedance (BIA). Panelists subsequently proceeded to sensory booths for bitterness sensitivity assessment using the PROP test. Panelists received gift card incentives after completing all four elements of the sensory panel.

4.2.9 Bitterness and Astringency Orientation. In preparation for evaluating the wine samples, panelists attended a brief orientation session on bitterness and astringency, as performed previously (Pickering, Simunkova, & DiBattista, 2004). In this training, panelists tasted a bitter aqueous solution (0.05 mM quinine sulfate, Sigma) and an astringent aqueous solution (4 mM Alum, McCormick). Panelists evaluated these solutions by holding them in their mouths for 5 seconds, swirling, and expectorating. A short discussion related to the sensations of each sample occurred following each evaluation. Once panelists felt that they could adequately distinguish bitterness and astringency, they were instructed to proceed to the sensory booths to evaluate the wine samples.

4.2.10 Wine Evaluation. Wine sample evaluation was conducted under red lights to mask the difference in color of the wine samples imparted by the added extracts. Without masking the color, the control wine appeared pale yellow, the GSE-amended wine appeared light pink, and the PBE-amended wine appeared red. Before beginning, the panelists were instructed to wear a nose clip (MD Spiro, Lewiston, ME, USA) while evaluating each sample in order to mask the differences in volatile compounds between the GSE- and PBE-amended wines and allow for the panelists to focus on bitterness and astringency with minimal distraction. The panelists evaluated each of the three wines (control Pinot Grigio wine, GSE-amended wine, PBE-amended wine) following a pre-determined randomized serving order. A 5-minute break between samples was used to prevent fatigue and carry-over, during which panelists were instructed to cleanse their palates with crackers and distilled water. Panelists were instructed to evaluate each wine by holding the sample in the mouth, swirling, and expectorating, multiple times if necessary. Panelists were then asked a series of questions including overall wine liking, bitterness liking, bitterness intensity, and astringency intensity. Panelists indicated liking with the hedonic scale and intensity with a 9-point horizontal line scale anchored on each end. Panelists were also asked a 'check-all-that-apply' (CATA) question to determine attributes associated with each sample. Terms included in the CATA were sourness/acidity, astringency, bitterness, sweetness, alcohol burn, mouthcoating, complex, silky, sandpaper, irritant, drying, abrasive, and rough and were selected based on terms previously validated by consumers to describe astringency (L. Vidal et al., 2018). After evaluating each sample, panelists were asked a ranking question in terms of overall product preference. Panelists were probed as to their liking of five bitter foods: tonic water, leafy greens, dark roasted coffee, sharp cheddar cheese, and dark chocolate. Subjects indicated their preference for these foods using the hedonic scale. Panelists were classified as "bitter preferring" if they scored an 8 (like very much) or higher for any three of the five foods. This classification was defined *a priori* based on a preliminary survey of 89 potential panelists at Washington State University.

4.2.11 Body Composition. Body composition assessment was performed at the beginning of the second day of sensory testing by BIA (Kyle, 2004). Upon arrival, panelists were asked to urinate before proceeding to the testing room. Panelists were then instructed to remove shoes, socks, heavy clothing, and personal effects. The height of each subject was measured and recorded for accurate programming of the BIA scale (SC-240, Tanita Corporation of America, Inc., Arlington Heights, IL, USA). Panelists were then asked to step onto the scale for body composition analysis. Once

complete, panelist weight, BMI, body fat percentage (BF%), and body water percentage were recorded. BMI data were used to classify panelists based on the ACSM BMI classification system: lean (BMI < 25 kg/m^2), overweight 25 ≤ BMI < 30 kg/m^2), or obese (BMI ≥ 30 kg/m^2). Panelists were also classified in terms of their BF% based on the ACSM guidelines for BF% as outlined in **Table B3**, which accounts for both age and sex. Panelists who achieved an "excellent" or "good" BF% using the ACSM table were classified as having below average BF%. Those who achieved an "average" BF% were classified as average. Those who achieved a "below average" or "poor" rating were classified as having an above average BF% classification (American College of Sports Medicine, 2013).

4.2.12 Bitterness Sensitivity. Following the BIA procedure, bitterness sensitivity of each panelist was assessed by a PROP test, as described previously (Pickering et al., 2004; Prescott & Tepper, 2004). A 1 M sodium chloride solution and a 0.32 mM PROP solution were prepared 2 hours before the start of the second day of sensory evaluation. The sodium chloride solution was prepared in distilled water by vortexing and 5 minutes of sonication. The PROP solution was prepared by first dissolving the PROP in 15 mL ethanol (200 proof, Decon Laboratories, King of Prussia, PA, USA), sonicating for 10 minutes, and then diluting to volume with distilled water. Panelists were presented with the sodium chloride solution and PROP solution in sequence, in 30 mL aliquots presented in plastic cups labeled with randomly assigned 3-digit codes. Panelists were instructed to hold the entire sample in their mouth, swirl it, and expectorate. After tasting, panelists indicated the intensity of each solution on a labeled magnitude scale (LMS). Panelists were required to wait for 2 minutes between samples and were instructed to cleanse their palate with crackers and distilled water. Using the LMS data, panelists were classified into three bitterness sensitivity categories (Ditschun & Guinard, 2004): "non-tasters" if they scored the PROP solution more than 0.2 LMS units lower than the sodium chloride solution, "tasters" if they scored PROP and sodium chloride within 0.2 LMS units, or "super-tasters" if they scored the PROP solution more than 0.2 LMS units higher than the sodium chloride solution.

4.2.13 Statistical Analysis. Statistical analysis of extract characterization procedures was performed by Student's T-tests using GraphPad Prism (v.7, La Jolla, CA, USA). Statistical analysis of the sensory test data was performed using XLStat Sensory (Addinsoft, New York, NY, USA). Significance was defined *a priori* as $p < 0.05$. ANCOVA models were used to determine relationships between all qualitative and quantitative variables (**Table B4**) with 2 levels of interaction on the individual sensory attributes. 2-way ANOVA was performed to look at the relationship between wine type, panelist phenotypes, and the interaction between these two variables on the sensory attributes. A series of 1-way ANOVA procedures were used to look at differences between phenotypes within individual wine samples, to look at differences between wine samples within individual phenotypes, and to look at differences between all wines and phenotypes together. Finally, to assess just the difference between GSE and PBE scores (the control wine was a control in the sense that it was not bitter or astringent, but not a true control in the sense that it was simply used as a delivery system for GSE and PBE), the difference between individual panelist values for GSE and PBE were taken for each sensory attribute within each phenotype and analyzed by 1-way ANOVA.

4.3 RESULTS AND DISCUSSION

4.3.1 Panelist Demographics and Phenotype Distributions. Demographic and phenotype classification information is presented in **Table 4.2** for the 102 panelists that participated in the sensory panel. Males and females comprised 40.2 and 59.8% of the panel, respectively. Average panelist age was 34.9 ± 13.1 years and the majority of the panel was white (64%). In terms of education, 88.3% of the panelists had at least a 4-year college degree. Income ranged from less than $19,999 to greater than $200,000 with the greatest percentage of panelists reporting income to be between $20,000 and $49,999 (37.3%). It was required that panelists like red wine to participate in the study. The average reported liking of red wine on the hedonic scale was 7.5, which corresponds to "like moderately".

Table 4.2 Demographic Information and Categorization of Panelists[a] Participating in Analysis of Wine

Characteristic	Category	
Gender	Male (%)	40.2
	Female (%)	59.8
Average Age (years)		34.9 ± 13.1
Race	White (%)	64.7
	Hispanic (%)	9.8
	American Indian or Alaskan Native (%)	2.0
	Asian or Asian American (%)	23.5
	Black, African American, or Non-Hispanic (%)	2.0
	Middle Eastern (%)	0.0
	Pacific Islander (%)	0.0
	Other (%)	2.0
Education Level	High-School Diploma (%)	3.9
	Some college or Associate Degree (%)	7.8
	Bachelor's Degree (%)	2W
	Master's Degree (%)	27.5
	Doctoral Degree (%)	20.6
	Advanced or Professional Degree (%)	2.9
Income	Less than $19,999 (%)	21.6
	$20,000 – $49,999 (%)	37.3
	$50,000 – $79,999 (%)	15.7
	$80,000 - $99,999 (%)	7.8
	$100,000 – $149,999 (%)	10.8
	$150,000 – $199,999 (%)	0.0
	$200,000 or more (%)	2.0
	Prefer not to answer (%)	4.9
Average hedonic liking of red wine (Scale of 1-9)		7.5 ± 1.3[b]
BMI	Lean (%)	55.9
	Overweight (%)	30.4
	Obese (%)	13.7
BF%	Below average (%)	13.7
	Average (%)	23.5
	Above average (%)	62.7
Bitterness Sensitivity	Non-taster (%)	35.3
	Taster (%)	43.1
	Super-taster (%)	21.6
Bitterness Preference	Non-Preferring (%)	59.8
	Preferring (%)	40.2

[a] n = 102 panelists

[b] equivalent to a hedonic score of "like moderately"

Panelists were segmented based on data collected during the panel based on four different phenotype characteristics: BF%, BMI, PROP sensitivity, and bitterness preference (**Table 4.2**). The population of lean individuals was slightly skewed compared to the total US population. According to the CDC, 39% of the US population is considered obese (BMI $\geq$ 30), however, in this study only 13% of the panelists were obese. PROP sensitivity follows a bimodal distribution, which was observed in this population (Drewnowski, Henderson, & Shore, 1997). The distribution of panelists classified as bitter preferring and bitter non-preferring was almost even, with 40% of the panelists being classified as bitter preferring. Additional demographic sorting was performed and reported for each phenotype (BMI, BF%, PROP sensitivity, bitterness preference) to determine trends (**Tables B4-B7**). Analyses of the panel attributes was performed based on both BF% and BMI, as it is clear in Tables S4-5 that BMI and BF% categorization do not completely align. Although all panelists classified as having below average BF% also were categorized as having a lean BMI, not all panelists classified as having lean BMI were also classified as having below average BF%, which coincides with previous reports of a nonlinear relationship of these attributes (Meeuwsen, Horgan, & Elia, 2010). It was also noted that the majority of PROP tasters and supertasters were female, but the distribution of PROP nontasters was roughly even between males and female (**Table B6**).

To determine whether any correlations existed between bitterness preferences and PROP intensity scores, a series of linear regression analyses were performed. PROP intensity rating of the panelists was not significantly correlated with liking scores of the 5 bitter foods rated by the panelists for bitterness preference classification (overall p value = 0.406), panelist BF% ($p = 0.706$), or panelist BMI ($p = 0.409$), data not shown. However, panelists who were classified as bitter non-preferring rated the PROP solution slightly but not significantly higher than panelists who were classified as bitter preferring (**Figure B1**). This suggested that while the panelists could be segmented in terms of their liking of bitter foods, preference did not relate to PROP sensitivity.

4.3.2 Overall Panel Results. Overall product liking, bitterness liking, bitterness intensity, and astringency intensity for each of the three wine samples is presented in **Figure 4.2**. In terms of overall liking and bitterness liking, the control sample was liked significantly more than the PBE-amended wine (**Figure 4.2A-B**). Subjects perceived the PBE-amended wine as significantly higher in terms of bitterness and astringency intensity compared to the control wine (**Figure 4.2C-D**). For all four parameters, GSE-amended wine was not significantly different from the other wine samples. It must be mentioned that it was hypothesized that the GSE-amended wine would score higher in overall bitterness intensity compared to the PBE-amended wine, but the PBE-amended wine scored higher in both cases.

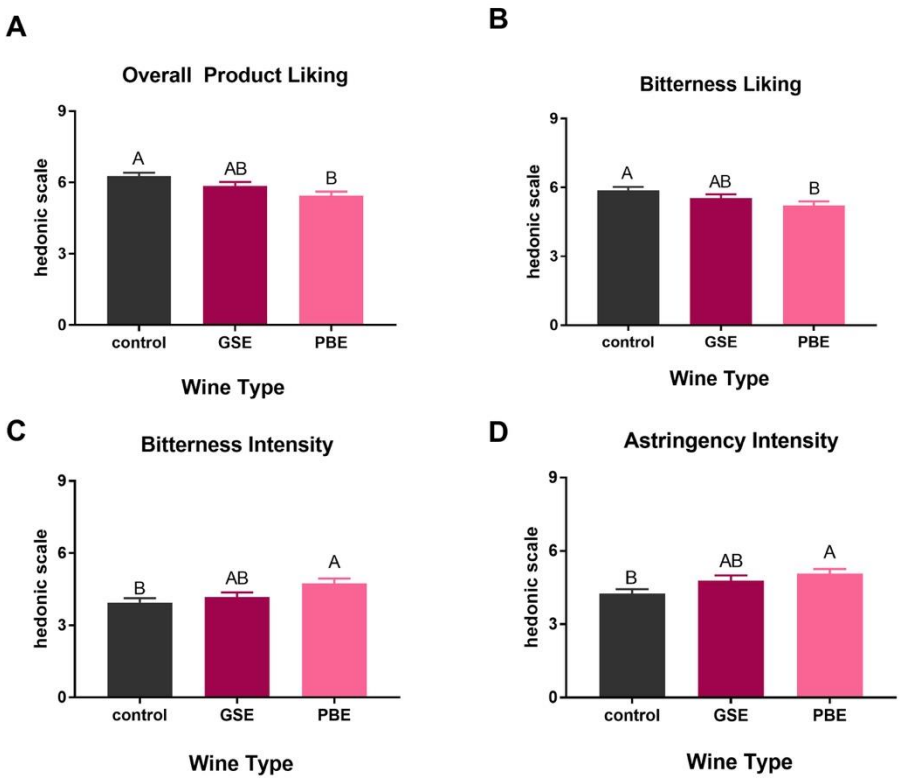

Figure 4.2. *Overall sensory panel results for each wine sample including overall product liking (A), bitterness liking (B), bitterness intensity (C), and astringency intensity (D). Values are expressed as means ± SEM based on hedonic scale values that range from 1 to 9, with 1 indicating "dislike extremely" and 9 indicating "like extremely". Significantly different values are indicated by different letters at the 95% confidence level based on 1-way ANOVA.*

Product ranking results are presented in **Table 4.3**. The trends in the ranking data generally aligned with the liking data, showing decreased liking (GSE > PBE) with increasing mDP (GSE < PBE); however, in this case all wines were ranked significantly different from each other. This suggests that when considering wines on an individual basis, panelists were not able to fully differentiate the three samples, but when asked to compare liking of all three samples together, they were able to significantly differentiate all samples.

Table 4.3 Overall Product Ranking

Product	Average Ranking
Control	1.77 ± 0.05a
GSE-amended wine	1.97 ± 0.04b
PBE-amended wine	2.25 ± 0.04c

Values are expressed as means ± SEM with different letters indicating significantly different rankings (1 = highest ranking, 3 = lowest ranking) based on the Friedman's test with Nemenyi's procedure. Significance was defined as $p < 0.05$.

A check-all-that-apply analysis (CATA) was performed to examine the distribution of attributes associated with the wine samples. **Figure 4.3A** depicts the distribution of responses for each of the CATA terms. CATA terms that were significantly associated with mean liking included sweetness ($p < 0.0001$), astringency ($p < 0.0001$), silky ($p < 0.0001$), bitterness ($p < 0.0002$), mouthcoating ($p < 0.007$), and drying ($p < 0.035$) were all terms that significantly increased mean

liking scores of the wines if present in the sample. No terms caused a significant decrease in mean liking. A possible explanation for this is that it is possible that the term drying is a more familiar term for wine consumers than astringency. **Figure 4.3B** depicts the terms that are associated with mean impact. Terms including irritant, abrasive, sandpaper, and rough were associated with decreased liking scores, but these did not significantly decrease liking.

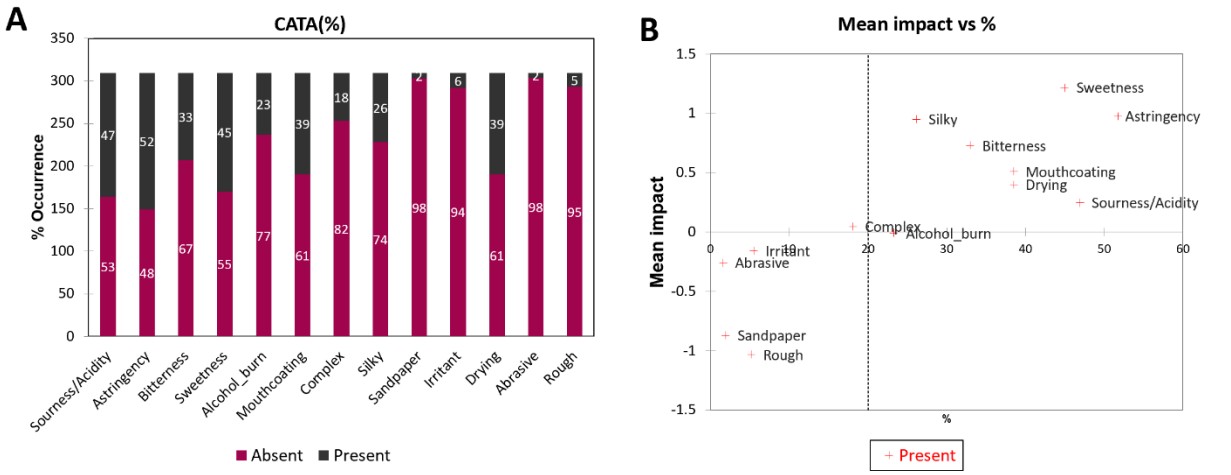

Figure 4.3 *Distribution of CATA responses, where percentage indicates the number of times the CATA term was used (grey) or not used (purple) by each panelist for each wine sample (n = 306) (A). Representation of attributes that are desired in products and attributes that are not desired in products (B).*

Prior to investigating individual phenotype and polyphenol type effects, a series of ANCOVA models were used to assess all phenotype effects simultaneously on overall liking, bitterness liking, bitterness intensity, and astringency intensity. Explanatory variables are listed in **Table B8** and significant results based on Type 3 sums of squares analysis are found in **Table 4.4**. The model for overall liking was significant ($p = 0.028$) and the 36 factors tested explained 35.7% of the variability. BF% as a continuous variable, age, the interaction between BMI as a continuous variable and age, the interaction between age and bitterness sensitivity, and the interaction between bitterness sensitivity and preference were significant variables in the model. None of the interactions between wine type and phenotype were significant. The model for bitterness liking was significant ($p = 0.001$) and the combined factors explained 40.4% of the variability, however, based on the type 3 sums of squares analysis, only the interaction between age and BMI as a categorical variable was significant ($p = 0.045$). The model for bitterness intensity was not significantly explained by the explanatory variables. The model for astringency intensity was highly significant ($p = 0.0001$) and 83.8% of the variability was explained by the factors. Significant explanatory factors by type 3 sums of squares analysis included BMI as a continuous variable, BMI as a categorical variable, wine type, bitterness preference category, bitterness sensitivity, the interaction between BF% as a categorical and continuous variable, the interaction between BF% as a continuous variable and bitterness sensitivity, the interaction between wine type and BF% as a categorical variable, the interaction between bitterness preference and wine type, and the interaction between bitterness preference and bitterness sensitivity. ANCOVA modeling with the same parameters was performed for each of the three individual wines separately but none of these models were significant in terms of overall liking, bitterness liking, bitterness intensity, or astringency intensity (data not shown). Overall, it would appear that astringency intensity was

the attribute most correlated with the explanatory variables used in this study, given that 10 out of the 36 explanatory variables tested were significant.

After assessing all explanatory variables in relation to the wine attributes, a series of secondary analyses were performed to examine the relationships within each phenotype category. Results are discussed below.

Table 4.4. ANCOVA Model Parameters and Results

Response Variable	**Overall Liking**	**Bitterness Liking**	**Bitterness Intensity**	**Astringency Intensity**
	Overall Model	Overall Model	Overall Model	Overall Model
Variability Explained (%)	**35.7**	**40.4**	34	**83.8**
p-value	**0.028**	**0.001**	0.081	**< 0.0001**
Explanatory Variables	Significance			
BF% (continuous)				
BMI (continuous)				**0.012**
age				
BMI (categorical)				**0.049**
BF% (categorical)				
wine type	**< 0.0001**			**0.014**
Bitterness preference				**0.007**
PROP sensitivity	**0.026**			**0.006**
BF%*BMI (continuous)				
BF%*age (continuous)				
BF% (continuous)*BMI (categorical)				
BF% (continuous) *BF% (categorial)				**0.008**
BF% (continuous) *wine type				
BF% (continuous)*Bitterness preference				
BF% (continuous)*PROP sensitivity				**0.017**
BMI (continuous)*age				
BMI (continuous)*BMI (categorical)				
BMI (continuous)*BF% (categorical)				
BMI (continuous)* wine type				
BMI (continuous)*Bitterness preference				
BMI (continuous)*PROP sensitivity				
age*BMI (categorical)		**0.045**		
age*BF% (categorical)				
age*wine type				
age*Bitter preference				

age* PROP sensitivity	**< 0.0001**		
BMI (categorical)*BF% (categorical)			
BMI (categorical)*wine type	**0.046**		
BMI (categorical)*Bitterness preference			
BMI (categorical)*PROP sensitivity			
BF% (categorical)*wine type			**0.001**
BF% (categorical)*Bitterness preference			
BF% (categorical)*PROP sensitivity			
Bitterness preference*wine type			**0.012**
PROP sensitivity*wine type			
Bitterness preference*PROP sensitivity	**0.040**		**0.023**

4.3.3 Analysis by Panelist BF% Classification. Panelists were classified into three BF% categories as described above. A series of analyses were performed on the panel outcomes based on segmenting the population into "below average", "average", and "above average" BF% classifications. In terms of product ranking (**Table 4.5**), panelists classified as having below average and average BF% ranked the control wine significantly higher than the PBE-amended wine and ranked the GSE-amended wine not significantly different from the other two wines. Panelists classified as having above average BF% ranked both the control wine and the GSE-amended wine higher than the PBE-amended wine but not significantly different from each other, suggesting that individuals with higher BF% had less tolerance or liking of the attributes.

Table 4.5 Overall Product Ranking by BF% Classification

	Average Ranking		
Product	**Below Average BF%**	**Average BF%**	**Above Average BF%**
Control	1.64 ± 0.1a	1.71 ± 0.1a	1.84 ± 0.06a
GSE-amended wine	2.05 ± 0.1ab	1.97 ± 0.09ab	1.95 ± 0.06a
PBE-amended wine	2.31 ± 0.1b	2.32 ± 0.09b	2.20 ± 0.06b

Values are expressed as means ± SEM with different letters indicating significantly different rankings (1 = highest ranking, 3 = lowest ranking) down columns based on the Friedman's test with Nemenyi's procedure. Significance was defined as $p < 0.05$.

To assess the individual factors of wine type, BF%, and the interaction between wine type and BF% on the main outcomes of overall liking, bitterness liking, bitterness intensity and astringency intensity, a two-way ANVOA was performed. The models for overall liking ($p = 0.040$) and astringency intensity ($p = 0.027$) were significant. However, in both cases, based on type 3 sums of squares analysis, only wine type significantly explained the variability in the model. As the primary interest in this study was related to the interaction between phenotypes and wine types, secondary analyses using one-way ANOVA were performed to differentiate between individual phenotypes within each wine and to differentiate between wines within each BF% category. No significant differences were observed when comparing different phenotypes within each wine sample (**Figure B2A-D**). Conversely, notable differences were observed when analyzing the sensory attributes within each BF% category (i.e. differences between wine samples within phenotype), rather than looking at differences between BF% category within each wine sample (as was performed in **Figure B2**). When assessing differences between wine samples within an individual BF% classification, it was observed that panelists with below average BF% liked the control and the GSE-amended wine significantly more than the PBE-amended wine while panelists classified with average BF% did not significantly prefer any sample, and panelists with above average BF% showed a trend of decreased liking with increasing mDP with the control sample being liked significantly more than the PBE-amended wine (**Figure 4.4A**). Differences were not observed within the bitterness liking and intensity attributes (**Figure 4.4B-C**), but panelists with below average BF% indicated that the PBE-amended wine was significantly more astringent than the control and GSE-amended wines, panelists with average BF% indicated that both the GSE-amended and PBE-amended wines were significantly more astringent than the control, while the above average BF% panelists did not detect any differences in astringency intensity (**Figure 4.4D**). This observation aligned with the ranking data (**Table 4.5**), again suggesting that ability to perceive differences in astringency decreases with increasing BF%.

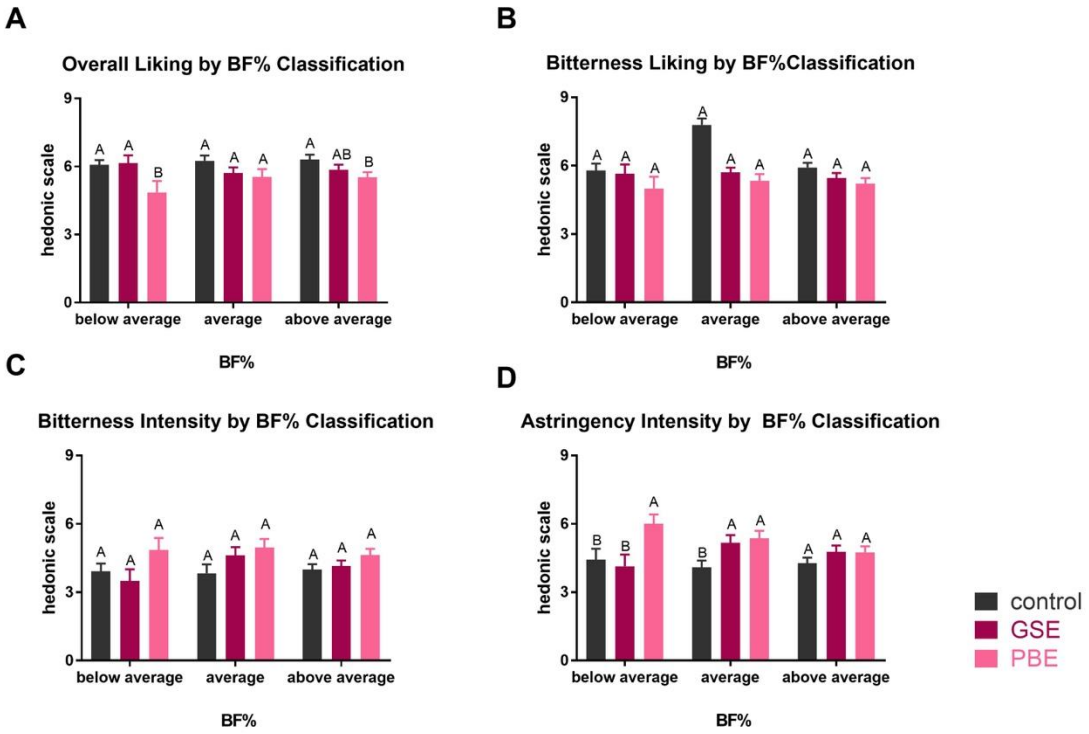

Figure 4.4. *Sensory panel results segmented based on panelist BF% classification and wine type for overall product liking (A), bitterness liking (B), bitterness intensity (C), and astringency intensity (D). Values are expressed as means ± SEM based on hedonic scale values that range from 1 to 9, with 1 indicating "dislike extremely" and 9 indicating "like extremely". Significantly different values within each phenotype category are indicated by different letters at the 95% confidence level based on 1-way ANOVA. BF% classifications were determined based on individual panelist gender, measured BF% scores, and the ACSM BF% guidelines.*

To assess the absolute differences in sensory attributes between the two flavanol-rich wine samples, the difference in scores for the panel attributes for GSE and PBE for each panelist were computed and analyzed by 1-way ANOVA within each BF% classification. By this method, no differences were observed between categories for overall liking, bitterness liking, or bitterness intensity (**Figure 4.5A-C**), but panelists with below average BF% classification indicated that the GSE-amended wine was significantly less astringent than the PBE-amended wine compared to the average and above average BF% panelists, who did not find the wines different in terms of astringency intensity (**Figure 4.5D**). Additionally, for every wine attribute, the panelists with below average BF% found greater, albeit not significant, differences in GSE and PBE wines compared to the average and below average BF% panelists. This aligns with previous data and indicates that perception of bitterness and especially astringency may be blunted by increasing BF%.

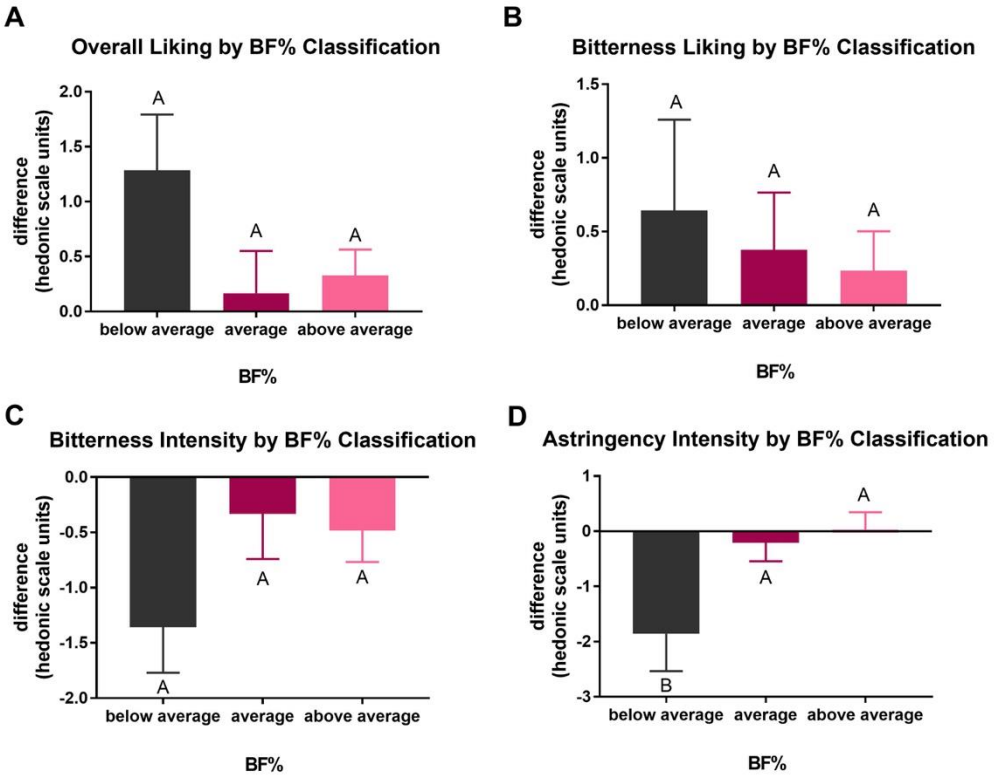

Figure 4.5. *Difference (GSE minus PBE) in sensory panel results for GSE and PBE wine samples when panelists were segmented by panelist BF% for overall product liking (A), bitterness liking (B), bitterness intensity (C), and astringency intensity (D). Values are expressed as means ± SEM based on hedonic scale values that range from 1 to 9, with 1 indicating "dislike extremely" and 9 indicating "like extremely". Differences in scores were calculated by subtracting the hedonic score of GSE and PBE. Significantly different values are indicated by different letters at the 95% confidence level based on 1-way ANOVA. BF% classifications were determined based on individual panelist gender, measured BF% scores, and the ACSM BF% guidelines.*

4.3.4 Analysis by Panelist BMI Classification. Individual panelist BMIs were recorded with the BF% data. Panelists were classified into the three BMI categories (lean, overweight, and obese) as outlined above. The results from the overall product ranking (**Table 4.6**) indicated that the lean panelists significantly preferred both the control and GSE-amended wines over the PBE-amended wine. The overweight panelists preferred the control wine over the PBE-amended wine, but the GSE-amended wine was not preferred over any other wine. Interestingly, there was no difference in wine ranking in the obese panelist category, suggesting a reduced ability to differentiate the wine samples and preferences for them.

Table 4.6 Overall Product Ranking by BMI Classification

	Average Ranking		
Product	**Lean**	**Overweight**	**Obese**
Control	1.82 ± 0.06a	1.75 ± 0.09a	1.71 ± 0.1a
GSE-amended wine	1.91 ± 0.106a	2.04 ± 0.08ab	2.07 ± 0.1a
PBE-amended wine	2.28 ± 0.06b	2.20 ± 0.08b	2.21 ± 0.1a

Values are expressed as means ± SEM with different letters indicating significantly different rankings (1 = highest ranking, 3 = lowest ranking) down columns based on the Friedman's test with Nemenyi's procedure. Significance was defined as $p < 0.05$.

When examining the panel results by 2-way ANOVA with BMI classification and wine type as the two factors explaining differences in overall liking, bitterness liking, bitterness intensity, and astringency intensity, the overall model for overall liking ($p = 0.011$), bitterness liking ($p = 0.006$), bitterness intensity ($p = 0.031$), and astringency intensity ($p = 0.031$) were significant. Based on the type 3 sums of squares analysis, no attributes were significant for bitterness intensity or astringency intensity, wine type was a significant factor for overall liking ($p = 0.019$). Only the interaction between BMI classification and wine type was significant for bitterness liking ($p = 0.049$) (**Figure B3**). No other terms were significant in the 2-way ANOVA model (data not shown). When making comparisons within each wine sample (**Figure B4A-D**), no differences were observed for overall liking, bitterness intensity, or astringency intensity. In terms of bitterness intensity, the overweight panelists had significantly lower liking for the PBE-amended wine ($p = 0.016$) compared to the lean and obese panelists (**Figure B4B**).

Most interestingly, differences were observed within all four panel attributes when analyzing the dataset by BMI phenotype (**Figure 4.6A-D**). In terms of overall liking (**Figure 4.6A**), both the lean and overweight panelists liked the control sample significantly more than the PBE-amended wine. The overweight panelists also liked the GSE-amended wine significantly more than the PBE-amended wine, but the lean panelists did not like the GSE-amended wine significantly more or less than any other sample. No differences in liking were observed in the obese panelists. In terms of bitterness liking (**Figure 4.6B**), no differences were observed in the lean or obese panelists. The overweight panelists liked the bitterness of the control and GSE-amended wine samples significantly more than the PBE-amended wine. Similarly, the overweight panelists found the PBE-amended wine to be significantly more bitter than the control and GSE-amended wines, but the lean and obese panelists did not find any significant differences between wine bitterness intensity (**Figure 4.6C**). However, a trend of increasing bitterness intensity with increasing wine flavanol mDP was observed in the lean group. Increased astringency intensity with increasing wine flavanol mDP was also observed in the lean and overweight groups, but only the lean group found the control wine to be significantly less astringent than the two other samples (**Figure 4.6D**). Overall, the lean panelists exhibited decreased liking with increased mDP with concurrent increases in perceived bitterness and astringency, but the obese group found no differences in liking or differences in perceived attribute intensity. This aligns with the findings from the BF% analysis, suggesting that increased fat mass is associated with a loss in ability to differentiate sensory attribute intensities. No significant differences were observed in bitterness liking, bitterness intensity or astringency intensity in the above average BF% group (**Figure 4.4B-D**), and no significant differences were observed for any attribute in the obese category when classifying panelists by BMI (**Figure 4.6)**.

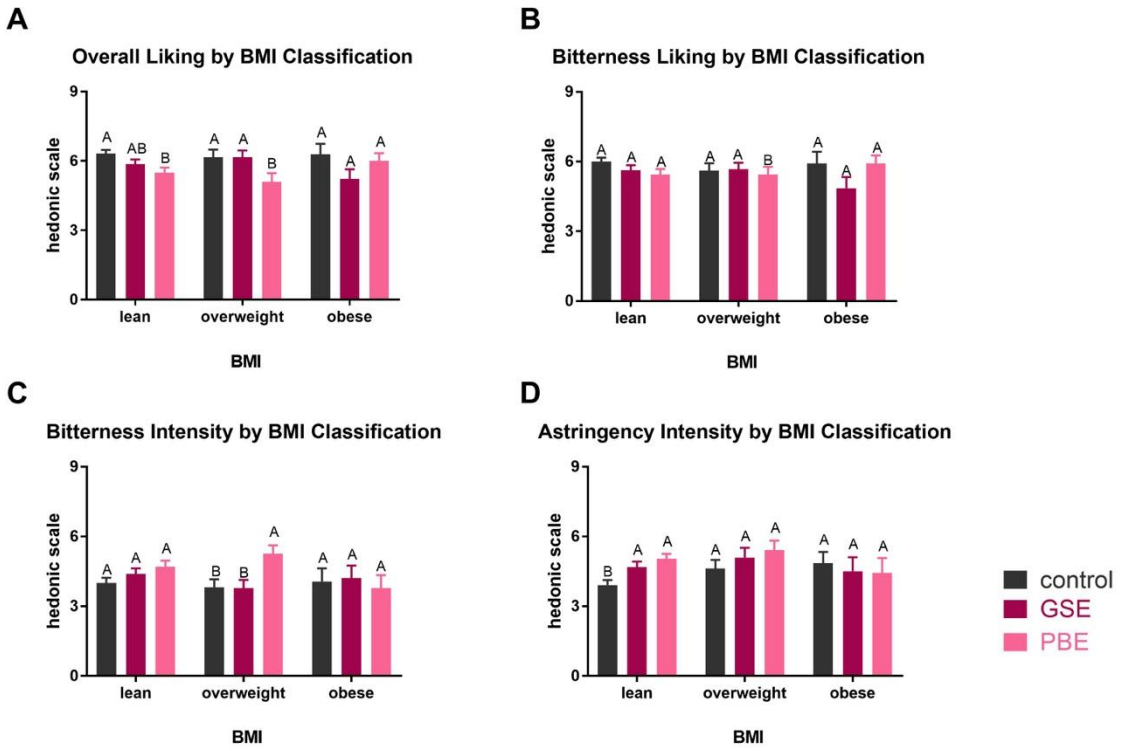

Figure 4.6 *Sensory panel results segmented based on panelist BMI classification and wine type for overall product liking (A), bitterness liking (B), bitterness intensity (C), and astringency intensity (D). Values are expressed as means ± SEM based on hedonic scale values that range from 1 to 9, with 1 indicating "dislike extremely" and 9 indicating "like extremely". Significantly different values within each phenotype category are indicated by different letters at the 95% confidence level based on 1-way ANOVA. Panelist BMI classifications were determined based on ACSM guidelines.*

When taking the absolute individual panelist score differences between GSE and PBE for the panel attributes, differences between BMI categories were observed in overall liking, bitterness liking, and bitterness intensity (**Figure 4.7A-C**). The obese panelists liked the PBE-amended wine significantly more than the GSE-amended wine compared with the lean and overweight panelists, which contrasted with the BF% classification results, in which all BF% groups liked the GSE-amended wine more than the PBE wine. Bitterness liking was significantly different for all BMI categories. The overweight panelists liked the bitterness of the GSE-amended wine over the PBE-amended wine, the lean panelists only preferred the GSE-amended wine slightly more than the PBE wine, but the obese panelists liked the PBE-amended wine bitterness more than the GSE-amended wine. The bitterness intensity data agreed with the trends in overall liking and bitterness liking. The obese panelists found the GSE-amended wine to be more bitter and astringent than the PBE-amended wine while the lean and overweight categories reported the opposite, with the overweight group finding the PBE-amended wine significantly more bitter compared to the lean and obese groups (**Figure 4.7C-D**). Overall, this indicates that within an individual BMI classification, increased astringency and bitterness perceptions were associated with decreased overall liking and bitterness liking, but the trends in terms of product preference were inconsistent across groups.

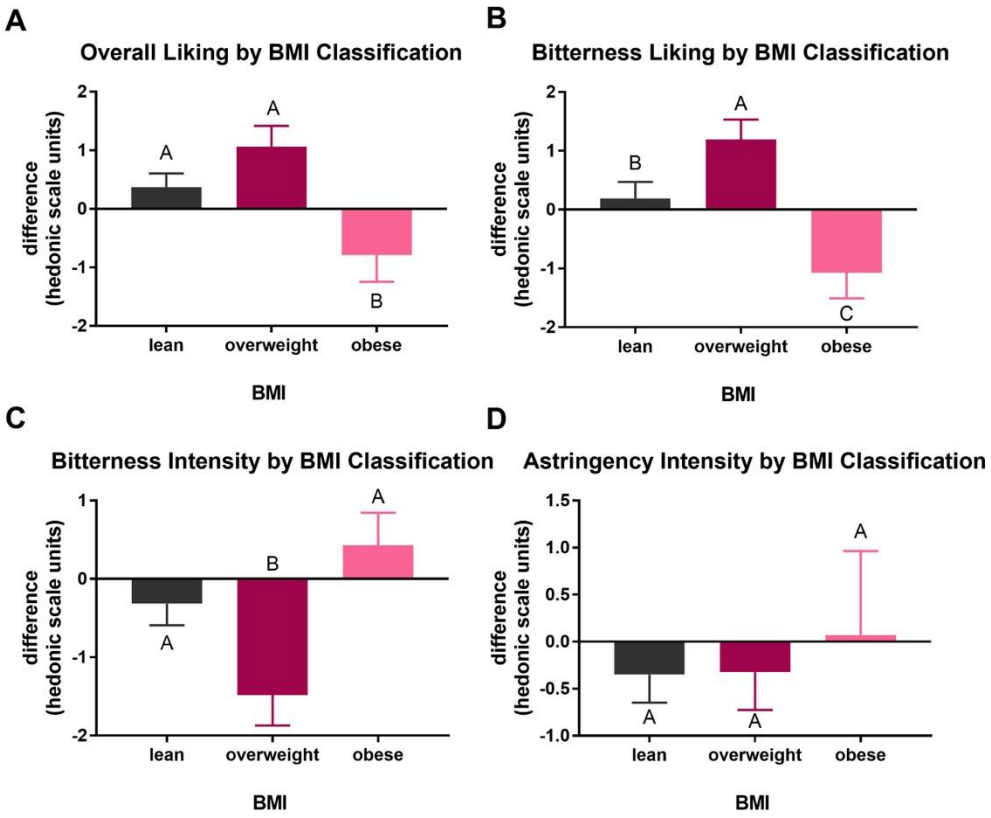

Figure 4.7 *Difference (GSE minus PBE) in sensory panel results for GSE and PBE wine samples when panelists were segmented by panelist BMI for overall product liking (A), bitterness liking (B), bitterness intensity (C), and astringency intensity (D). Values are expressed as means ± SEM based on hedonic scale values that range from 1 to 9, with 1 indicating "dislike extremely" and 9 indicating "like extremely". Differences in scores were calculated by subtracting the hedonic score of GSE and PBE. Significantly different values are indicated by different letters at the 95% confidence level based on 1-way ANOVA. Panelist BMI classifications were determined based on ACSM guidelines.*

4.3.5 Analysis by Panelist PROP Taster Status Classification. Bitterness sensitivity was assessed by a PROP test as outlined above. Panelists were separated into "nontaster", "taster", and "supertaster" categories. Product ranking followed the same trend within each bitterness sensitivity category (**Table 4.7**). Regardless of PROP sensitivity, product ranking decreased with increasing mDP and the control wine was ranked significantly higher than the PBE-amended wine, but the GSE-amended wine was not ranked differently than any other sample.

Table 4.7 Overall Product Ranking by PROP Sensitivity Status

	Average Ranking		
Product	**Nontaster**	**Taster**	**Supertaster**
Control	1.79 ± 0.08a	1.79 ± 0.07a	1.79 ± 0.1a
GSE-amended wine	2.02 ± 0.07ab	1.96 ± 0.07ab	1.91 ± 0.1ab
PBE-amended wine	2.20 ± 0.07b	2.25 ± 0.07b	2.30 ± 0.1b

Values are expressed as means ± SEM with different letters indicating significantly different rankings (1 = highest ranking, 3 = lowest ranking) down columns based on the Friedman's test with Nemenyi's procedure. Significance was defined as $p < 0.05$.

By two-way ANOVA, the models examining the significance of wine type, bitterness sensitivity, and their interactions on overall liking ($p = 0.011$), bitterness liking (0.033), bitterness intensity ($p = 0.018$), and astringency intensity ($p = 0.040$) were significant. Wine type was a significant factor in all of the models, bitterness sensitivity was significant in the model for astringency intensity, but the interaction of bitterness sensitivity and wine type was not significant for any panel attribute (data not shown). When comparing bitterness sensitivity results within each wine sample (**Figure B5A-D**), no differences were observed for overall liking, bitterness intensity, or astringency intensity. Nontasters liked the bitterness of the GSE-amended wine significantly more than supertasters, while tasters did not like the bitterness of any product more than the others (**Figure B5B**).

Analysis of main outcomes within phenotypes (i.e. only within each sensitivity category) indicated that no significant differences in product attributes were observed within taster or supertaster categories (**Figure 4.8A-D**). Nontasters however indicated significant differences in overall liking for all wines with a trend of decreased liking with increased mDP (**Figure 4.8A**). Nontasters also disliked the bitterness and found the PBE-amended wine to be significantly more intense than the control and GSE-amended wines (**Figure 4.8B-C**). No difference in astringency was detected by any of the bitter sensitivity categories, but all showed a clear trend of increasing astringency intensity with increasing mDP (**Figure 4.8D**).

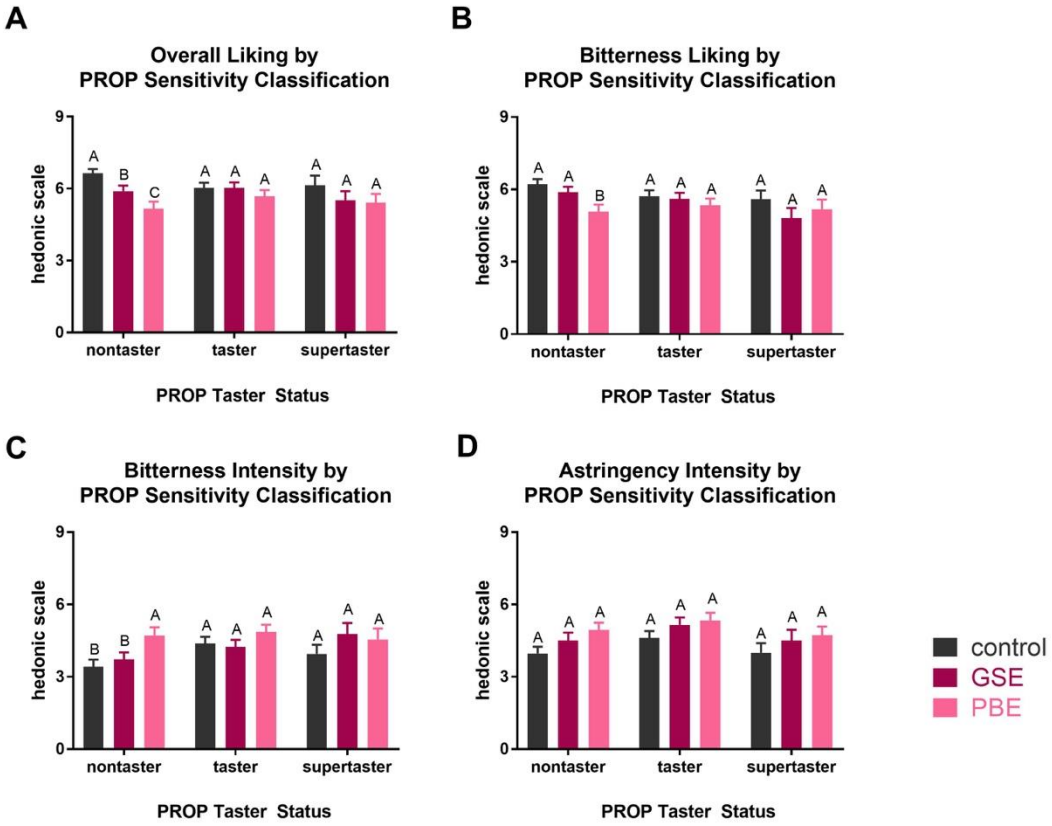

Figure 4.8 *Sensory panel results segmented based on panelist bitterness sensitivity classification and wine type for overall product liking (A), bitterness liking (B), bitterness intensity (C), and astringency intensity (D). Values are expressed as means ± SEM based on hedonic scale values that range from 1 to 9, with 1 indicating "dislike extremely" and 9 indicating "like extremely". Significantly different values within each phenotype category are indicated by different letters at the 95% confidence level based on 1-way ANOVA. PROP sensitivity classifications were determined based on LMS intensity ratings of a PROP solution against a sodium chloride solution. Non-tasters rated PROP lower*

than sodium chloride, tasters rated PROP equal to sodium chloride, and supertasters rated PROP higher than sodium chloride.

Overall, these data suggest that PROP sensitivity is an unreliable indicator of liking and sensitivity of flavanols of different mDP in a wine-like matrix. No significant differences between sensitivity categories were observed when comparing the difference in absolute differences in panelist product attribute ratings of the GSE- and PBE-amended wines (**Figure 4.9A-D**). However, several potentially useful albeit non-significant trends were observed. The nontasters preferred the GSE-amended wine (more bitter) compared with the PBE-amended wine (more astringent) than the tasters or supertasters, and the supertasters did not score the wines very differently in terms of overall liking (**Figure 4.9A**). Additionally, the nontasters and tasters liked the bitterness of the GSE-amended wine more than the PBE-amended wine, while the supertasters preferred the PBE-amended wine (**Figure 4.9B**). The supertasters correspondingly found the GSE-amended wine to be slightly more bitter than the PBE-amended wine while the nontasters and tasters found the PBE-amended wine to be more bitter than the GSE-amended wine (**Figure 4.9C**). All sensitivity categories found the PBE-amended wine to be more astringent than the GSE-amended wine (**Figure 4.9D**).

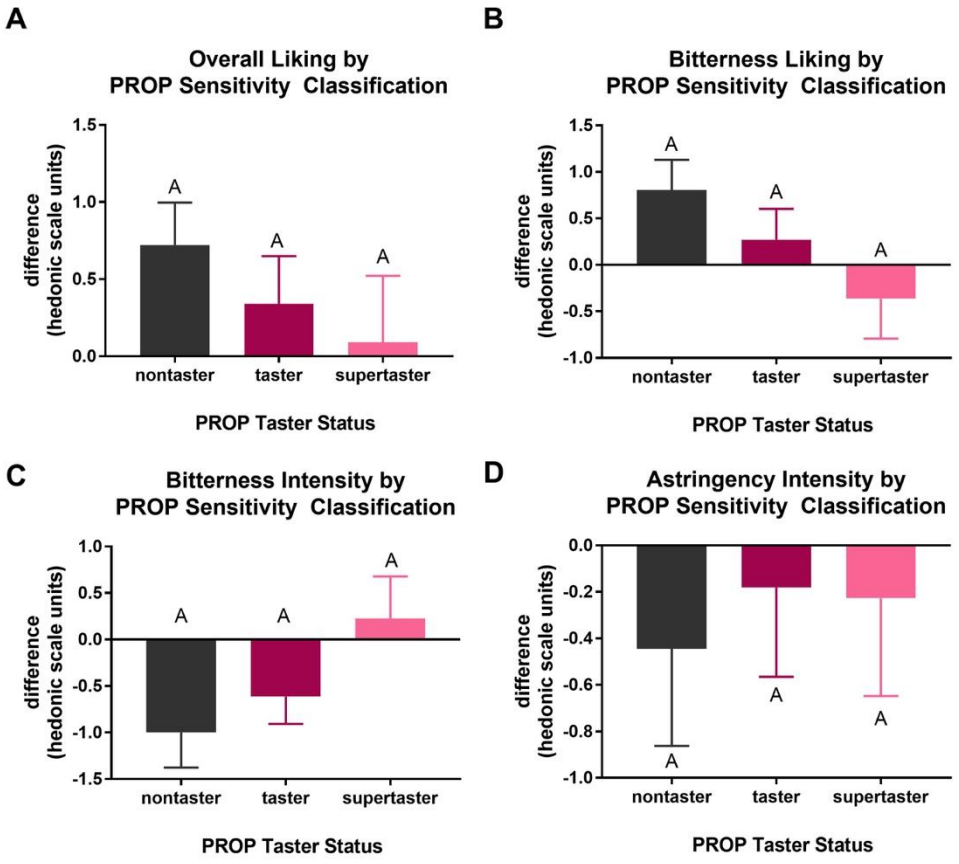

Figure 4.9 *Difference (GSE minus PBE) in sensory panel results for GSE and PBE wine samples when panelists were segmented by panelist bitterness sensitivity for overall product liking (A), bitterness liking (B), bitterness intensity (C), and astringency intensity (D). Values are expressed as means ± SEM based on hedonic scale values that range from 1 to 9, with 1 indicating "dislike extremely" and 9 indicating "like extremely". Differences in scores were calculated by subtracting the hedonic score of GSE and PBE. Significantly different values are indicated by different letters at the 95% confidence level based on 1-way ANOVA. PROP sensitivity classifications were determined based on LMS*

intensity ratings of a PROP solution against a sodium chloride solution. Non-tasters rated PROP lower than sodium chloride, tasters rated PROP equal to sodium chloride, and supertasters rated PROP higher than sodium chloride.

4.3.6 Analysis by Panelist Bitterness Preference Classification. Bitterness preference was assessed quantitatively by collecting panelist liking data on bitter foods qualitatively as described above. ANCOVA models were used to assess wine attributes. Reported hedonic liking of the five bitter foods were entered into the model as quantitative variables and wine type was entered as a qualitative variable. Two levels of interaction were used. The overall model for wine liking was significant ($p = 0.025$), with the interaction of leafy green liking and sharp cheddar cheese liking as a significant factor ($p = 0.047$). The model for bitterness liking was also significant ($p = 0.019$) with wine type ($p = 0.011$), the interaction between dark chocolate liking and dark roast coffee liking ($p = 0.038$), and the interaction between leafy greens liking and tonic water liking ($p = 0.002$) contributing significantly to the model. Neither the model for bitterness intensity nor the model for astringency intensity was significant.

The series of ranking and ANOVA tests as performed previously was also performed based on the qualitative assignment of panelist bitterness preference. The ranking data (**Table 4.8**) indicated that bitter non-preferring panelists ranked the control wine significantly higher than either flavanol-rich wine sample but the bitter preferring panelists ranked both the control and GSE-amended wine (more bitter sample) significantly higher than the PBE-amended wine and not significantly different from each other. By two-way ANOVA the models for overall liking ($p = 0.017$), bitterness intensity ($p = 0.015$), and astringency intensity ($p = 0.011$) were significant (data not shown). In the cases of overall liking and bitterness liking, wine type was the only significant factor ($p = 0.003$ and 0.02, respectively). No interactions between bitter preference and wine type were significant factors.

Table 4.8 Overall Product Ranking by Bitterness Preference

	Average Ranking	
Product	Non-Preferring	Preferring
Control	1.75 ± 0.06a	1.83 ± 0.08a
GSE wine	2.01 ± 0.06b	1.92 ± 0.07a
PBE wine	2.23 ± 0.06b	2.26 ± 0.07b

Values are expressed as means ± SEM with different letters indicating significantly different rankings (1 = highest ranking, 3 = lowest ranking) down columns based on the Friedman's test with Nemenyi's procedure. Significance was defined as $p < 0.05$.

No differences were observed between the bitterness preference categories when looking at results from 1-way ANOVA within each wine sample individually (**Figure B6A-D**). When looking at differences between wine samples within a preference category, no differences were observed in the bitter preferring panelists for wine attributes (**Figure 4.10A-D**). Bitter non-preferring panelists, however, liked the PBE-amended wine significantly less than the control wine. However, the bitter non-preferring group found the PBE-amended wine significantly more bitter than both the control and the GSE-amended wine. Despite a lack of significant differences in bitterness liking and astringency intensity of the products, both bitter preferring and bitter non-preferring panelists indicated decreased bitterness liking with increasing flavanol mDP and increased astringency and bitterness intensity with increasing flavanol mDP. No differences were observed between bitter preferring and non-preferring panelists when looking at the differences in scores for GSE- and PBE-amended wines (**Figure 4.11A-D**). Both groups scored the GSE-

amended wine higher in terms of overall liking, bitterness liking, and bitterness intensity. The bitter non-preferring group found the PBE-amended wine to be much more astringent than the GSE-amended wine while the bitter preferring group detected no difference.

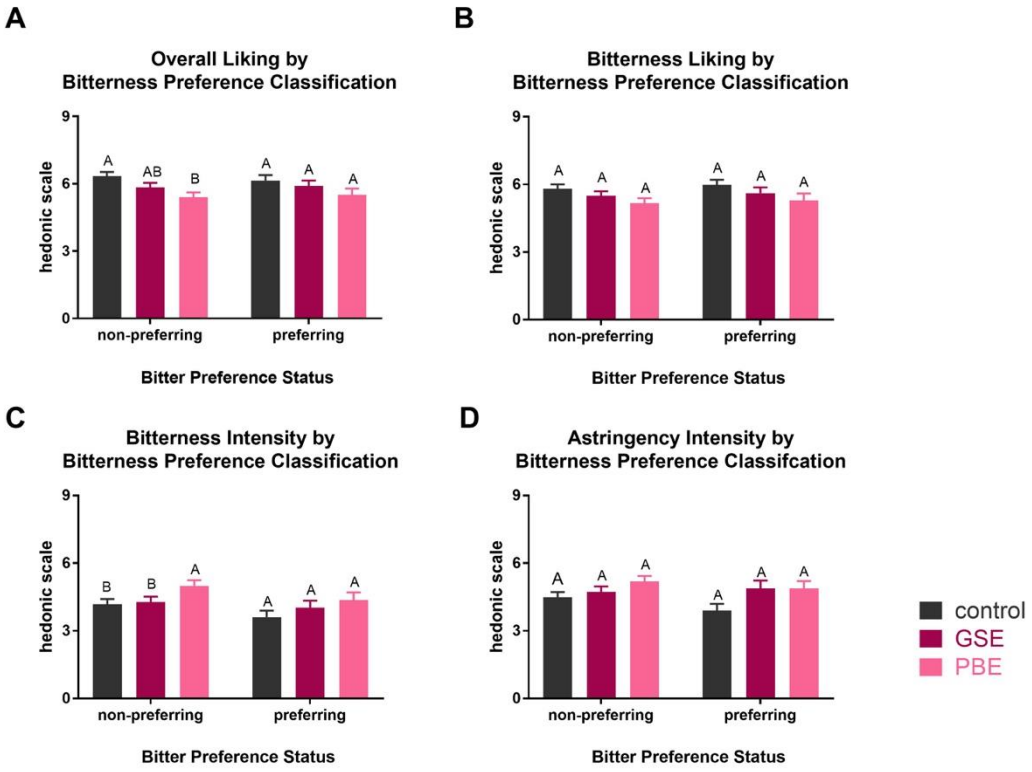

Figure 4.10 *Sensory panel results segmented based on panelist bitterness preference classification and wine type for overall product liking (A), bitterness liking (B), bitterness intensity (C), and astringency intensity (D). Values are expressed as means ± SEM based on hedonic scale values that range from 1 to 9, with 1 indicating "dislike extremely" and 9 indicating "like extremely". Significantly different values within each phenotype category are indicated by different letters at the 95% confidence level based on 1-way ANOVA. Bitter preference was determined on an individual level based on panelist hedonic scores for 5 bitter foods. To be classified as bitter preferring, panelists had to score 3 out of the 5 bitter foods at an 8 (like very much) or higher.*

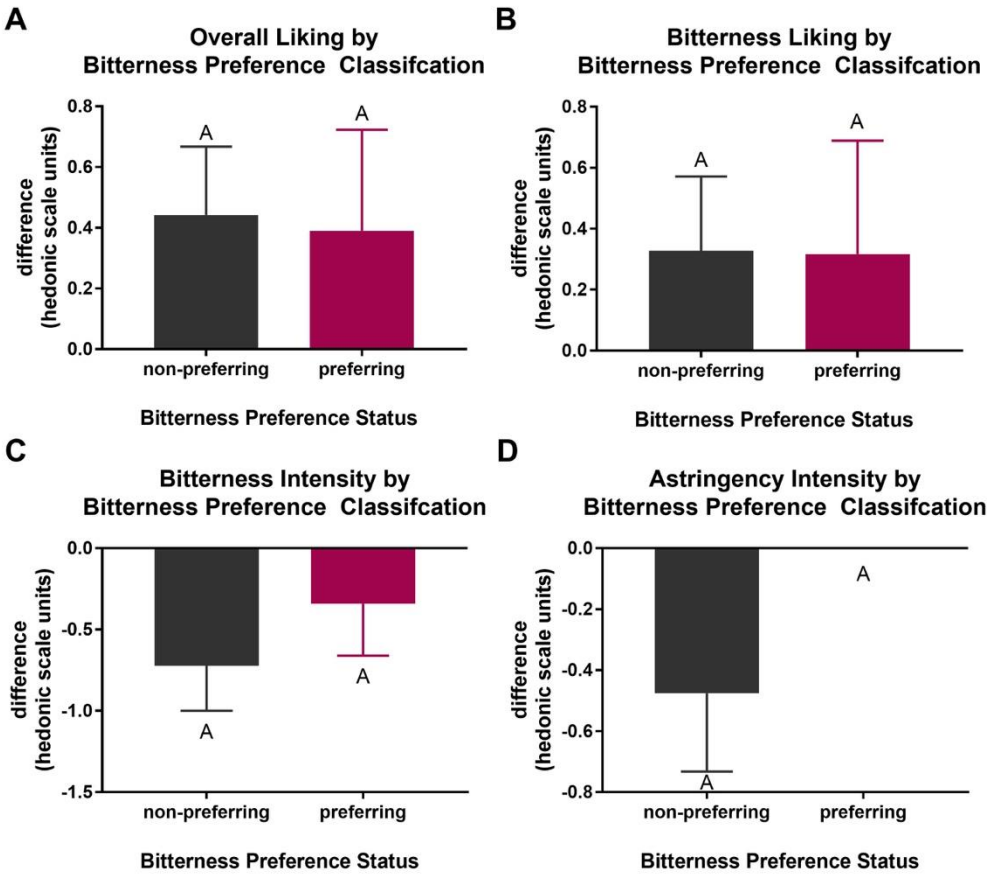

Figure 4.11 *Difference (GSE minus PBE) in sensory panel results for GSE and PBE wine samples when panelists were segmented by panelist bitterness preference for overall product liking (A), bitterness liking (B), bitterness intensity (C), and astringency intensity (D). Values are expressed as means ± SEM based on hedonic scale values that range from 1 to 9, with 1 indicating "dislike extremely" and 9 indicating "like extremely". Differences in scores were calculated by subtracting the hedonic score of GSE and PBE. Significantly different values are indicated by different letters at the 95% confidence level based on 1-way ANOVA. Bitter preference was determined on an individual level based on panelist hedonic scores for 5 bitter foods. To be classified as bitter preferring, panelists had to score 3 out of the 5 bitter foods at an 8 (like very much) or higher.*

4.3.7 Discussion. Overall, this study demonstrated that differences in phenotype are generally correlated with differences in liking of flavanol-rich foods and perception of bitterness and astringency. Considering all wine samples and all factors related to phenotype in the series of ANCOVA models, overall liking and astringency intensity were most explained by the variables. For both overall liking and astringency intensity, wine type and PROP sensitivity were individually significant factors, indicating that differences in flavanol mDP and bitterness sensitivity significantly contributed to liking and perceived astringency, as expected. It was also observed that the interaction between age and BMI category significantly explained the model for bitterness liking, and the interaction between age and PROP sensitivity significantly explained the model for overall liking. Previous studies on taste perception and age have indicated declining basic taste sensitivity with age, which could explain the correlation between age, PROP sensitivity, and overall liking observed here (Fukunaga, Uematsu, & Sugimoto, 2005). When examining the relationships between wine type and phenotype, it was observed that the interaction between BF% and wine type, and the interaction between bitterness preference and wine type significantly

explained astringency intensity. The interaction between BMI category and wine type significantly explained overall liking and the interaction between bitterness preference and PROP sensitivity significantly explained both liking and perceived astringency intensity. These findings indicate that body composition, preference for bitter taste, and bitterness sensitivity influence liking and astringency perception of foods that differ in bitterness and astringency due to differences in flavanol mDP. Despite previous reports indicating associations between PROP sensitivity, liking of bitter foods, and perceived bitterness intensity, in this study bitterness intensity was not significantly associated with any factors and bitterness liking was only associated with the interaction between age and BMI (Guinard et al., 1996; Keller, Steinmann, Nurse, & Tepper, 2002). It is possible that the reason for the discrepancy between our results and others is due to the fact that this study was performed using adults and model food systems (specifically model wine, in which some degree of bitterness is expected), rather than simple solutions of bitter tasting compounds (Cox et al., 2016; Tepper et al., 2009).

The CATA data provided further insight as to the attributes associated with certain wine samples and consumer perception of astringency and bitterness. Results from the mean impact analysis (**Figure 4.3B**) indicated that astringency, bitterness, mouthcoating, and drying were all terms associated with an increase in sample liking, along with sweetness and silkiness. This indicated that neither astringency nor bitterness were terms associated with negative connotation in terms of wine liking, despite reduced liking of the most astringent wine in the dataset. Moreover, the inclusion of mouthcoating, drying, and astringency on the list indicate that perhaps panelists do not completely understand the term astringency, seeing as it increased overall product liking in the CATA dataset, yet there was a trend towards decreased product liking with increased astringency due to flavanol mDP across phenotypes. It also suggests that "drying" may be a more consumer friendly term when referring to wine attributes. Previous research on consumer astringency vocabulary in the context of red wine has also indicated that a variety of terms related to astringency are used by consumers interchangeably (L. Vidal, Giménez, Medina, Boido, & Ares, 2015). Nonetheless, the panelists were able to differentiate intensities of bitterness and astringency of the wines and associated different taste attributes with different wines, but still found the PBE wine to be the most intense in terms of bitterness and astringency overall.

Notable differences within each phenotype category were observed based on the ANOVA procedures and segmentation of the population. As seen in the ANCOVA models, overall liking and astringency liking were most differentiated within the BF% phenotype. Regardless of BF% status, the PBE-amended wine was the least liked, an observation that could be attributed to increased bitterness and astringency of the wine. Panelists with higher BF%, however, were not able to differentiate between the bitterness or astringency intensity of any sample (**Figure 4.4**). The below average BF% panelists detected significantly larger differences in astringency intensity and larger, but not significantly larger, differences in overall liking, bitterness liking, and bitterness intensity between the GSE and PBE amended wines compared to panelists of average or above average BF% (**Figure 4.5**). These observations are suggestive of a reduced ability to perceive bitter and astringent sensations due to increased BF%, which aligns with the results of previous work (Overberg et al., 2012; Simchen et al., 2006). These mechanisms are not entirely understood but could be due to endocrine influence of taste perception, genetics, or repetitive exposure to specific taste stimuli (Overberg et al., 2012). Conversely, these results could also imply that lean individuals have increased sensitivity to and liking for bitter and astringent foods, perhaps due to a difference in the types of foods frequently consumed (less high-fat, high-sugar substances), or

increased mindfulness while eating (O'Reilly, Cook, Spruijt-Metz, & Black, 2014). Trends were somewhat similar when classifying panelists based on BMI categories, where the lean category indicated decreased wine liking and increased perceived bitterness and astringency with increasing mDP while the obese panelists found no differences in wine liking, bitterness, or astringency (**Figure 4.6**). Interestingly though, when looking at the difference in sensory attributes between GSE and PBE only, the obese panelists reported the opposite findings compared to the lean and overweight panelists (i.e. obese panelists found the PBE-amended wine to be less bitter and astringent than the GSE-amended wine, while the lean and overweight panelists thought that the PBE-amended wine was more bitter and astringent than the GSE-amended wine) (**Figure 4.7**). These results suggest that elevated BMI changes perception of and preference of flavanol-rich foods, rather than just blunting taste sensations as suggested by the BF% data. Moreover, these results appear to be independent of PROP sensitivity, as all BMI categories had only a small proportion of PROP supertasters (**Table B5**). For the lean, overweight, and obese BMI groups, 24.6%, 19.4%, and 14.3% of the respective populations were also PROP supertasters. It would be useful to perform follow up analyses with more equal sample-size distributions to validate these findings.

Unexpectedly, PROP sensitivity and bitterness preference categorization did not differentiate liking and perception of sensory attributes to the same extent as categorization based on BF% and BMI. Based on PROP sensitivity, only nontasters indicated significant differences in liking and bitterness intensity (**Figure 4.8**), though supertasters were the only phenotype group to correctly identify the GSE-amended wine to be the more bitter sample (**Figure 4.9**). Similarly, bitter non-preferring individuals indicated significantly decreased wine liking and significantly increased bitterness intensity with increasing mDP (**Figure 4.10**). Previous studies have indicated that the PROP nontaster phenotype is associated with adiposity, which would imply, based on our findings, that PROP nontasters would have reduced sensitivity for bitterness and astringency (Goldstein, Daun, & Tepper, 2012; Tepper et al., 2012). Our results, however, indicate the exact opposite. In fact, of the 63 panelists classified as having an above average BF%, only 18 of them (25%) were also nontasters (**Table B4**). Other reports have indicated correlations between PROP taster status and greater acceptance of bitter foods such as beer or vegetables in nontaster categories (Bell & Tepper, 2006; Intranuovo & Powers, 2006). The findings from this study suggest that PROP taster status may not be the best predictor of liking and intensity of bitterness and astringency derived from GSE or PBE flavanols at the dose used in this study. Sensitivity to other compounds such as quinine sulfate or PTC could be more associated with liking and intensity of the attributes presented here and more investigation is warranted to establish consensus (Tepper et al., 2009).

This study indicated that differences in phenotype were associated with differences in flavanol preferences, but several limitations must be acknowledged. While it was possible to probe phenotype using survey methods before the study to ascertain the available population distribution, it was not possible to pre-screen panelists in terms of phenotype during study recruitment and therefore, study treatment groups were unequal. Moreover, the methods used to segment panelists based on bitterness preference and bitterness sensitivity did not indicate large differences in product liking or attribute sensitivity. It is possible that pre-screening panelists, while difficult to do for sensitive topics including body composition, would give a better indication of preference trends in a future study, as would devising a different method of assessment for preference for and sensitivity of bitter foods. It must be mentioned, that the substances used in this study were model-solutions, not true consumer products. Trends in liking may have been different if more traditional

wine products were used. However, we believe that this model was the ideal way to probe differences in liking by phenotype in a controlled setting, as other attributes in a real wine (residual sugar, ethanol content, anthocyanins, wine vintage, grape varietal) could confound the study outcomes (Boulet et al., 2016; McRae & Kennedy, 2011). Moreover, although these products were not real wines, in some cases, panelists rated the GSE-amended wine as highly as the control wine, indicating they believed this model product to be within the sensory specifications for red wine. The use of nose clips to blind panelists from atypical wine aromatics in the PBE sample and the use of red lighting to blind panelists from color difference also helped ensure that the consumers believe that they were tasting actual wine products. Additionally, the overall goal of this study was to determine whether different consumer phenotypes prefer and perceive wines of variable mDP differently, particularly in terms of bitterness and astringency. The panelists as a whole were able to significantly detect differences in all attributes of the wines indicating that we successfully created model wine solutions that were perceived differently, most likely due to differences in flavanol mDP. However, as a whole, panelists found the PBE-amended wine to be the most bitter and most astringent sample, despite the expectation for the GSE-amended wine to be perceived as more bitter due to the overwhelming presence of smaller DP flavanols. These findings could potentially be attributed, in part, to the fact that minimally trained panelists were used in this study. Panelists reported that the orientation to bitterness and astringency training was helpful in preparation for tasting the wine samples, but bitterness is still a complex taste that can be difficult for untrained consumers do detect and differentiate (Drewnowski & Gomez-Carneros, 2000). It must be mentioned that even though different phenotypes indicated variable preferences and sensitivities to taste attributes, this study did not address the question as to whether factors related to phenotype (genetics, diet, lifestyle) influenced taste preferences, or whether inherent taste preferences contributed to phenotype. Previous work related to taste preference and weight loss has indicated shifts in preference away from typical Western diet foods, but the present study can neither support nor refute this observation (Andriessen et al., 2018). Finally, it must also be mentioned that even though flavanol mDP impacts astringency, other factors can also influence this taste attribute due to variations in protein binding capacity, including flavanol structural variation and galloylation (Brossaud et al., 2001; Cheynier, 2005; McRae & Kennedy, 2011). These factors may also influence preference for and sensitivity to bitter and astringent foods. Future work should be directed towards linking phenotypes with preference trends and gene expression related to obesity and bitterness sensitivity, as well as looking at variation in preferences when mDP differences are greater (Goldstein et al., 2012; Tepper et al., 2012).

4.4 CONCLUSION

This study demonstrated that differences in phenotype are correlated with differences in preference for model wines containing flavanols with different mDPs and associated sensory attribute intensities. Regardless of phenotype, the wine made from flavanols with the highest mDP was the least liked and perceived as the most bitter and astringent, which was likely due to the presence of oligomer and polymer flavanols. Overall product liking and astringency intensity were the attributes where the most differences were observed. Classification of panelists based on BF% and BMI showed greater phenotype effects compared to classification based on preference for bitter foods or PROP sensitivity. These findings strongly suggest that weight status has a greater influence on taste perception and preference for flavanols of different mDPs, and that increasing

adiposity is associated with decreased sensitivity to small differences in bitter and astringent sensations.

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REFERENCES

American College of Sports Medicine. (2013). *ACSM's health-related physical fitness assessment manual*. Lippincott Williams & Wilkins.

Andriessen, C., Christensen, P., Vestergaard Nielsen, L., Ritz, C., Astrup, A., Meinert Larsen, T., … Raben, A. (2018). Weight loss decreases self-reported appetite and alters food preferences in overweight and obese adults: Observational data from the DiOGenes study. *Appetite*, *125*, 314–322. https://doi.org/10.1016/j.appet.2018.02.016

Beecher, G. R. (2003). Overview of dietary flavonoids: nomenclature, occurrence and intake. *The Journal of Nutrition*, *133*(10), 3248S–3254S.

Bell, K. I., & Tepper, B. J. (2006). Short-term vegetable intake by young children classified by 6- n-propylthoiuracil bitter-taste phenotype. *The American Journal of Clinical Nutrition*, *84*(1), 245–251. https://doi.org/10.1093/ajcn/84.1.245

Boulet, J.-C., Trarieux, C., Souquet, J.-M., Ducasse, M.-A., Caillé, S., Samson, A., … Cheynier, V. (2016). Models based on ultraviolet spectroscopy, polyphenols, oligosaccharides and polysaccharides for prediction of wine astringency. *Food Chemistry*, *190*, 357–363. https://doi.org/10.1016/j.foodchem.2015.05.062

Breitmaier, E. (2006). Terpenes: Importance, General Structure, and Biosynthesis. In *Terpenes* (pp. 1–9). Wiley-VCH Verlag GmbH & Co. KGaA. https://doi.org/10.1002/9783527609949.ch1

Brossaud, F., Cheynier, V., & Noble, A. C. (2001). Bitterness and astringency of grape and wine polyphenols. *Australian Journal of Grape and Wine Research*, *7*(1), 33–39.

Cheynier, V. (2005). Polyphenols in foods are more complex than often thought. *The American Journal of Clinical Nutrition*, *81*(1), 223S–229S.

Cheynier, V., Dueñas-Paton, M., Salas, E., Maury, C., Souquet, J.-M., Sarni-Manchado, P., & Fulcrand, H. (2006). Structure and properties of wine pigments and tannins. *American Journal of Enology and Viticulture*, *57*(3), 298–305.

Cox, D. N., Hendrie, G. A., & Carty, D. (2016). Sensitivity, hedonics and preferences for basic tastes and fat amongst adults and children of differing weight status: A comprehensive review. *Food Quality and Preference*, *48*, 359–367. https://doi.org/10.1016/j.foodqual.2015.01.006

Ditschun, T. L., & Guinard, J.-X. (2004). Comparison of new and existing methods for the classification of individuals according to 6-n propylthiouracil (PROP) taster status. *Journal of Sensory Studies*, *19*(2), 149–170. https://doi.org/10.1111/j.1745-459X.2004.tb00142.x

Dorenkott, M. R., Griffin, L. E., Goodrich, K. M., Thompson-Witrick, K. A., Fundaro, G., Ye, L., … Neilson, A. P. (2014). Oligomeric Cocoa Procyanidins Possess Enhanced Bioactivity Compared to Monomeric and Polymeric Cocoa Procyanidins for Preventing the Development of

Obesity, Insulin Resistance, and Impaired Glucose Tolerance during High-Fat Feeding. *Journal of Agricultural and Food Chemistry*, *62*(10), 2216–2227. https://doi.org/10.1021/jf500333y
Drewnowski, A., & Gomez-Carneros, C. (2000). Bitter taste, phytonutrients, and the consumer: a review. *The American Journal of Clinical Nutrition*, *72*(6), 1424–1435. https://doi.org/10.1093/ajcn/72.6.1424
Drewnowski, A., Henderson, S. A., & Shore, A. B. (1997). Genetic Sensitivity to 6-n-Propylthiouracil (PROP) and Hedonic Responses to Bitter and Sweet Tastes. *Chemical Senses*, *22*(1), 27–37. https://doi.org/10.1093/chemse/22.1.27
Fukunaga, A., Uematsu, H., & Sugimoto, K. (2005). Influences of Aging on Taste Perception and Oral Somatic Sensation. *The Journals of Gerontology: Series A*, *60*(1), 109–113. https://doi.org/10.1093/gerona/60.1.109
Fulcrand, H., Dueñas, M., Salas, E., & Cheynier, V. (2006). Phenolic reactions during winemaking and aging. *American Journal of Enology and Viticulture*, *57*(3), 289–297.
Goldstein, G. L., Daun, H., & Tepper, B. J. (2012). Adiposity in Middle-aged Women is Associated with Genetic Taste Blindness to 6-n-Propylthiouracil. *Obesity Research*, *13*(6), 1017–1023. https://doi.org/10.1038/oby.2005.119
Guinard, J.-X., Zoumas-Morse, C., Dietz, J., Goldberg, S., Holz, M., Heck, E., & Amoros, A. (1996). Does consumption of beer, alcohol, and bitter substances affect bitterness perception? *Physiology & Behavior*, *59*(4), 625–631. https://doi.org/10.1016/0031-9384(95)02124-8
Intranuovo, L. R., & Powers, A. S. (2006). The Perceived Bitterness of Beer and 6-n-Propylthiouracil (PROP) Taste Sensitivity. *Annals of the New York Academy of Sciences*, *855*(1), 813–815. https://doi.org/10.1111/j.1749-6632.1998.tb10665.x
Kallithraka, S., Bakker, J., & Clifford, M. N. (1997). Evaluation of bitterness and astringency of (+)-catechin and (-)-epicatechin in red wine and in model solution. *Journal of Sensory Studies*, *12*(1), 25–37.
Keller, K. L., Steinmann, L., Nurse, R. J., & Tepper, B. J. (2002). Genetic taste sensitivity to 6-n-propylthiouracil influences food preference and reported intake in preschool children. *Appetite*, *38*(1), 3–12. https://doi.org/10.1006/appe.2001.0441
Kennedy, J. A., Saucier, C., & Glories, Y. (2006). Grape and wine phenolics: history and perspective. *American Journal of Enology and Viticulture*, *57*(3), 239–248.
Kyle, U. (2004). Bioelectrical impedance analysis-part I: review of principles and methods. *Clinical Nutrition*, *23*(5), 1226–1243. https://doi.org/10.1016/j.clnu.2004.06.004
Lesschaeve, I., & Noble, A. C. (2005). Polyphenols: factors influencing their sensory properties and their effects on food and beverage preferences. *The American Journal of Clinical Nutrition*, *81*(1), 330S–335S.
McRae, J. M., & Kennedy, J. A. (2011). Wine and grape tannin interactions with salivary proteins and their impact on astringency: a review of current research. *Molecules*, *16*(3), 2348–2364.
Meeuwsen, S., Horgan, G. W., & Elia, M. (2010). The relationship between BMI and percent body fat, measured by bioelectrical impedance, in a large adult sample is curvilinear and influenced by age and sex. *Clinical Nutrition*, *29*(5), 560–566. https://doi.org/10.1016/j.clnu.2009.12.011
Meyerhof, W., Batram, C., Kuhn, C., Brockhoff, A., Chudoba, E., Bufe, B., … Behrens, M. (2010). The molecular receptive ranges of human TAS2R bitter taste receptors. *Chemical Senses*, *35*(2), 157–170.

Mueller, K. L., Hoon, M. A., Erlenbach, I., Chandrashekar, J., Zuker, C. S., & Ryba, N. J. P. (2005). The receptors and coding logic for bitter taste. *Nature*, *434*(7030), 225–229. https://doi.org/10.1038/nature03352
Neilson, A. P., O'Keefe, S. F., & Bolling, B. W. (2016). High-Molecular-Weight Proanthocyanidins in Foods: Overcoming Analytical Challenges in Pursuit of Novel Dietary Bioactive Components. *Annual Review of Food Science and Technology*, *7*(1), 43–64. https://doi.org/10.1146/annurev-food-022814-015604
O'Reilly, G. A., Cook, L., Spruijt-Metz, D., & Black, D. S. (2014). Mindfulness-Based Interventions for Obesity-Related Eating Behaviors: A Literature Review. *Obesity Reviews : An Official Journal of the International Association for the Study of Obesity*, *15*(6), 453–461. https://doi.org/10.1111/obr.12156
Overberg, J., Hummel, T., Krude, H., & Wiegand, S. (2012). Differences in taste sensitivity between obese and non-obese children and adolescents. *Archives of Disease in Childhood*, archdischild–2012.
Pickering, G. J., Simunkova, K., & DiBattista, D. (2004). Intensity of taste and astringency sensations elicited by red wines is associated with sensitivity to PROP (6-n-propylthiouracil). *Food Quality and Preference*, *15*(2), 147–154. https://doi.org/10.1016/S0950-3293(03)00053-3
Prescott, J., & Tepper, B. J. (2004). *Genetic Variation in Taste Sensitivity*. CRC Press.
Pronin, A. N., Xu, H., Tang, H., Zhang, L., Li, Q., & Li, X. (2007). Specific Alleles of Bitter Receptor Genes Influence Human Sensitivity to the Bitterness of Aloin and Saccharin. *Current Biology*, *17*(16), 1403–1408. https://doi.org/10.1016/j.cub.2007.07.046
Ryan, C. M., Khoo, W., Ye, L., Lambert, J. D., O'Keefe, S. F., & Neilson, A. P. (2016). Loss of Native Flavanols during Fermentation and Roasting Does Not Necessarily Reduce Digestive Enzyme-Inhibiting Bioactivities of Cocoa. *Journal of Agricultural and Food Chemistry*, *64*(18), 3616–3625. https://doi.org/10.1021/acs.jafc.6b01725
Simchen, U., Koebnick, C., Hoyer, S., Issanchou, S., & Zunft, H.-J. (2006). Odour and taste sensitivity is associated with body weight and extent of misreporting of body weight. *European Journal of Clinical Nutrition*, *60*(6), 698.
Soares, S., Silva, M. S., García-Estévez, I., Groβman, P., Brás, N. F., Brandão, E., … Meyerhof, W. (2018). Human bitter taste receptors are activated by different classes of polyphenols. *Journal of Agricultural and Food Chemistry*. https://doi.org/10.1021/acs.jafc.8b03569
Stewart, J. E., & Keast, R. S. J. (2012). Recent fat intake modulates fat taste sensitivity in lean and overweight subjects. *International Journal of Obesity*, *36*(6), 834.
Stewart, Jessica E., Feinle-Bisset, C., Golding, M., Delahunty, C., Clifton, P. M., & Keast, R. S. (2010). Oral sensitivity to fatty acids, food consumption and BMI in human subjects. *British Journal of Nutrition*, *104*(1), 145–152.
Tepper, B. J., Koelliker, Y., Zhao, L., Ullrich, N. V., Lanzara, C., D'Adamo, P., … Gasparini, P. (2012). Variation in the Bitter-taste Receptor Gene TAS2R38, and Adiposity in a Genetically Isolated Population in Southern Italy. *Obesity*, *16*(10), 2289–2295. https://doi.org/10.1038/oby.2008.357
Tepper, B. J., White, E. A., Koelliker, Y., Lanzara, C., D'Adamo, P., & Gasparini, P. (2009). Genetic Variation in Taste Sensitivity to 6-n-Propylthiouracil and Its Relationship to Taste Perception and Food Selection. *Annals of the New York Academy of Sciences*, *1170*(1), 126–139. https://doi.org/10.1111/j.1749-6632.2009.03916.x

Vidal, L., Antúnez, L., Giménez, A., Medina, K., Boido, E., & Ares, G. (2018). Astringency evaluation of Tannat wines: Comparison of assessments from trained assessors and experts. *Journal of Sensory Studies*, e12330.
Vidal, L., Giménez, A., Medina, K., Boido, E., & Ares, G. (2015). How do consumers describe wine astringency? *Food Research International*, *78*, 321–326. https://doi.org/10.1016/j.foodres.2015.09.025
Vidal, S., Meudec, E., Cheynier, V., Skouroumounis, G., & Hayasaka, Y. (2004). Mass spectrometric evidence for the existence of oligomeric anthocyanins in grape skins. *Journal of Agricultural and Food Chemistry*, *52*(23), 7144–7151.

CHAPTER 5:

Processing Strategies to Enhance the Health-Promoting Properties of Wine

ABSTRACT: Grapes are rich in dietary flavanols, which are potent antioxidants that can be extracted into a wine matrix during wine production. Dietary flavanols naturally exist in a wide variety of sizes and structures. It is believed that individual flavanol degrees of polymerization (DP) and total mean degrees of polymerization (mDP) of wines are related to bioactivity, as is the dosage ingested. In this pilot study, the effects of different common enological processing conditions on wine mDP, flavanol DP diversity, and total concentrations were determined. Traditional red and rosé wines were produced in addition to a cold-soaked red wine, a rosé wine made with added grape seeds, a rosé wine with added grape skins, and a rosé wine made with crushed grape seeds. No significant differences were observed among mDP, but significantly higher levels of flavanol monomers were found in the wines made with added grape seeds. Additionally, the flavanol diversity and flavanol concentration in the rosé wines with added seeds were significantly greater than all other wines. Cold soaking did not increase mDP, flavanol diversity, or concentration compared to the control red wine. It is possible that aging could produce changes in these wines especially those made with added seeds due to the abundance of monomer flavanols. Overall, in young wines, none of these processing treatments altered initial mDP, but concentration and flavanol diversity were vastly different. Moreover, the continued study of enhanced rosé wine, in terms of flavanol profile manipulation and subsequent health outcomes, is justified.

5.1 INTRODUCTION

Grapes and wine are rich sources of bioactive dietary flavanols, a subset of polyphenolic flavonoids found in plant tissue (Y. Xu et al., 2011). These compounds are extracted from grape tissue during winemaking and enhance the sensory characteristics of wine by impacting taste and mouthfeel. It is well documented that these compounds are correlated with health promoting effects when wine is consumed in moderation. Possible outcomes include reduced risk of hypertension, atherosclerosis, diabetes, and inflammation (Athyros et al., 2008; Han, Shen, & Lou, 2007; Rasmussen, Frederiksen, Struntze Krogholm, & Poulsen, 2005).

Dietary flavanols exist as catechin or epicatechin (EC) monomers, or procyanidins (PCs), which are oligomers and polymers of the monomer units (**Figure 5.1**) (Cheynier et al., 2006; Dorenkott et al., 2014). Given the wide variety in size and structure, individual flavanols can be characterized by their degrees of polymerization (DP), and flavanol-rich foods can be characterized by their mean degrees of polymerization (mDP) (Dorenkott et al., 2014; Neilson, O'Keefe, & Bolling, 2016). Numerous studies have indicated that mDP plays a role in *in vivo* bioactivity with certain sized PCs exerting greater effects depending on disease state and target tissue (Neilson et al., 2016).

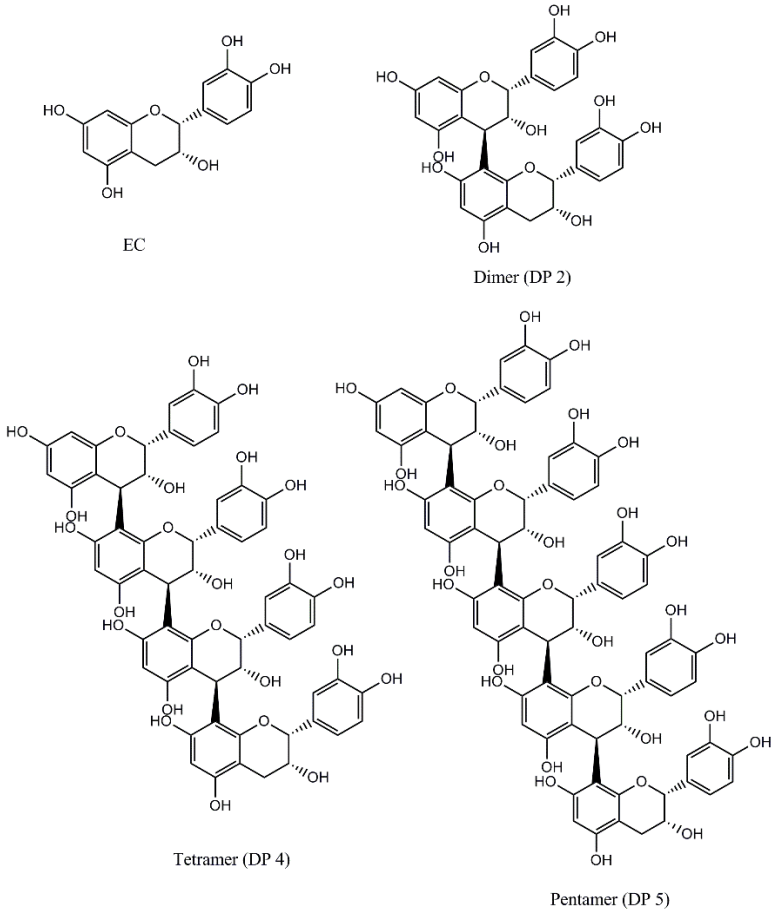

Figure 5.1 *Structural and size variation of different flavanol compounds.*

The flavanol composition of wine can vary greatly due to the complexity of winemaking and viticultural practices used. Flavanols of varying DP are extracted from grapes during the winemaking process (Boulton, Singleton, Bisson, & Kunkee, 2013). The concentration and DP of PCs vary depending on location in grape tissues. Grape seeds are believed to contain smaller PCs than grape skins (suggesting that inclusion of skins would likely raise mDP while seeds would have a lowering effect), but overall the seeds contain higher concentrations of PCs (Monagas, Gómez-Cordovés, Bartolomé, Laureano, & Ricardo da Silva, 2003). These variations in starting materials, whose composition are also influenced by environmental factors and fruit maturity, lead to a great variety of PCs found in different wines (Manach, Scalbert, Morand, Rémésy, & Jiménez, 2004; Pérez-Magariño & González-San José, 2004). Common wine processing techniques employed to increase wine color and improve sensory characteristics can further influence the PC profiles of wines, since flavanols are extracted in wine must during crushing, maceration, and fermentation. Cold soaking of grape must prior to fermentation, punching down of the must cap during fermentation, and extended maceration of must after fermentation and prior to pressing, are techniques employed by winemakers to increase phenolic extraction (Boulton et al., 2013; Koyama, Goto-Yamamoto, & Hashizume, 2007; Sacchi, Bisson, & Adams, 2005). Evaluation of these techniques with respect to polyphenol composition have thus far been somewhat limited (Lerno et al., 2015; Panprivech, Lerno, Brenneman, Block, & Oberholster, 2015; Sacchi et al., 2005). It has been shown that cold soaking reduces flavanol concentration and the addition of grape seeds to a red wine matrix increases flavanol concentration, but the impacts on mDP in these instances have not been determined (Casassa, Bolcato, & Sari, 2015; Kovac, Alonso, & Revilla, 1995; Sacchi et al., 2005). Little attention has been paid to the health effects and flavanol profile of rosé wine. Moreover, polymerization of small flavanols into larger PCs can also occur during aging, which could impact the flavanol profile of wines (Monagas et al., 2003; Pérez-Magariño & González-San José, 2004). Collectively, these factors allow for a wide variety of mDPs and flavanol concentrations to exist in wines (Cheynier et al., 2006). The study of these compounds, their occurrence, and how specific wine processing techniques impact mDP is of interest due to their apparent bioactivity and potential disease prevention properties (Arnous, Makris, & Kefalas, 2001; Griffin et al., 2017; Han et al., 2007; Rasmussen et al., 2005).

Numerous studies have examined the differences in flavanol concentration, DP, and mDP in grapes and wine (Jordão, Ricardo-da-Silva, & Laureano, 2001; Monagas et al., 2003; Prieur, Rigaud, Cheynier, & Moutounet, 1994; Sun, Leandro, Ricardo da Silva, & Spranger, 1998). However, it is difficult to make comparisons and note trends between studies because wines were made from different grapes, came from different vineyards, and/or different vintages. The purpose of this pilot study was to examine in-depth how flavanol concentration, mDP, and qualitative flavanol profile in wines is influenced by variables including processing methods, wine style, and fermentation substrate addition. Grapes from a common vineyard block were used for this study so direct comparisons across treatments were possible.

5.2 MATERIALS AND METHODS

5.2.1 Chemicals and Standards. Standards for thiolysis included (±)-catechin (C), (−)-epicatechin (EC), (−)-epigallocatechin, (−)-epicatechin gallate, and (−)-epigallocatechin gallate (Sigma, St. Louis, MO, USA), PC dimer B_2 and PC trimer C_1 (ChromaDex, Irvine, CA, USA), PC dimer B_1, dimer B_2 gallate, dimer B_5, trimer T_2, tetramer A_2, and pentamers through decamers

from Planta Analytica (Danbury, CT, USA). UPLC solvents were of LC-MS grade (VWR, Radnor, PA, USA). HPLC solvents were of ACS grade (Fisher, Pittsburgh, PA, USA, or VWR).

5.2.2 Grapes. Cabernet Sauvignon (clone ENTAV-INRA 337) grapes were obtained from nine-year-old, field-grown grapevines at the AHS Jr. Agricultural Research and Extension Center (AREC) in Virginia's Shenandoah Valley AVA in 2015. Vineyard rows were oriented northeast [28°]/southwest on a 2% slope, ~ 300 meters above sea level. Rows were spaced 3.0 m apart with 1.50 m in-row vine spacing. Vines were trained to bi-lateral cordons, spur-pruned, and canopies were trained upright to a vertical shoot positioned system. Canopy management included shoot thinning and positioning, fruit zone leaf removal, lateral and basal shoot removal, and shoot hedging. Canopies were shoot-thinned to ~ 12 shoots per m (of row) soon after bud burst. East-side leaves, lateral shoots, and late-emerging basal shoots in the fruit zone were removed immediately post fruit set. Removal of vegetation at this time aimed to retain 1-2 leaf layers in the fruiting zone as outlined in local cultural recommendations (Wolf, 2008). The grapes were obtained from the 2015 season, and were sourced from non-root-manipulated treatment plots of a larger experiment described elsewhere (Hickey, Hatch, Stallings, & Wolf, 2016). Grapes were harvested on 6 Oct 2015 on the basis of commercial standards of maturity. On harvest, the following parameters were observed: soluble solids averaged 21.8 °Brix, titratable acidity averaged 6.02 g/L, and pH averaged 3.43 across the plots from which grapes were sourced (Hill, 2017). Heat summation for the 2015 season (Apr-Oct., inclusive, base 10°C) was 2014 heat units, somewhat greater than long-term average (~1890 units). Average total rainfall from Apr-Oct. was 500 mm.

5.2.3 Red wines. Shortly after harvest**,** Cabernet Sauvignon grapes were separated into two groups for winemaking. Both wines were made by destemming the grapes and filling 19 L buckets with 11 L of must. Potassium metabisulfite (Carolina Wine Supply, Yadkinville, NC, USA) was added to both wines at 50 mg/L. The first batch of wine was subjected to a cold soaking procedure at 4°C for two days prior to inoculation with EC1118 yeast (Lallemand, Montreal, Canada) and supplementation with Fermaid K yeast nutrient (Scott Laboratories, Petaluma, CA, USA). Yeast was added to the must at 0.24 g/L and Fermaid K was added to the must at 0.25 g/L, as per manufacturer specifications. The second batch was inoculated shortly after potassium metabisulfite addition and was not subjected to cold soaking. Both wines were fermented for 9 days in an 18°C temperature-controlled room until residual sugar fell below 0.2%. The caps on all wines were punched down daily, and must temperature and brix were monitored throughout the fermentation period. The wines were pressed and bottled once fermentation was complete and samples were stored at −80°C to preserve the flavanol profiles for analyses.

5.2.4 Rosé wines. Four experimental treatments of rosé wines were made from Cabernet Sauvignon grapes. The juice from the 2015 vintage received potassium metabisulfite (Carolina Wine Supply) additions at 50 mg/L. Additionally, seeds and skins were collected from the pressed grapes via manual separation. A subset of grape seeds was crushed using a mortar and pestle. Rosé wines were made in 250 mL flasks and fermented in an 18°C temperature-controlled room. The first treatment consisted of 200 mL juice and served as the control. The second treatment was made by weighing 21 g of skins into a flask and then bringing the volume to 200 mL with juice (rosé + Sk). The third treatment was made by weighing 21 g of seeds and then bringing the volume up to 200 mL with juice (rosé + Sd). The fourth treatment was made by weighing 21 g of crushed seeds

and then bringing the volume up to 200 mL with juice (rosé + CrSd). Potassium metabisulfite was added to all treatments at a concentration of 50 mg/L. Once the juices reached 19°C, all treatments were inoculated with EC1118 yeast (Lallemand) and supplemented with Fermaid K yeast nutrient (Scott Laboratories) as described previously. A stir bar was placed into each of the flasks, sealed with airlocks, and placed on a hot plate (Corning, PC-351, Cole-Parmer, Vernon Hills, IL, USA) at 250 rpm. Flasks were stirred at 400 rpm twice a day and weighed twice a day to monitor fermentation. The wines fermented for 14 days until residual sugar fell below 0.2%. All wines were racked and samples stored at -80°C until analysis.

5.2.5 Flavanol Extraction. In preparation for flavanol characterization of the wine samples, total flavanol concentration of the wines was needed. First, wines were removed from the freezer and allowed to thaw before 4 mL aliquots were transferred to freeze drying vessels. The samples were then frozen at -80°C before being freeze dried (FreeZone, Labconco, Kansas City, MO, USA) until no water remained. To ensure that the ethanol remained frozen, sample vessels were surrounded by dry ice for the duration of the freeze-drying period. Once complete, freeze-dried residues were then resolubilized to 1 mg/mL in methanol as needed for subsequent analyses.

5.2.6 Estimation of mDP Using Thiolysis. The mDP of the flavanols in the wine samples were estimated using the thiolysis procedures outlined in Dorenkott et al. (Dorenkott et al., 2014) and Ryan et al. (Ryan et al., 2016) with modifications. Samples (n=5 per wine) were thiolyzed by combining 50 µL of sample (1 mg extract/mL methanol) with 50 µL HCl (3.3% w/v) and 100 µL benzyl mercaptan (5% w/v in MeOH). Controls for each sample were prepared using 50 µL of sample and 150 mL of MeOH. All samples and controls were heated at 90ºC for 1 min, cooled on ice for 5 min, and transferred to HPLC vials in a 1:9 v/v ratio with 0.1% formic acid in water/0.1% formic acid in acetonitrile (95:5). Monomers and benzylthioether derivatives were quantified using a UPLC-MS/MS method described in Dorenkott et al. (Dorenkott et al., 2014). Full methodological details can be found in Supplementary Information.

5.2.7 Qualitative Analysis of PCs by Normal Phase HPLC. Profiling of the flavanols in the wine extracts (n=1) was performed on an Agilent Technologies 1260 Infinity HPLC (Santa Clara, CA, USA) as outlined previously in Bitzer et al. (Bitzer et al., 2015). The wine extracts were analyzed at 10 mg/mL in methanol. Full methodological details can be found in Supplementary Information.

5.2.8 Estimation of Flavanol Concentration. Total polyphenols and total PC concentration of the wine samples were estimated by the Folin-Ciocalteu (FC) and 4-dimethylaminocinnamaldehyde (DMAC) assays, respectively, as previously described (Ainsworth & Gillespie, 2007; Payne et al., 2010; Ryan et al., 2016). For the FC assay, wine extracts were diluted in triplicate to 10 mg/mL in MeOH and compared to a gallic acid standard curve (0-1 mg/mL in 40% EtOH). Sample absorbance was measured using a BioTek Synergy 2 spectrophotometer (Winoosky, VT, USA) at 765 nm and polyphenol concentration was calculated based on a linear regression equation. For the DMAC assay, wine extracts were diluted to 1 mg/mL in MeOH and compared to a PC B_2 standard curve (0 to 100 ppm in 1:1 MeOH:water). Sample absorbance was measured using a BioTek Synergy 2 set at 640 nm and PC concentration was determined using a linear regression equation.

5.2.9 Statistical Analysis. Results were analyzed using GraphPad Prism v. 6 (La Jolla, CA, USA). Statistical significance between wine samples was determined using 1-way ANOVA with Tukey's HSD post-hoc test for all measures except the normal-phase HPLC chromatogram results (qualitative analysis only). Significance was defined as $p < 0.05$.

5.3 RESULTS AND DISCUSSION

5.3.1 mDP. All samples were thiolyzed by UPLC-MS and mDP for each wine was calculated based on equations (Appendix C, Equation C1 and C2) including and excluding native monomers (**Figure 5.2A,B**). Average native monomer abundance (**Figure 5.2C**) and net monomers, which represent the quantity of derivatives generated, (**Figure 5.2D**) are also reported. No significant differences in mDP were observed between any samples using either mDP calculation. The mDP for all samples based on the calculation including native monomers (**Figure 5.2A**) was approximately 2, indicating that the average compound size across samples was dimers; note that this value accounts for the monomer content. These observations suggest that cold soaking treatments did not alter the ratio of small and large compounds extracted into the wine matrix beyond traditional red wine production methods, nor did the addition of seeds or skins. These findings suggest that when the same grape variety is used to make different types of wine with different enology practices, traditional Cabernet Sauvignon vs. rosé techniques in this case, mDP may not differ.

The mDPs for the wines examined here, which were all made from the same vintage and grape variety, were not significantly different from each other. Since it has been reported that different grape varieties contain different concentrations of flavanols, a rosé and red wine made from different grape varieties could have different mDPs, which could ultimately yield different mDPs in finished products (Fang et al., 2008). It is also possible that the wines produced from the same grapes in this study could have different mDPs after aging. Flavanols undergo various chemical reactions including polymerization which could lead to different mDPs among samples (Cheynier et al., 2006; Fulcrand, Dueñas, Salas, & Cheynier, 2006). Given the difference in flavanol concentration of the wines (**Figure 5.4**), it seems plausible that some flavanols would polymerize to a greater extent than others in time.

While grape seeds and skins both contain high concentrations of flavanol PCs, grape skins tend to be more abundant in polymeric (i.e. larger) PCs than seeds (Rodríguez Montealegre, Romero Peces, Chacón Vozmediano, Martínez Gascueña, & García Romero, 2006). Moreover, mDP values for grape seeds have been reported to be as high as 7 and mDP values for grape skins have been reported to range from 33 to 85 (Monagas et al., 2003). It would not appear from these results that grape skins contain an excess of polymer PCs, as the mDPs were not significantly higher in the wine made with added skins or the cold soaked wine compared with any other wine produced from these grapes. The rosé wine made with added skins slightly, but not significantly, increased mDP compared to the other wines, and the cold soaked wine had a slightly, but not significantly, lower mDP than all other wines (**Figure 5.2A,B**).

Despite the observation that mDP values were not significantly different in any of the wines, the results from thiolysis also provided some insight as to the composition of the wines analyzed. **Figures 5.2C and 5.2D** show the abundance of native monomers and monomers generated during the thiolytic reaction. The rosé wines made with added seeds and crushed seeds contained significantly higher levels of net monomers and native monomers compared to all other wines. These results are consistent with other studies and suggest that grape seeds are abundant

in small flavanols (Rodríguez Montealegre et al., 2006) and that those flavanols are efficiently extracted from the seeds into the wine matrix. Again, it is possible that if these wines made with added seeds are allowed to age, due to the high abundance of monomer compounds, polymerization reactions may produce larger PC compounds, driving the mDP upward (Fang et al., 2008).

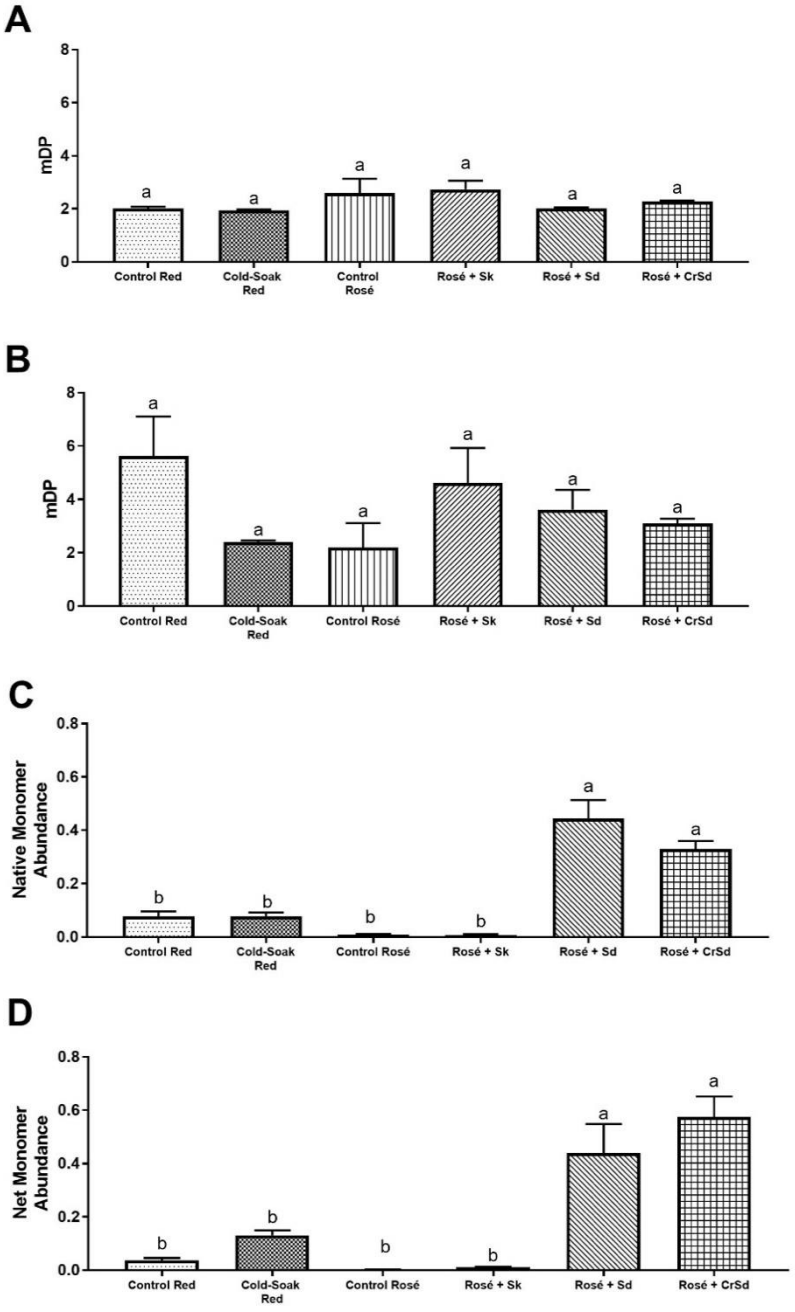

Figure 5.2 *mDP values including baseline monomers (A), without baseline monomer values (B), abundance of native monomers (C), and net monomers (D). Values are expressed as means (n = 5) ± SEM. Samples with different letters are significantly different from each other. Significance was defined as p < 0.05.*

5.3.2 Polyphenol Profile. While the thiolysis procedure indicates the average size of compounds in a sample and abundance of monomers, it gives no indication of compound DPs in the sample. A follow up analysis employing normal-phase HPLC was used specifically to determine the flavanol profile for the wine samples. The chromatographic representation of the polyphenols found in each wine have been enlarged and are depicted in **Figure 5.3**. The raw, unaltered chromatograms can be found in **Figure C1**. The qualitative chromatograms indicated the presence of PCs in increasing DP order. Many peaks clustered near the origin were indicative of a wine high in monomer compounds, while peaks farther from the origin indicated a higher presence of oligomers and polymers. It must be noted that these fluorescence chromatograms do not quantitatively portray PC concentration, as relative response factors decrease with increasing DP (Robbins et al., 2012).

It is clear from the chromatograms (**Figure 5.3**), which have been enlarged to show detail, that the control red wine (**Figure 5.3A**) and the rosé wines made with added whole (**Figure 5.3E**) and crushed seeds (**Figure 5.3F**) had the most diverse flavanol profile, while the control rosé (**Figure 5.3C**) and the rosé with added skins (**Figure 5.3D**) were abundant in small PCs only. These results suggest that large PC compounds were not extracted from the grape skins. In terms of processing, these chromatograms further indicate that cold soaking does not increase extraction of larger PCs, an observation that has been made previously (Marais, 2003a, 2003b). Additionally, even though the mDP of the control red and control rosé wines were not significantly different from each other, the red wine processing methods appeared to increase extraction of larger PCs. This is likely due to the fundamental difference between red and rosé wine making techniques. Red wine is fermented on the must, which contains grape seeds, skins, and sometimes stems, while rosé wine is fermented on the pressed juice only, where extraction time is limited (Boulton et al., 2013).

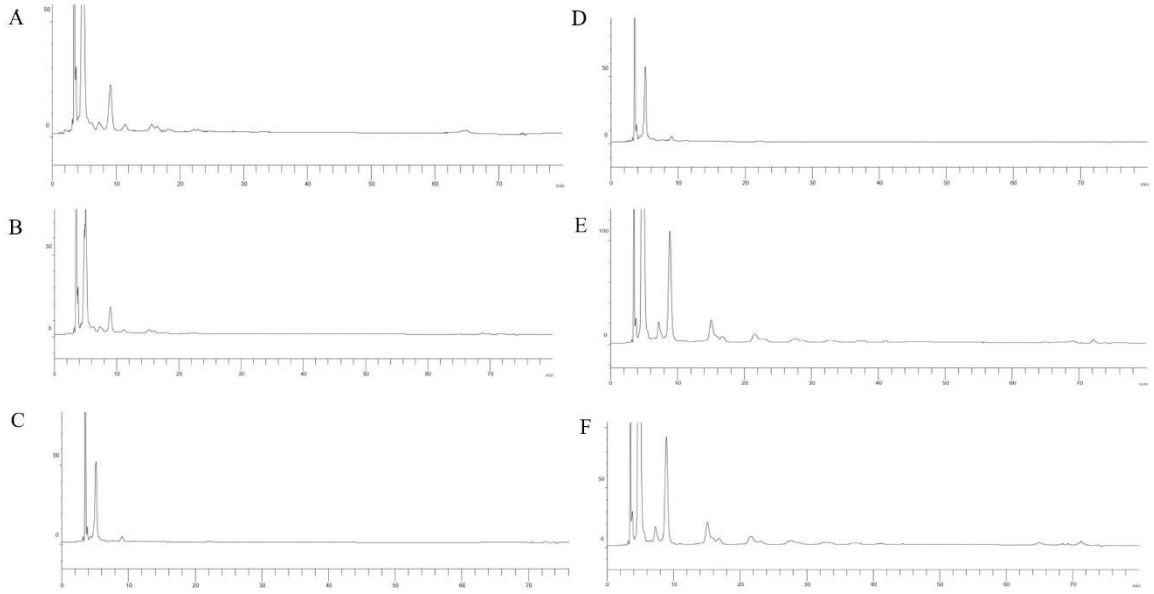

Figure 5.3 *Qualitative chromatograms from the normal phase HPLC analysis of all wine extracts (n=1). Chromatograms have been enlarged to show detail for A) Control red wine B) Cold-Soaked red wine C) Control rosé D) Rosé with added skins E) Rosé with added seeds F) Rosé with added crushed seeds. All chromatograms are expressed in fluorescence units (LU) over time (min).*

Flavanol profile diversity was much greater in the wines made with added seeds (**Figure 5.3E,F**) compared to the wine made with added skins (**Figure 5.3D**). These findings further suggest that grape seed compounds are extracted more efficiently than those in skins. Another possible explanation is that abundance of polymer PCs is higher in seeds compared to skins, although this is in contrast to previously reported results (Rodríguez Montealegre et al., 2006). The wine made with added skins was almost identical to the control rosé wine in terms of flavanol profile (**Figure 5.3C**), so this does not appear to be a viable option to increase the abundance of large PCs extracted into a young wine.

Interestingly, the rosé wines made with added seeds (**Figure 5.3E,F**) contained large polymer PCs, yet the mDP was low (**Figure 5.2A,B**) and not significantly different than the other wines. The calculation for mDP is based on a weighted average of monomers and PC derivatives, as shown above. It would appear that despite their presence in these wines, the oligomer and polymer PCs were not nearly as abundant as the monomers (**Figure 5.2C,D**). Moreover, as the mDP calculation is based on a weighted average, the large concentration of monomers in these samples would make it difficult to increase mDP even with a high concentration of polymer compounds (Neilson et al., 2016). The larger PCs, although present, were essentially diluted out of the mDP calculation based on the overwhelming majority of monomers extracted from the seeds into these wines. Still, it can be inferred from these datasets that grape seeds possess monomer, oligomer, and polymer PCs that can easily be extracted into a wine matrix that could potentially increase mDP due to polymerization reactions during aging (Fang et al., 2008; Fulcrand et al., 2006).

While it seems possible for mDP to increase over time in these wines, it must also be acknowledged that large PC compounds are poorly soluble in most organic solvents including ethanol (Bucić-Kojić, Planinić, Tomas, Bilić, & Velić, 2007). Polymerization reactions could possibly cause PCs to precipitate out of the wine matrix following polymerization reactions rather than remaining in solution and increasing mDP. In the future, it may be worth exploring alternative methods to increase PC concentration in wines to ensure solubility and saturation. An extraction procedure could be applied on grape seeds or grape skins separately from the wine fermentation using food-grade solvents, and the extract could subsequently be added to the wine matrix to potentially increase mDP without the need for aging (Bucić-Kojić et al., 2007; Nawaz, Shi, Mittal, & Kakuda, 2006). Different extraction solvents and mixtures of solvents could be employed to optimize extraction of target PCs to manipulate mDP and also possibly bioactivity (B. j. Xu & Chang, 2007).

5.3.3 Flavanol Concentration. To examine the concentrations of flavanols in the wine samples, which could not be derived from the thiolysis or HPLC chromatograms, FC and DMAC assays were performed (**Figure 5.4**). Similar trends were observed in both assays, but the responses were slightly different as the FC assay measures total polyphenols while the DMAC assay measures total PCs (Ainsworth & Gillespie, 2007; Payne et al., 2010).

As expected, the control red wine contained significantly higher total polyphenols and PCs compared to the control rosé wine (**Figure 5.4A,B**). This is again likely linked to the fundamental processing differences between these two wine types. Red wine must has more contact time with the flavanol-rich grape materials than does the pressed rosé juice (Boulton et al., 2013). Cold soaking significantly reduced total polyphenols and slightly reduced total PCs compared to the control red wine processing. These findings again suggest that cold soaking reduced, rather than

increased, extraction of flavanols into the wine matrix, in addition to not increasing flavanol diversity or mDP (**Figure 4.2,3**). Moreover, the DMAC assay results indicate that the cold soaking of red wine yielded only slightly higher total PCs than the control rosé and rosé with skins, which had essentially no contact time with the grape must compared to the extended contact time of the cold soaked red wine. In terms of increasing extraction of flavanols or increasing their diversity in a young wine, cold-soaking does not appear to be necessary nor desirable. One possible explanation for these results is that the cold soaking procedure allows for released PCs to bind with fiber or other materials in the must matrix, causing them to precipitate out of solution (Palafox-Carlos, Ayala-Zavala, & González-Aguilar, 2011).

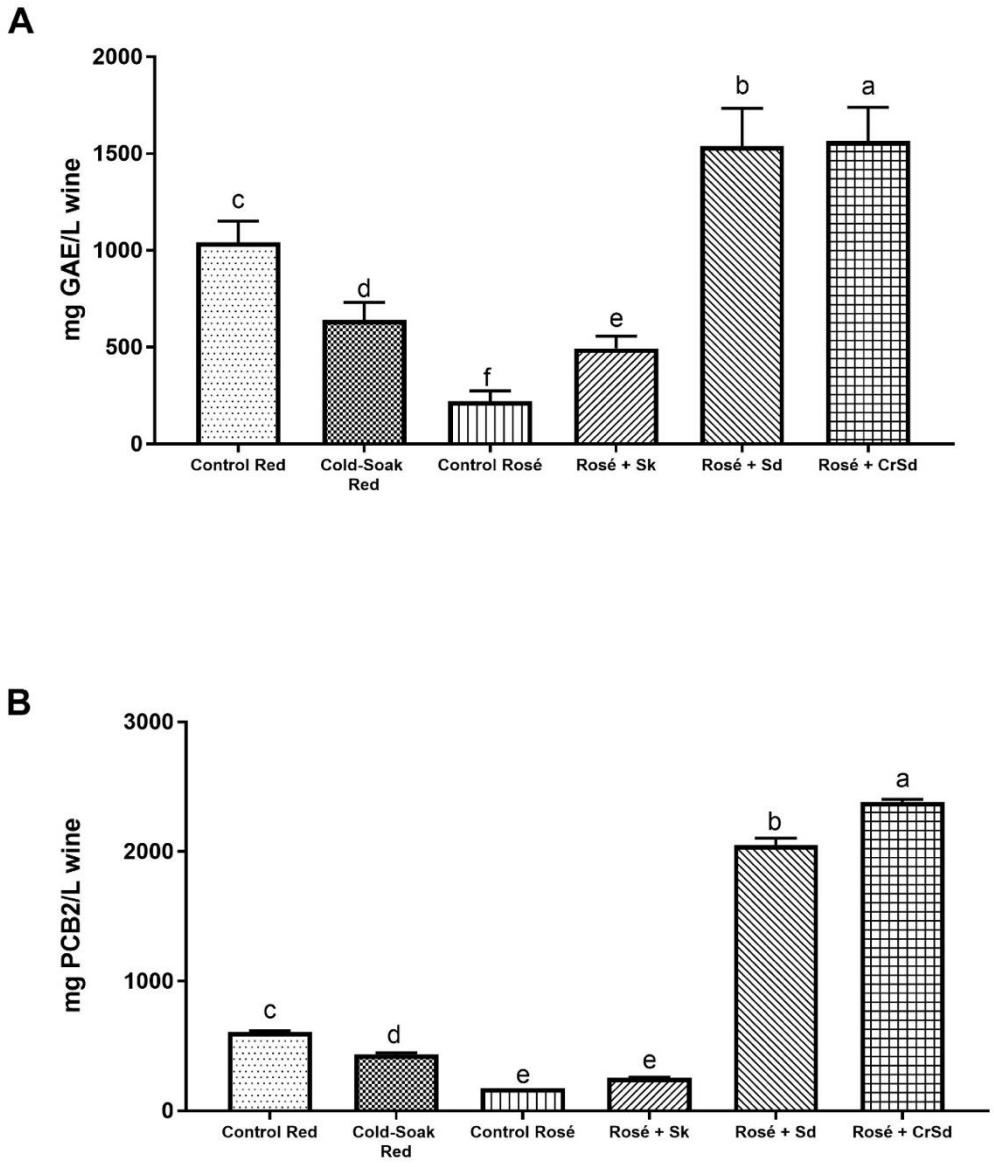

Figure 5.4 *Results from A) the Folin-Ciocalteu assay and B) the DMAC assay for all wines. Values are expressed as means (n = 3) ± SEM and indicate the quantity of total polyphenols (A) or total PCs (B) in each sample compared to a standard curve. Values with different letters are significantly different from each other. Significance was defined as p < 0.05.*

The rosé wines made with seeds and crushed seeds contained significantly higher levels of total polyphenols and total PCs compared to all other wines. Crushing the seeds did not appear to increase extraction of flavanols from the grape seeds into the wine matrix. Interestingly, the rosé wines with added grape seeds contained significantly higher levels of polyphenols and PCs compared to the control red wine, so it is possible that the addition of grape seeds to a red wine must in excess could also increase flavanol concentration and perhaps PC diversity in young red wine (**Figure 5.3**).

The rosé wine with added skins significantly increased FC response compared to rosé control (**Figure 5.4A**) but did not significantly increase DMAC response compared to control rosé (**Figure 5.4B**). Furthermore, the control rosé and the rosé made with added skins had the lowest concentrations of flavanols of all wines. It would appear that the addition of grape skins into a young wine matrix will not increase concentration of PCs or increase diversity of flavanols beyond control levels in the wine styles produced here. It is possible that other processing techniques, such as extended maceration, could improve the extraction of PCs from grape skins (Boulton et al., 2013; González-Manzano, Rivas-Gonzalo, & Santos-Buelga, 2004). These observations also indicate that grape seeds inherently contain higher levels of flavanols compared to skins. It has been determined that grape skins contain less than 300 mg flavanols/kg, while grape seeds contain up to 1400 mg flavanols/kg (Monagas et al., 2003; Rodríguez Montealegre et al., 2006). Moreover, grape skins are abundant in anthocyanins, which give rise to the rich color of wine, but it is not believed that anthocyanins are responsible for the associated health benefits of wine (Kennedy, Saucier, & Glories, 2006). Overall, grape skin addition to a wine matrix will likely have little effect on the flavanol profile, mDP, or flavanol concentration, at least in the early stages of production when using Cabernet Sauvignon grapes.

When comparing the concentrations of flavanols in these wines to other wines, several key observations were made. Total polyphenol analysis of other young red wines has indicated that the content can range from 1100 to 2100 mg GAE/L depending on grape variety, vintage, and processing conditions, while rosé wine reportedly contains 650 mg GAE/L (Paixão, Perestrelo, Marques, & Câmara, 2007; Pellegrini et al., 2000). The control red wine produced in this study contained 1350 mg GAE/L while the control rosé wine contained 372 mg GAE/L. The cold-soaked wine only contained 900 mg GAE/L, less than other reported values for young red wines. In contrast, the rosé wines made with added seeds contained approximately 2100 mg GAE/L, a 7-fold increase from control levels, and into the range of a young red wine. If added seeds were also utilized in the red wine must, it is possible that the young red wine would have a polyphenol concentration similar to one of an aged red wine (aged red wines range from 1200 to 3700 mg GAE/L) without actually aging the wine (Arnous et al., 2001). In any case, the polyphenol concentration of the rosé wine with added seeds was much more potent than traditional rosé and Cabernet Sauvignon wines.

Positive health outcomes have been observed with moderate wine consumption. In mice, a red wine containing 2000 mg GAE/L, consumed in an amount equivalent to moderate wine consumption in humans, decreased energy intake and subsequently weight gain (Bargalló et al., 2006). In another study, rats on a high-fructose diet were supplemented with a red wine containing 1300 mg GAE/L at a dosage equivalent to 0.5 L/d day in humans. This treatment reduced cardiac hypertrophy and reactive oxygen species in the insulin resistant animals (Al-Awwadi et al., 2004). Several of the wines made in this study meet or exceed these doses in which positive effects have been observed. Many other reports of the positive effect of moderate wine consumption on human

health have been reported, but most are epidemiological in nature and they do not examine flavanol content and composition of the wines consumed. Moreover, these studies tend to focus on red or white wines and rosé wines are generally neglected (Athyros et al., 2008; Koppes, Dekker, Hendriks, Bouter, & Heine, 2005; Liu, Wang, Lam, & Xu, 2008). It is clear from this study that not all wines contain the same amount, or type, of flavanols, so simply consuming a moderate amount of red wine may not produce desired health outcomes. Wine style, processing conditions, and grape variety should be considered where possible, as these parameters may affect polyphenol composition and subsequently human health benefits. With a better understanding of how processing parameters influence wine flavanol content, we can work towards optimization of a wine product for maximum health benefits.

5.4 CONCLUSION

To our knowledge, this is the first study to examine the effects of wine processing on mDP, flavanol profile, and concentration in young wines made from the same grapes. It was clear from this study that the processing methods and substrate additions utilized here did not produce changes in mDP, but the flavanol profiles of the wines were quite different, and the addition of grape seeds into the wine matrix greatly increased the amount of flavanols in the finished product while grape skin addition and cold soaking procedures did not. It must be noted that many other practices, such as extended maceration, must freezing, and manipulation of fermentation temperature, are routinely utilized by winemakers that may also impact mDP, flavanol profile, and concentration (Sacchi et al., 2005). Moreover, even though some of the techniques studied here did not produce favorable results in terms of potential bioactivity, they still may be desirable for other reasons. Cold soaking, which does not appear to improve the flavanol profile of red wine, reportedly improves quality aspects of wine including color and sensory characteristics (Parenti, Spugnoli, Calamai, Ferrari, & Gori, 2004; Sacchi et al., 2005).

It must be mentioned that the wines produced in this study were not evaluated in terms of their sensory characteristics. The wines made with added seeds or skins could have a vastly different flavor profile compared to traditional wines, as flavanols are primarily responsible for the bitter flavor and astringent sensations of wine (Brossaud, Cheynier, Asselin, & Moutounet, 1999). It is possible that even with a superior flavanol profile, the rosé wine with added seeds could be decidedly unacceptable to consumers of rosé wine. Such products may require development and marketing outside the wine category or serve a purely research function as substrates for dietary intervention studies investigating polyphenol structure-function relationships.

Currently, there is great interest in the polyphenol mDP of foods, as mDP is reportedly related to bioactivity. Flavanols of different DP appear to have different effects *in vivo* depending on disease model in question and target tissue (Neilson et al., 2016). It would appear that the processing methods tested here, in the short term, were unable to significantly manipulate mDP for the purpose of generating wines that could produce different bioactive effects. Before it can be concluded that these processing methods cannot manipulate mDP, the effect of wine aging must also be explored. In any case, it would appear from this study that certain processing techniques may increase flavanol diversity and concentration in young wines, while mDP remains similar, at least until aging occurs. Furthermore, certain processing treatments did increase flavanol concentration and profile specifically in the rosé wines, which is an important consideration in terms of dosage required to achieve desired effects. Overall, it would appear that rose wine, which

has largely been underrepresented in health promotion studies deserves attention given the highly significant increase in polyphenol concentration observed due to substrate addition.

REFERENCES

Ainsworth, E. A., & Gillespie, K. M. (2007). Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin-Ciocalteu reagent. *Nature Protocols*, *2*(4), 875–877. https://doi.org/10.1038/nprot.2007.102

Al-Awwadi, N. A., Bornet, A., Azay, J., Araiz, C., Delbosc, S., Cristol, J.-P., … Teissedre, P.-L. (2004). Red wine polyphenols alone or in association with ethanol prevent hypertension, cardiac hypertrophy, and production of reactive oxygen species in the insulin-resistant fructose-fed rat. *Journal of Agricultural and Food Chemistry*, *52*(18), 5593–5597.

Arnous, A., Makris, D. P., & Kefalas, P. (2001). Effect of principal polyphenolic components in relation to antioxidant characteristics of aged red wines. *Journal of Agricultural and Food Chemistry*, *49*(12), 5736–5742.

Athyros, V. G., Liberopoulos, E. N., Mikhailidis, D. P., Papageorgiou, A. A., Ganotakis, E. S., Tziomalos, K., … Elisaf, M. (2008). Association of Drinking Pattern and Alcohol Beverage Type With the Prevalence of Metabolic Syndrome, Diabetes, Coronary Heart Disease, Stroke, and Peripheral Arterial Disease in a Mediterranean Cohort. *Angiology*, *58*(6), 689–697. https://doi.org/10.1177/0003319707306146

Bargalló, M. V., Grau, A. A., de Dios Fernández-Larrea, J., Anguiano, G. P., Segarra, M. C. B., Rovira, M. J. S., … Olivé, M. B. (2006). Moderate red-wine consumption partially prevents body weight gain in rats fed a hyperlipidic diet. *The Journal of Nutritional Biochemistry*, *17*(2), 139–142.

Bitzer, Z. T., Glisan, S. L., Dorenkott, M. R., Goodrich, K. M., Ye, L., O'Keefe, S. F., … Neilson, A. P. (2015). Cocoa procyanidins with different degrees of polymerization possess distinct activities in models of colonic inflammation. *The Journal of Nutritional Biochemistry*, *26*(8), 827–831. https://doi.org/10.1016/j.jnutbio.2015.02.007

Boulton, R. B., Singleton, V. L., Bisson, L. F., & Kunkee, R. E. (2013). *Principles and Practices of Winemaking*. Springer Science & Business Media.

Brossaud, F., Cheynier, V., Asselin, C., & Moutounet, M. (1999). Flavonoid compositional differences of grapes among site test plantings of Cabernet franc. *American Journal of Enology and Viticulture*, *50*(3), 277–284.

Bucić-Kojić, A., Planinić, M., Tomas, S., Bilić, M., & Velić, D. (2007). Study of solid–liquid extraction kinetics of total polyphenols from grape seeds. *Journal of Food Engineering*, *81*(1), 236–242. https://doi.org/10.1016/j.jfoodeng.2006.10.027

Casassa, L. F., Bolcato, E. A., & Sari, S. E. (2015). Chemical, chromatic, and sensory attributes of 6 red wines produced with prefermentative cold soak. *Food Chemistry*, *174*, 110–118.

Cheynier, V., Dueñas-Paton, M., Salas, E., Maury, C., Souquet, J.-M., Sarni-Manchado, P., & Fulcrand, H. (2006). Structure and properties of wine pigments and tannins. *American Journal of Enology and Viticulture*, *57*(3), 298–305.

Dorenkott, M. R., Griffin, L. E., Goodrich, K. M., Thompson-Witrick, K. A., Fundaro, G., Ye, L., … Neilson, A. P. (2014). Oligomeric Cocoa Procyanidins Possess Enhanced Bioactivity Compared to Monomeric and Polymeric Cocoa Procyanidins for Preventing the Development of Obesity, Insulin Resistance, and Impaired Glucose Tolerance during High-Fat Feeding. *Journal of Agricultural and Food Chemistry*, *62*(10), 2216–2227. https://doi.org/10.1021/jf500333y

Fang, F., Li, J.-M., Zhang, P., Tang, K., Wang, W., Pan, Q.-H., & Huang, W.-D. (2008). Effects of grape variety, harvest date, fermentation vessel and wine ageing on flavonoid concentration in red wines. *Food Research International*, *41*(1), 53–60. https://doi.org/10.1016/j.foodres.2007.09.004

Fulcrand, H., Dueñas, M., Salas, E., & Cheynier, V. (2006). Phenolic reactions during winemaking and aging. *American Journal of Enology and Viticulture*, *57*(3), 289–297.

González-Manzano, S., Rivas-Gonzalo, J. C., & Santos-Buelga, C. (2004). Extraction of flavan-3-ols from grape seed and skin into wine using simulated maceration. *Analytica Chimica Acta*, *513*(1), 283–289. https://doi.org/10.1016/j.aca.2003.10.019

Griffin, L. E., Witrick, K. A., Klotz, C., Dorenkott, M. R., Goodrich, K. M., Fundaro, G., … Neilson, A. P. (2017). Alterations to metabolically active bacteria in the mucosa of the small intestine predict anti-obesity and anti-diabetic activities of grape seed extract in mice. *Food & Function*, *8*(10), 3510–3522. https://doi.org/10.1039/C7FO01236E

Han, X., Shen, T., & Lou, H. (2007). Dietary polyphenols and their biological significance. *International Journal of Molecular Sciences*, *8*(9), 950–988.

Hickey, C. C., Hatch, T. A., Stallings, J., & Wolf, T. K. (2016). Under-trellis cover crop and rootstock alter growth, components of yield, and fruit composition of Cabernet Sauvignon. *American Journal of Enology and Viticulture*, ajev–2016.

Hill, B. T. (2017). *Root restriction, under-trellis cover cropping, and rootstock modify vine size and berry composition of Cabernet Sauvignon*. Virginia Tech.

Jordão, A. M., Ricardo-da-Silva, J. M., & Laureano, O. (2001). Evolution of proanthocyanidins in bunch stems during berry development (Vitis vinifera L.). *VITIS-Journal of Grapevine Research*, *40*(1), 17.

Kennedy, J. A., Saucier, C., & Glories, Y. (2006). Grape and wine phenolics: history and perspective. *American Journal of Enology and Viticulture*, *57*(3), 239–248.

Koppes, L. L., Dekker, J. M., Hendriks, H. F., Bouter, L. M., & Heine, R. J. (2005). Moderate Alcohol Consumption Lowers the Risk of Type 2 Diabetes A meta-analysis of prospective observational studies. *Diabetes Care*, *28*(3), 719–725.

Kovac, V., Alonso, E., & Revilla, E. (1995). The Effect of Adding Supplementary Quantities of Seeds During Fermentation on the Phenolic Composition of Wines. *American Journal of Enology and Viticulture*, *46*(3), 363–367.

Koyama, K., Goto-Yamamoto, N., & Hashizume, K. (2007). Influence of maceration temperature in red wine vinification on extraction of phenolics from berry skins and seeds of grape (Vitis vinifera). *Bioscience, Biotechnology, and Biochemistry*, *71*(4), 958–965.

Lerno, L., Reichwage, M., Ponangi, R., Hearne, L., Block, D. E., & Oberholster, A. (2015). Effects of cap and overall fermentation temperature on phenolic extraction in cabernet sauvignon fermentations. *American Journal of Enology and Viticulture*, *66*(4), 444–453.

Liu, L., Wang, Y., Lam, K. S. L., & Xu, A. (2008). Moderate Wine Consumption in the Prevention of Metabolic Syndrome and its Related Medical Complications. *Endocrine, Metabolic & Immune Disorders - Drug Targetsg Targets - Immune, Endocrine & Metabolic Disorders)*, *8*(2), 89–98. https://doi.org/10.2174/187153008784534385

Manach, C., Scalbert, A., Morand, C., Rémésy, C., & Jiménez, L. (2004). Polyphenols: food sources and bioavailability. *The American Journal of Clinical Nutrition*, *79*(5), 727–747.

Marais, J. (2003a). Effect of different wine-making techniques on the composition and quality of Pinotage wine. I. Low-temperature skin contact prior to fermentation. *South African Journal for Enology and Viticulture*, *24*(2), 70–75.

Marais, J. (2003b). Effect of different wine-making techniques on the composition and quality of Pinotage wine. II. Juice/skin mixing practices. *South African Journal for Enology and Viticulture*, *24*(2), 76–79.
Monagas, M., Gómez-Cordovés, C., Bartolomé, B., Laureano, O., & Ricardo da Silva, J. M. (2003). Monomeric, oligomeric, and polymeric flavan-3-ol composition of wines and grapes from Vitis vinifera L. Cv. Graciano, Tempranillo, and Cabernet Sauvignon. *Journal of Agricultural and Food Chemistry*, *51*(22), 6475–6481.
Nawaz, H., Shi, J., Mittal, G. S., & Kakuda, Y. (2006). Extraction of polyphenols from grape seeds and concentration by ultrafiltration. *Separation and Purification Technology*, *48*(2), 176–181. https://doi.org/10.1016/j.seppur.2005.07.006
Neilson, A. P., O'Keefe, S. F., & Bolling, B. W. (2016). High-Molecular-Weight Proanthocyanidins in Foods: Overcoming Analytical Challenges in Pursuit of Novel Dietary Bioactive Components. *Annual Review of Food Science and Technology*, *7*(1), 43–64. https://doi.org/10.1146/annurev-food-022814-015604
Paixão, N., Perestrelo, R., Marques, J. C., & Câmara, J. S. (2007). Relationship between antioxidant capacity and total phenolic content of red, rosé and white wines. *Food Chemistry*, *105*(1), 204–214.
Palafox-Carlos, H., Ayala-Zavala, J. F., & González-Aguilar, G. A. (2011). The Role of Dietary Fiber in the Bioaccessibility and Bioavailability of Fruit and Vegetable Antioxidants. *Journal of Food Science*, *76*(1), R6–R15. https://doi.org/10.1111/j.1750-3841.2010.01957.x
Panprivech, S., Lerno, L. A., Brenneman, C. A., Block, D. E., & Oberholster, A. (2015). Investigating the effect of cold soak duration on phenolic extraction during Cabernet Sauvignon fermentation. *Molecules*, *20*(5), 7974–7989.
Parenti, A., Spugnoli, P., Calamai, L., Ferrari, S., & Gori, C. (2004). Effects of cold maceration on red wine quality from Tuscan Sangiovese grape. *European Food Research and Technology*, *218*(4), 360–366. https://doi.org/10.1007/s00217-003-0866-1
Payne, M. J., Hurst, W. J., Stuart, D. A., Ou, B., Fan, E., Ji, H., & Kou, Y. (2010). Determination of total procyanidins in selected chocolate and confectionery products using DMAC. *Journal of AOAC International*, *93*(1), 89–96.
Pellegrini, N., Simonetti, P., Gardana, C., Brenna, O., Brighenti, F., & Pietta, P. (2000). Polyphenol content and total antioxidant activity of vini novelli (young red wines). *Journal of Agricultural and Food Chemistry*, *48*(3), 732–735.
Pérez-Magariño, S., & González-San José, M. L. (2004). Evolution of Flavanols, Anthocyanins, and Their Derivatives during the Aging of Red Wines Elaborated from Grapes Harvested at Different Stages of Ripening. *Journal of Agricultural and Food Chemistry*, *52*(5), 1181–1189. https://doi.org/10.1021/jf035099i
Prieur, C., Rigaud, J., Cheynier, V., & Moutounet, M. (1994). Oligomeric and polymeric procyanidins from grape seeds. *Phytochemistry*, *36*(3), 781–784.
Rasmussen, S. E., Frederiksen, H., Struntze Krogholm, K., & Poulsen, L. (2005). Dietary proanthocyanidins: occurrence, dietary intake, bioavailability, and protection against cardiovascular disease. *Molecular Nutrition & Food Research*, *49*(2), 159–174.
Robbins, R. J., Leonczak, J., Li, J., Johnson, J. C., Collins, T., Kwik-Uribe, C., & Schmitz, H. H. (2012). Determination of Flavanol and Procyanidin (by Degree of Polymerization 1–10) Content of Chocolate, Cocoa Liquors, Powder(s), and Cocoa Flavanol Extracts by Normal Phase High-Performance Liquid Chromatography: Collaborative Study. *Journal of AOAC International*, *95*(4), 1153–1160. https://doi.org/10.5740/jaoacint.12-162

Rodríguez Montealegre, R., Romero Peces, R., Chacón Vozmediano, J. L., Martínez Gascueña, J., & García Romero, E. (2006). Phenolic compounds in skins and seeds of ten grape Vitis vinifera varieties grown in a warm climate. *Journal of Food Composition and Analysis*, *19*(6–7), 687–693. https://doi.org/10.1016/j.jfca.2005.05.003

Ryan, C. M., Khoo, W., Ye, L., Lambert, J. D., O'Keefe, S. F., & Neilson, A. P. (2016). Loss of Native Flavanols during Fermentation and Roasting Does Not Necessarily Reduce Digestive Enzyme-Inhibiting Bioactivities of Cocoa. *Journal of Agricultural and Food Chemistry*, *64*(18), 3616–3625. https://doi.org/10.1021/acs.jafc.6b01725

Sacchi, K. L., Bisson, L. F., & Adams, D. O. (2005). A Review of the Effect of Winemaking Techniques on Phenolic Extraction in Red Wines. *American Journal of Enology and Viticulture*, *56*(3), 197–206.

Sun, B., Leandro, C., Ricardo da Silva, J. M., & Spranger, I. (1998). Separation of grape and wine proanthocyanidins according to their degree of polymerization. *Journal of Agricultural and Food Chemistry*, *46*(4), 1390–1396.

Wolf, T. K. (2008). Wine grape production guide for eastern North America.

Xu, B. j., & Chang, S. k. c. (2007). A Comparative Study on Phenolic Profiles and Antioxidant Activities of Legumes as Affected by Extraction Solvents. *Journal of Food Science*, *72*(2), S159–S166. https://doi.org/10.1111/j.1750-3841.2006.00260.x

Xu, Y., Simon, J. E., Welch, C., Wightman, J. D., Ferruzzi, M. G., Ho, L., … Wu, Q. (2011). Survey of polyphenol constituents in grapes and grape-derived products. *Journal of Agricultural and Food Chemistry*, *59*(19), 10586–10593.

CHAPTER 6:

Conclusions and Future Work

Ultimately, the goal of this body of work was to determine the extent to which mDP of dietary flavanols influences metabolic health, how mDP influences consumer preference and perception of flavanol-rich foods, and to explore the potential to manipulate mDP of flavanol-rich wines. A variety of techniques including animal and human models were employed to achieve this goal.

Previous studies have indicated a correlation between dietary flavanol consumption and reduced symptoms of chronic diseases associated with lifestyle. The study described in Chapter 3 was designed to examine whether flavanol size influences the ability of flavanols to reduce the symptoms of the metabolic syndrome. The study was designed as a follow up procedure to the study described in Appendix D, which indicated that GSE flavanols, delivered at a low, physiologically relevant dose reduced the symptoms of metabolic syndrome to a greater extent than higher dosages. The results from Chapter 3 indicated that small to medium sized (DP1 – DP4) flavanols delivered at low dose and regardless of mDP, prevented increased levels of inflammatory cytokines. Improvements to fasting blood glucose and weight gain were not observed as they were in the study described in Appendix D. This discrepancy could be due to the fact that the rodent diets were different in terms of fat content (45% in Appendix D vs 60% kcals/fat in Chapter 3), or it could suggest that flavanols may at times exert health benefits independently of their roles in development of obesity. Future mechanistic studies should be directed towards examining how flavanols influence the gut microbiota and metabolic pathways affected by inflammatory cytokines. It would also be worth investigating whether satiety mechanisms are impacted by flavanol supplementation, given that the lower mDP supplemented treatment group ate significantly more calories on a weekly basis compared to the other groups.

The results from Chapter 3 suggest that flavanol consumption may protect against lifestyle-induced disease risk but given that flavanols elicit bitter and astringent sensations when consumed, this could be a barrier to consumption for some individuals. By way of a consumer sensory panel (Chapter 4), preferences and perception of wine-like products made from flavanols with different degrees of bitterness and astringency were probed. It was observed that the panelists overall were able to detect the differences in bitterness and astringency intensity due to differences in mDP and wine liking decreased with increasing mDP (and therefore increasing astringency). This trend was also observed when considering only the panelists classified as having a low BF% or lean BMI. When considering the panelists who were classified as obese or with an above average BF% based on ACSM guidelines, however, the ability to differentiate the wines was significantly reduced. This suggests that obesity influences taste sensitivity thus leading to decreased consumption of nutrient dense products, or that consumers that have reduced taste sensitivity to nutrient dense products may be predisposed to becoming obese. In either case, future efforts should be directed towards discerning how taste attributes associated with flavanols can be optimized to increase consumption in populations who could benefit from the health benefits associated with dietary flavanols.

There are many processing techniques employed by winemakers when producing different wine styles. These techniques are typically utilized to impact the sensory characteristics of the finished product. The final study described here (Chapter 5) was aimed at exploring the possibility of utilizing winemaking techniques to manipulate flavanol mDP so that health benefits and sensory

characteristics can be optimized. Although the techniques employed in this study utilizing the same grape variety did not yield significant differences in mDP, they did yield significant differences in flavanol concentration. This suggests that differences in mDP could arise during the aging process, or between wines made from different grape varieties or species, grapes grown under different conditions, or harvested at different stages of maturity. One limitation of this study that must be acknowledged is that no sensory work was performed to confirm palatability of the wine samples made here. Future work should be performed to determine how mDP can be manipulated during processing while also optimizing the sensory profile of the wines.

Overall, this study has increased our understanding of how mDP of dietary flavanols influence markers of the metabolic syndrome and taste perception of flavanol-rich products. These findings could be expanded upon through continued research into the influence of dietary flavanols in different segments of the gut, a highly novel emerging area of research. Also, these results provide motivation to further examine consumer perceptions of flavanol taste attributes and the relationship between perception and physiology. While myriad questions remain with regards to flavanol bioactivity, these studies motivate continued investigation to advance the understanding of the complex interactions of dietary flavanols, health benefits, and taste.

Appendix A: Supplementary Information for Chapter 3

A1. SUPPLEMENTARY METHODS

Thiolysis. The GSE and PBE were first diluted in MeOH to 1mg/mL. Extracts (n = 5) were derivatized using 100 uL benzyl mercaptan (5% w/v in MeOH), 50 uL HCl (3.3% w/v), and 50 uL of sample. Controls which were used to determine the quantity of unthiolyzed (native) monomers in the extracts, were prepared using 50 uL of sample and 150 uL of MeOH. Samples and controls were thiolyzed in a water bath at 90°C for 1 min, cooled on ice for 10 minutes, and transferred to HPLC vials with 95:5 0.1% formic acid in water/0.1% formic acid in acetonitrile in a 1:9 ratio. UPLC-MS/MS was used to quantify benzylthioether derivatives and corresponding monomers. Samples were analyzed using a Waters Acuity H-class separations module with a Waters Acquity UPLC HSS T3 column (2.1 mm x 100 mm, 1.8 µm particle size) at 40°C. A binary elution gradient consisting of 0.1% formic acid in water (Phase A) and 0.1% formic acid in acetonitrile (Phase B) was used. Sample injection volume was set at 20 µL and solvent flow rate was 0.6 mL/min. A linear elution gradient program described in Table S2 was used. Sample temperature was maintained at 10°C. The UPLC effluent was analyzed on a Waters Acuity triple quadrupole (TQD) MS using (−)-electrospray ionization (ESI) coupled with tandem mass spectrometry (MS/MS). (−) mode was employed for ionization, with capillary voltage set at -4.25 kV, cone voltage set at 30.0 V, extractor voltage set at 3.0 V, source temperature set at 150°C, and desolvation temperature set at 400°C. N_2 was the cone and desolvation gas. Respective flow rates of 75 and 900 L/h were used. Argon was the collision gas for MS/MS with the flow rate set at 0.25 mL/min in the collision cell. Multi-reaction monitoring (MRM) parameters for parent ions $[M\text{-}H]^-$ and collision-induced dissociated daughter ions (Table S3) were established with the Waters MassLynx program. The mDP for each sample was calculated from the concentration data obtained using an external standard curve generated from EC and ECG standards. mDP was calculated based on the equations outlined below that include (Equation 1) or exclude baseline monomers (Equation 2). Baseline monomers were defined as the native monomers present in the unthiolyzed control samples, whereas thiolyzed monomers were those generated during the thiolytic reaction. Net monomers refer to the difference in monomer concentrations between thiolyzed samples and samples prior to thiolysis.

$$\text{Equation 1: } mDP = \frac{\Sigma\, monomers\ (native+thiolyzed) + \Sigma\, derivatives}{\Sigma\, monomers}$$

$$\text{Equation 2: } mDP = \frac{\text{net } monomers + \Sigma\, derivatives}{\text{net } monomers}$$

Normal Phase HPLC. Samples of each extract were diluted in methanol to 1 mg/mL and inserted into HPLC vials. An Agilent Technologies 1260 Infinity HPLC (Santa Clara, CA, USA) outfitted with a temperature controlled autosampler, thermostat column compartment, solvent degasser, quaternary pump, and fluorescence detector was used for the analysis. Samples were chilled to 5°C in the autosampler and 5 µL aliquots were injected into the Develosil Diol Column (100Å, 250 x 4.6 mm, 5 µm particle size) (Phenomenex, Torrance, CA, USA) set at 35°C with a Luna HILIC guard column (4 x 3.0 mm ID SecurityGuard cartridge and cartridge holder) (Phenomenex). Mobile phase A consisted of 2% acetic acid (v/v) in ACN and mobile phase B consisted of 2% acetic acid (v/v), 3% ddH_2O (v/v), and 95% MeOH (v/v). Solvent flow rate was

set at 1 mL/min with a binary elution gradient program (Table S4). The FLC excitation wavelength was 230 nm and the emission wavelength was 321 nm. Chemical standards (0.1 mg/mL MeOH) with DPs ranging from DP 1 to DP 10 were also analyzed for the purpose of qualitatively determining the DP of the peaks detected in the GSE and PBE samples.

Folin Ciocalteu Assay. GSE and PBE samples were diluted in 40% ethanol to 0.2 mg/mL. A gallic acid standard curve ranging from 0 to 1000 ppm in 40% ethanol was also prepared. 100 µL aliquots of sample (n = 7) or standard (n = 4) were combined with 900 µL of distilled ddH_2O and 2.5 mL FC reagent (0.2 N) (Sigma, St. Louis, MO, USA) in a test tube. Samples and standards were vortexed, pipetted (250 µL) onto a 96-well plate, and allowed to sit at room temperature in the dark for 2 hr. Absorbance was measured on a BioTek Synergy 2 (Winooski, VT, USA) plate reader set at 765 nm. Total phenolics in the samples were calculated based on a linear regression equation generated from the gallic acid external standard curve. Additionally, PC standards (DP 1 to DP 10) prepared at 0.1 mg/mL in methanol were analyzed (n = 3) using the same methods to examine the variability in FC assay response based on DP. Several standards were combined in order to change mDP; for instance, EC was mixed with equal parts PC B_2 to create a solution with mDP = 1.5.

DMAC Assay. GSE and PBE samples were diluted to 0.01 mg/mL with pure ethanol. A standard curve of Procyanidin B_2 ranging from 0 to 100 ppm diluted with 1:1 ethanol:water was also prepared. DMAC solution was prepared by combining 3 mL stock HCl with 27 mL pure ethanol, chilling for 15 minutes to 4°C, adding 0.03 g DMAC, and mixing. 50 µL aliquots of sample (n = 7), standard (n = 4), or ethanol blank (n = 4) were pipetted into a 96-well plate. 250 µL DMAC solution was pipetted into each well, and the plate was analyzed with the BioTek Synergy 2 plate reader set at 640 nm. Total procyanidins in the samples were calculated based on a linear regression equation generated from the Procyanidin B_2 standard curve. Additionally, PC standards (DP 1 to DP 10) were prepared at 0.01 mg/mL and analyzed (n = 3) using the same methods to determine the variability in DMAC assay response based on DP. Again, several standards were combined in order to change mDP; for instance, EC was mixed with equal parts PC B_2 to create a solution with mDP = 1.5.

Glucose and Insulin Tolerance Tests. Mice were fasted overnight for 12 hours prior to test administration. Following fasting, glucose (USP-grade, Hospira, Inc., Lake Forest, IL) was delivered to mice via oral gavage (weeks 6 and 10) or i.p. injection (week 11). For OGTTs, a 20% dextrose solution was used and was administered to provide 2 g glucose/kg* body wt. For ipGTTs, a 15% dextrose solution was prepared and administered to provide 1.5 g glucose*kg body wt. Blood was collected by pricking the tail vein just prior to glucose administration (t = 0 min) and subsequent 30-minute intervals up to 180 minutes. One Touch Ultra Blue test strips (LifeScan, Inc., Milpitas, CA, USA) and a standard glucometer were used to measure blood glucose levels. The maximum blood glucose value able to be read by the glucometer was 600 mg/dL; readings above this range were recorded as 600 mg/dL. Insulin tolerance tests (ITTs) were performed during week 12. Before beginning, mice were fasted for 4 hours. Insulin (Humulin R, Cardinal Health, Dublin, OH, USA) was delivered (0.65 U insulin/kg*body wt in saline) via i.p. injection. Blood was collected from the tail just prior to insulin administration (t = 0 min) and at subsequent 15-minute time intervals up to t = 60 min. Blood glucose measurement was collected as it was for GTTs.

A2. SUPPLEMENTARY DISCUSSION

While the focus of this study was on dietary flavanols, not terpenes, the possible role of terpenes must be mentioned. PBE was selected as the larger mDP substance for use in this study rather than a grape-skin extract to eliminate the possibility of grape skin anthocyanins producing an effect. The possible effect of terpenes on study results was accounted for by using two PBE experimental groups (**Table 1**); one dose based on an extract weight equivalent to G, and one dose based on an adjustment for terpene content of the PBE as determined by FC (**Figure 1**). To date, there has been very little research on terpenes and metabolic syndrome. Terpenes have exhibited *in vitro* anti-inflammatory effects in RAW 264.7 macrophages and *in vivo* anti-inflammatory effects in mouse liver [1,2]. It did not appear from this study that the terpenes present in PBE significantly improved or worsened the results compared to dietary flavanols, as the PBE supplemented groups were overall not significantly different than the GSE supplemented group.

B3. SUPPLEMENTARY TABLES

Table A1. DRT Product Specifications

Parameter	Vitaflavin	Oligopin
Procyanidin Content (%)	75.6	68.9
Monomers (%)	24.4	31.1
Dimers (%)	NA	16.8
Dimers + Trimers (%)	43.6	NA
Timers + Tetramers (%)	NA	52.1
Procyanidin-Porter content (%)	67.0	66.0

Table A2. Linear elution gradient program for thiolysis

Time (min)	Phase A (%)
0.0-0.5	95
6.5	65
7.5-8.6	20
8.7–10.5	95

Table A3. MRM detection settings for quantification of flavanols and respective thiolyzed derivatives by UPLC-MS/MS

Analyte	Retention Time (min)	MW (g/mol)	$[M-H]^-$ (m/z)	Daughter Ion (m/z)	Cone Voltage (V)	Collision Energy (eV)
C/EC	3.45-4.09	290.142	288.98	245.05	40	14
ECG	4.92-5.10	441.952	440.92	169	38	16
C/EC benzylthioether derivative	7.60-8.31	412.031	410.94	124.97	30	18

ECG benzylthioether derivative	7.68-8.01	563.824	563.05	287.06	38	16

Table A4. Normal phase HPLC binary elution gradient program

Time (min)	Phase A (%)	Phase B (%)
0	93	7
3	93	7
60	62.4	37.6
63	0	100
70	0	100
76	93	7

Table A5. MS/MS Settings for MRM Detection of Monomers and PCs.

Compound	Retention Time (min)	MW (g/mol)	$[M-H]^{-a}$ (m/z)	Daughter Ion (m/z)	Cone Voltage (V)	Collision Energy (eV)
PC dimer B_1	3.14	578.136	577.136	289.105	38	24
(−)-epigallocatechin	3.21	306.038	305.038	124.977	40	22
Unknown dimer 1[b]	3.37	578.136	577.136	425.102	36	16
(±)-catechin	3.46	290.028	289.028	245.057	36	14
Unknown dimer 2[b]	3.75	578.136	577.136	425.102	34	16
PC dimer B_2	3.80	578.136	577.136	425.102	36	16
(−)-epicatechin	4.10	290.092	289.092	245.056	42	12
(−)-epigallocatechin gallate	4.20	458.038	457.038	168.982	34	16
PC trimer C_1	4.28	866.218	865.218	287.085	46	32
cinnamtannin tetramer A_2	4.45	1154.808	576.404	125.02	26	34
PC dimer B_2 gallate	4.48	730.164	729.164	407.129	42	32
PC pentamers	4.60	1442.82	720.41	125.022	26	44
PC nonamers	4.70	2586.36	864.12	125.17	28	46
PC hexamers	4.78	1731.038	864.519	125.02	32	56
PC heptamers	4.83	2018.8	1008.4	125.17	36	56
PC octamers	4.90	2307.17	1152.58	125.17	48	68
(−)-epicatechin gallate	5.14	442.076	441.076	168.968	38	18
PC decamers	5.18	2883.55	960.18	125.17	30	52
PC dimer B_5	5.10	578.136	577.136	289.107	30	26

[a]all MRMs used singly-charged parent ions except for cinnamtannin tetramer A_2, pentamers, hexamers, heptamers, octamers, which were doubly-charged ($[M-2H]^{2-}$), and nonamers and decamers, which were triply-charged ($[M-3H]^{3-}$)
[b]likely PC dimers B_3, B_4, B_6, B_7 or B

Table A6. Quantification of Individual Flavanols by Reverse-Phase HPLC

	mg flavanols/g extract	
Compound	**GSE**	**PBE**
(+)-catechin	7.33[a] ± 0.804	2.03[b] ± 0.039
(−)-epicatechin	7.19[a] ± 0.767	0.024[b] ± 0.012
(−)-epicatechin gallate	3.42[a] ± 0.421	0.00[b] ± 0.00
(−)-epigallocatechin	0.014[a] ± 0.006	0.00[a] ± 0.00
PC dimer B1	3.79[a] ± 0.210	4.00[a] ± 0.142
PC dimer B2	5.38[a] ± 0.345	0.00[b] ± 0.00
PC dimer B5	0.615[a] ± 0.085	0.00[b] ± 0.00
Unknown PC Dimer 1	2.49[b] ± 0.300	3.37[a] ± 0.037
Unknown PC Dimer 2	0.779[a] ± 0.040	0.883[a] ± 0.033
PC dimer B2 Gallate	5.88[a] ± 0.606	0.00[b] ± 0.00
Unknown PC Dimer Gallate	1.18[a] ± 0.170	0.00[b] ± 0.00
PC trimer C1	2.47[a] ± 0.211	1.82[a] ± 0.144
Unknown PC Trimer 1	0.641[a] ± 0.065	0.00[b] ± 0.00
Unknown PC Trimer 2	2.79[b] ± 0.604	5.53[a] ± 0.185
Unknown PC Trimer 3	0.384[a] ± .0342	0.179[a] ± 0.077
Cinnamtannin tetramer A2	0.748[a] ± 0.090	0.722[a] ± 0.057
Unknown PC Tetramer 1	0.078[a] ± 0.021	0.123[a] ± 0.012
PC Pentamer	0.686[a] ± 0.0365	0.385[b] ± 0.087
Unknown PC Pentamer 1	0.251[b] ± 0.029	0.559[a] ± 0.015
Unknown PC Pentamer 2	0.229[a] ± 0.054	0.260[a] ± 0.044
PC Hexamer	0.343[a] ± 0.078	0.174[a] ± 0.051
Unknown PC Hexamer 1	2.52[a] ± 0.256	1.17[b] ± 0.283
Unknown PC Hexamer 2	0.182[a] ± 0.053	0.268[a] ± 0.044
PC Heptamer	0.517[a] ± 0.219	0.962[a] ± 0.090
Unknown PC Heptamer 1	0.105[a] ± 0.030	0.680[a] ± 0.209
Unknown PC Heptamer2	0.152[a] ± 0.021	0.251[a] ± 0.088
PC Octamer	0.301[a] ± 0.050	0.123[b] ± 0.022
PC Nonamer	1.20[b] ± 0.170	1.94[a] ± 0.200
PC Decamer	0.00[b] ± 0.00	0.348[a] ± 0.130
Total Monomers	18.0[a] ± 2.00	2.05[b] ± 0.049
Total Dimers	20.1[a] ± 1.62	8.24[b] ± 0.173
Total Trimers	6.28[a] ± 0.632	7.53[a] ± 0.404
Total Tetramers	0.826[a] ± 0.110	0.845[a] ± 0.066
Total Pentamers	1.17[a] ± 0.084	1.20[a] ± 0.076
Total Hexamers	3.05[a] ± 0.306	1.62[b] ± 0.334
Total Heptamers	0.774[a] ± 0.243	1.89[a] ± 0.356
Total Oligomers, DP 3-6	11.3[a] ± 1.00	11.2[a] ± 0.078
Total Polymers, DP 7-10	2.27[b] ± 0.393	4.30[a] ± 0.486

Pairwise comparisons were performed using Students T-Test. Values are expressed as means (n = 3) ± SEM. Different letters across rows indicate significantly different values at $p < 0.05$.

A4. SUPPLEMETNARY FIGURES

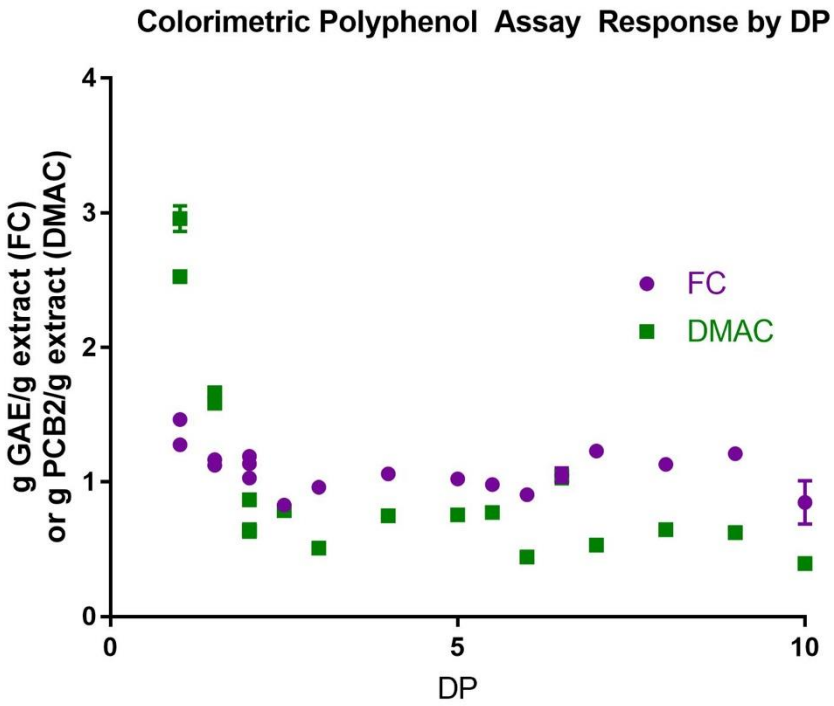

Figure A1. *FC and DMAC assay response as a function of flavanol DP. PC standards were analyzed at 0.1 mg/mL for FC and at 0.01 for DMAC. Monomers though decamers were analyzed for each assay. 1:1 mixtures of EC + PCB_1, PCB_2 + PCC_1, PC pentamer + PC hexamer, and PC hexamer + PC heptamer were also analyzed to look at the assay responses of mDP 1.5, 2.5, 5.5, and 6.5, respectively. Each data point represents the mean (n = 3) ± SEM for each PC standard in each assay.*

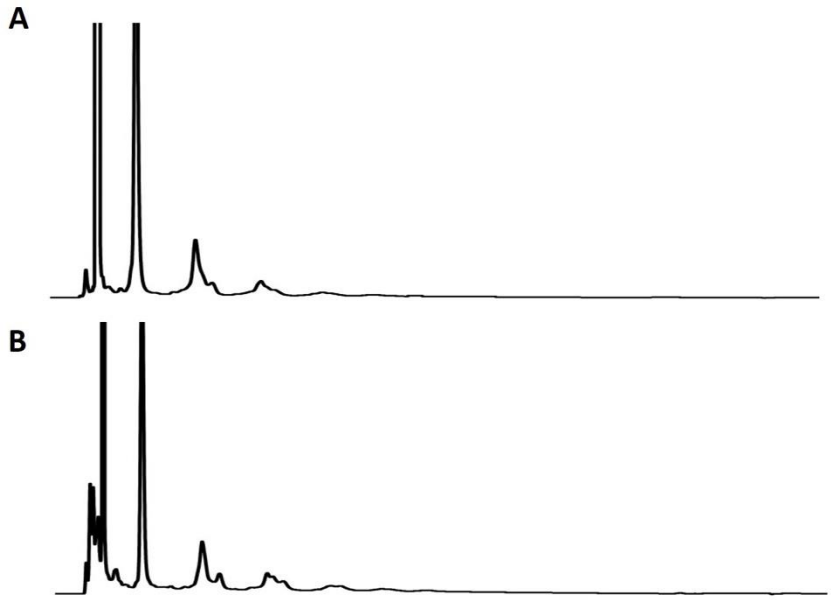

Figure A2. *Representative GSE (A) and PBE (B) normal phase HPLC chromatograms magnified to the same scale to show detail, as measured by HPLC with fluorescence detection at excitation wavelength of 230 nm and emission wavelength of 321.*

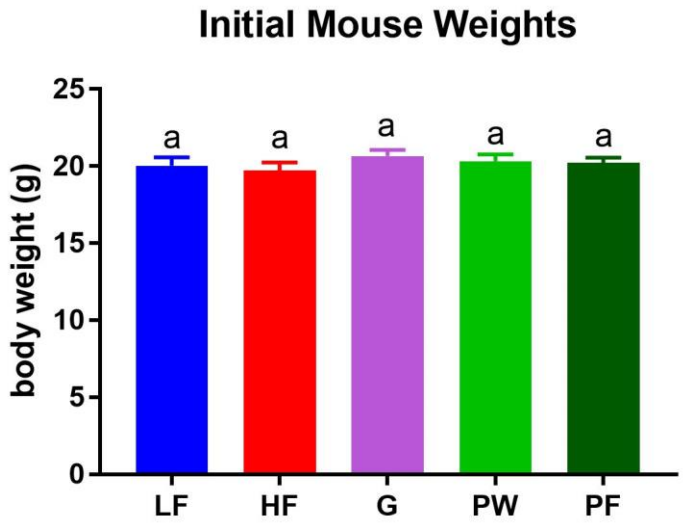

Figure A3. *Mouse body weights at the study onset expressed as means (n = 12/treatment) ± SEM. Significance was determined by 1-way ANOVA with Fisher's LSD post hoc test to determine differences between treatments. Significance is defined as $p < 0.05$ with different letters designating significantly different values.*

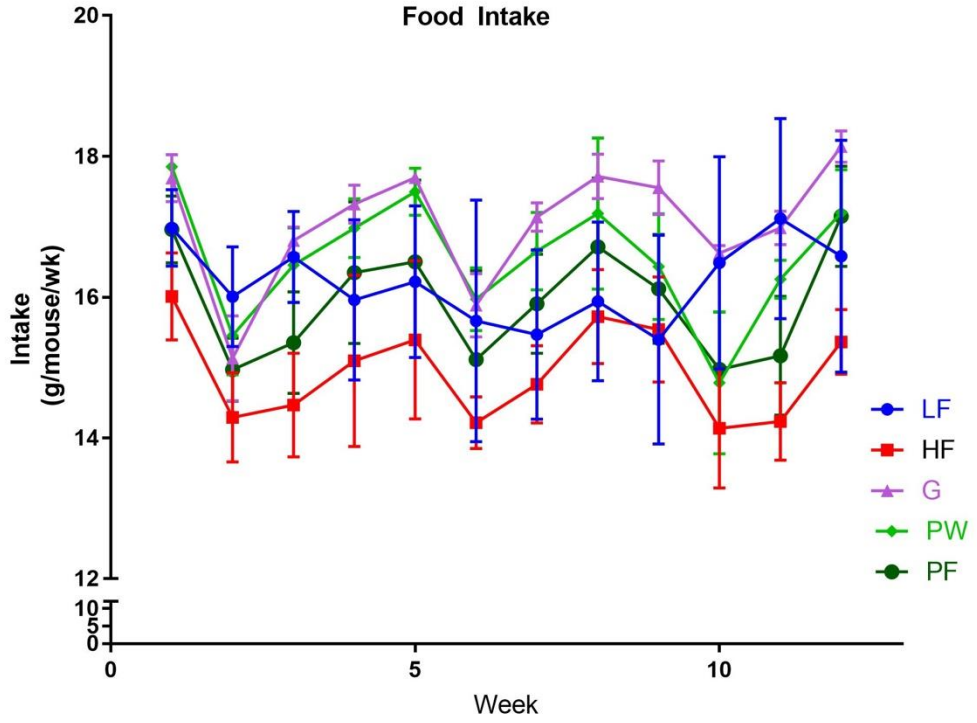

Figure A4. *Weekly food intake in grams/mouse/week. Values are expressed as means from 3 cages ± SEM.*

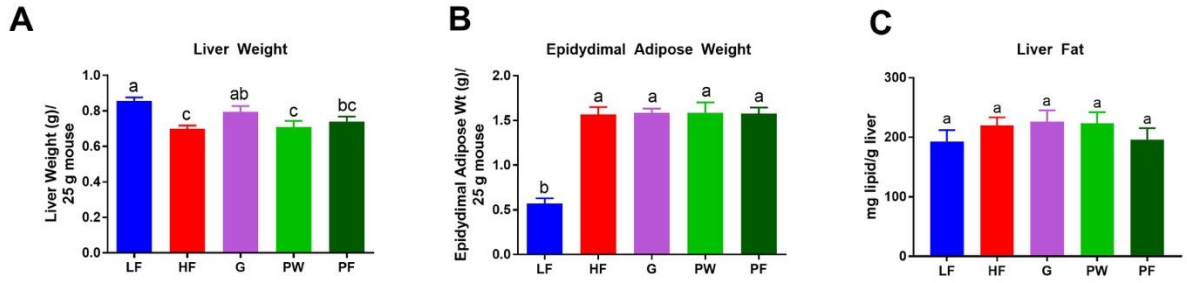

Figure A5. *A) Average mouse liver mass normalized to a 25-g mouse. B) Average mouse epididymal fat mass normalized to a 25-g mouse. C) Average liver fat mass. Values are expressed as means (n = 12/trt for HF, G, and PF, n = 9 for LF, and n = 11 for PW) ± SEM. Significance was determined by 1-way ANOVA with Fisher's LSD post hoc test to determine differences between treatments. Significance is defined as p < 0.05 with different letters designating significantly different values.*

REFERENCES

[1] Leyva-López N, Nair V, Bang WY, Cisneros-Zevallos L, Heredia JB. Protective role of terpenes and polyphenols from three species of Oregano (Lippia graveolens, Lippia palmeri and Hedeoma patens) on the suppression of lipopolysaccharide-induced inflammation in RAW 264.7 macrophage cells. Journal of Ethnopharmacology 2016; 187: 302–312

[2] Ma J-Q, Liu C-M, Qin Z-H, Jiang J-H, Sun Y-Z. Ganoderma applanatum terpenes protect mouse liver against benzo(α)pyren-induced oxidative stress and inflammation. Environmental toxicology and pharmacology 2011; 31: 460–468

APPENDIX B: SUPPLEMENTARY FILE FOR CHAPTER 4

B1. SUPPLEMENTARY METHODS

Wine Selection and Flavanol Dosage Determination. A variety of commercially available wines including dry Riesling, Pinot Grigio, Sauvignon Blanc, and Rosé were screened for usage as a base wine in the sensory evaluation. These wines were tasted by a small group (n = 8) of volunteers familiar with fermented foods and beverages and assessed in terms of sweetness, bitterness, astringency, acidity, and overall neutrality, with the goal of finding a neutral wine that did not exhibit taste characteristics expected to influence the results of the study and did not have high levels of flavanols. Beringer Main & Vine Pinot Grigio (St. Helena, CA, USA) matched the target profile for the study and was selected as the base wine for experimental sample formulation. GSE and PBE were added to the control wine at a range of concentrations to make preliminary assessments of palatability and determine the proper dosage for the consumer panel. According to the literature, red wine polyphenol concentration can range from 1500 to upwards of 4000 mg GAE/L based on the Folin-Ciocalteu assay (Burns et al., 2000; Sato et al., 1996; Simonetti, Pietta, & Testolin, 1997). As such, extracts were added to the base wine at concentrations of 1000-2500 mg GAE/L and were evaluated by the same small panel used to select the base wine. It was determined that the higher dosages of GSE and PBE were too intense in terms of bitterness and astringency to be acceptable for consumer evaluation and it was decided to present the extracts to the consumer panel at 1000 mg GAE/L. While this concentration was slightly lower than literature reported values for red wine, these concentrations were palatable and fell within the normal boundaries for the taste and mouthfeel of red wine and were also notably different in terms of their bitterness and astringency. Preliminary screening evaluation by the same small panel determined that at this concentration, the GSE-amended wine was noticeably more bitter (likely due to the greater presence of small DP flavanols) while the PBE-amended wine was noticeably more astringent (likely due to the greater presence of large DP flavanols) (Lesschaeve & Noble, 2005).

B2. SUPPLEMENTARY TABLES

Figure B1. Characterization of Control Wine

Alcohol	12.1 ± 0.0 % (v/v)
malic acid	1.22 ± 0.01 g/L
residual sugars	8.0 ± 0.0 g/L
pH	3.42 ± 0.003
Free Sulfur Dioxide	19.0 ± 1.0 mg/L
Total Sulfur Dioxide	62.7 ± 0.88 mg/L
Total Acidity as Tartaric Acid	4.95 ± 0.03 g/L
Volatile Acidity as Acetic Acid	0.35 ± 0.02 g/L

Results are represented as means (n = 3 separate bottles) ± SEM.

Table B2. Preparation of Flavanol-Rich Wines

	Control Wine	GSE-Amended Wine	PBE-Amended Wine
Target mg GAE/L based on Extract Addition	0	1000	1000
Extract Total Polyphenols (mg GAE/g extract)	NA	0.99 ± 0.01	0.68 ± 0.02
Extract Addition Amount (g/L)[a]	0	1.01	1.47
Wine Sample Total Polyphenols (mg GAE/L)	141.0 ± 2.3	994.7 ± 7.05	997.5 ± 17.9

[a] calculated based on Folin-Ciocalteu response for GSE and PBE

Table B3. ACSM Guidelines for Body Fat Percentage Classification

		Age (years)				
		20-29	30-39	40-49	50-59	60+
Fitness Category	**Male**	**Body Fat %**				
Excellent		7.1-9.3	11.3-13.8	13.6-16.2	15.3-17.8	15.3-18.3
Good		9.4-24	13.9-17.4	16.3-19.5	17.9-21.2	18.4-21.9
Average		14.1-17.5	17.5-20.4	19.6-22.4	21.3-24	22-25
Below Average		17.4-22.5	20.5-24.1	22.5-26	24.1-27.4	25-28.4
Poor		>22.4	>24.2	>26.1	>27.5	>28.5
	Female					
Excellent		14.5-17	15.5-17.9	18.5-21.2	21.6-24.9	21.1-25
Good		17.1-20.5	18-21.5	21.3-24.8	25-28.4	25.1-29.2
Average		20.6-23.6	21.6-24.8	24.9-28	28.5-31.5	29.3-32.4
Below Average		23.7-27.6	24.9-29.2	28.1-32	31.6-35.5	32.5-36.5
Poor		>27.7	>29.3	>32.1	>35.6	>36.6

Adapted from the American Council of Sports Medicine guidelines (American College of Sports Medicine, 2013)

Table B4. Demographics Separated by BF% Characterization

		Gender		BMI			PROP Sensitivity			Bitterness Preference	
	Number of Participants	%Male	%Female	%Lean	%Overweight	%Obese	%nontaster	%taster	%supertaster	%Preferring	%Nonpreferring
Below average BF%	14	50.0	50.0	100.0	0.0	0.0	57.1	28.6	14.3	64.3	35.7
Average BF%	24	66.7	33.3	66.7	33.3	0.0	41.7	41.7	16.7	62.5	37.5
Above average BF%	64	28.1	71.9	42.2	35.9	21.9	28.1	46.9	25.0	57.8	42.2

Table B5. Demographics Separated by BMI Categorization

		Gender		BF%			PROP Sensitivity			Bitterness Preference	
	Number of Participants	%Male	%Female	%below average	%average	%above average	%nontaster	%taster	%supertaster	%Preferring	%Nonpreferring
Lean BMI	57	26.3	73.7	24.6	28.1	47.4	31.6	43.9	24.6	61.4	38.6
Overweight BMI	31	67.7	32.3	0.0	25.8	74.2	41.9	38.7	19.4	54.8	45.2
Obese BMI	14	35.7	64.3	0.0	0.0	100.0	35.7	50.0	14.3	64.3	35.7

Table B6. Demographics Separated by PROP Taster Status

		Gender		BMI			BF%			Bitterness Preference	
	Number of Participants	%Male	%Female	%Lean	%Overweight	%Obese	%below average	%average	%above average	%Preferring	%Nonpreferring
Nontaster	36	52.8	47.2	50.0	36.1	13.9	22.2	27.8	50.0	41.7	58.3
Taster	44	34.1	65.9	56.8	33.3	15.9	9.1	22.7	68.2	40.9	59.1
Supertaster	22	31.8	68.2	63.6	27.3	9.1	9.1	18.2	72.7	36.4	63.6

Table B7. Demographics Separated by Bitterness Preference Categorization

		Gender		BMI			BF%			PROP Sensitivity		
	Number of Participants	%Male	%Female	%Lean	%Overweight	%Obese	%below average	%average	%above average	%nontaster	%taster	%supertaster
Nonpreferring	61	39.3	60.7	57.4	26.2	14.8	14.8	24.6	60.7	34.4	42.6	23.0
Preferring	41	41.5	58.5	53.7	34.1	12.2	12.2	22.0	65.9	36.6	43.9	19.5

Table B8. ANCOVA Explanatory Variables and Levels

Quantitative Variables		
BF%		
BMI		
Age		

Qualitative Variables	Number of Levels	Categories
BMI	3	lean, overweight, obese
BF%	3	below average, average, above average
PROP Sensitivity	3	nontaster, taster, supertaster
Bitterness Preference	2	bitter preferring, bitter nonpreferring
Wine type	3	control, GSE-amended (low mDP), PBE-amended (high mDP)

B3. SUPPLEMENTARY FIGURES

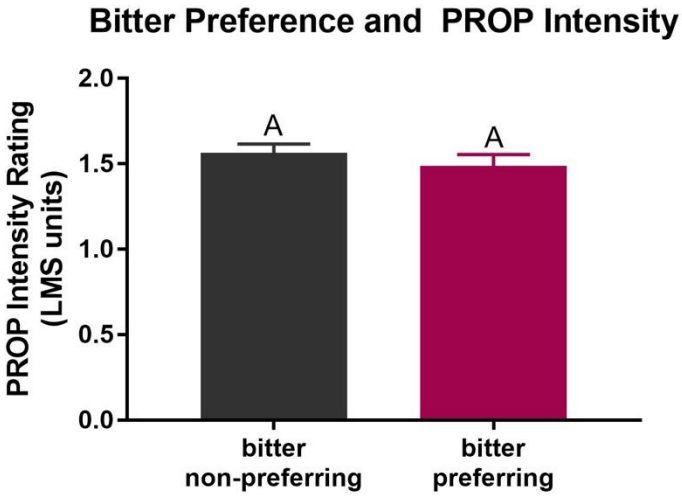

Figure B1. *Average PROP intensity rating for panelists classified as bitter non-preferring or bitter preferring. Values are expressed as means ± SEM with significantly different values having different letters based on a t-test. Bitter preference was determined on an individual level based on panelist hedonic scores for 5 bitter foods. To be classified as bitter preferring, panelists had to score 3 out of the 5 bitter foods at an 8 (like very much) or higher.*

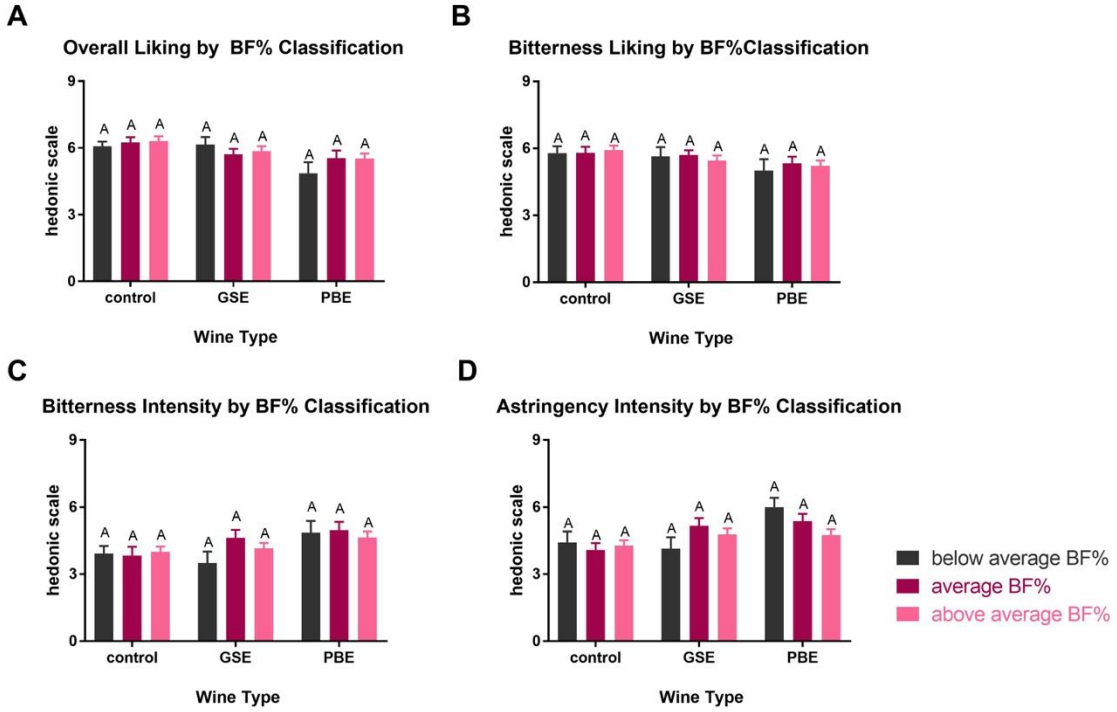

Figure B2. *Sensory panel results segmented based on panelist BF% classification and wine type for overall product liking (A), bitterness liking (B), bitterness intensity (C), and astringency intensity (D). Values are expressed as means ± SEM based on hedonic scale values that range from 1 to 9, with 1 indicating "dislike extremely" and 9 indicating "like extremely". Significantly different values* ***within each wine sample*** *are*

indicated by different letters at the 95% confidence level based on 1-way ANOVA. BF% classifications were determined based on individual panelist gender, measured BF% scores, and the ACSM BF% guidelines.

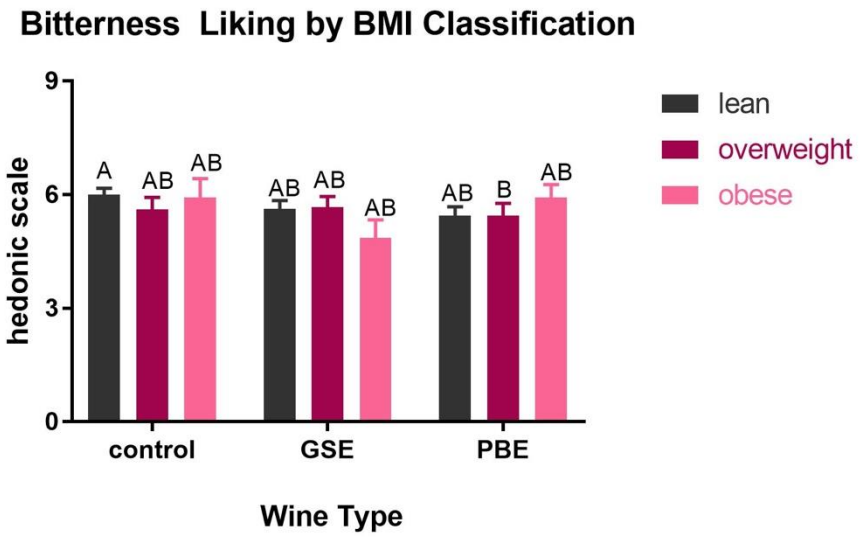

Figure B3. *Sensory panel results segmented based on panelist BMI classification and wine type for bitterness liking. Values are expressed as means ± SEM based on hedonic scale values that range from 1 to 9, with 1 indicating "dislike extremely" and 9 indicating "like extremely". Significantly different values across all wines and phenotypes are indicated by different letters at the 95% confidence level based on the interaction between wine type and phenotype by 2-way ANOVA. Panelist BMI classifications were determined based on ACSM guidelines.*

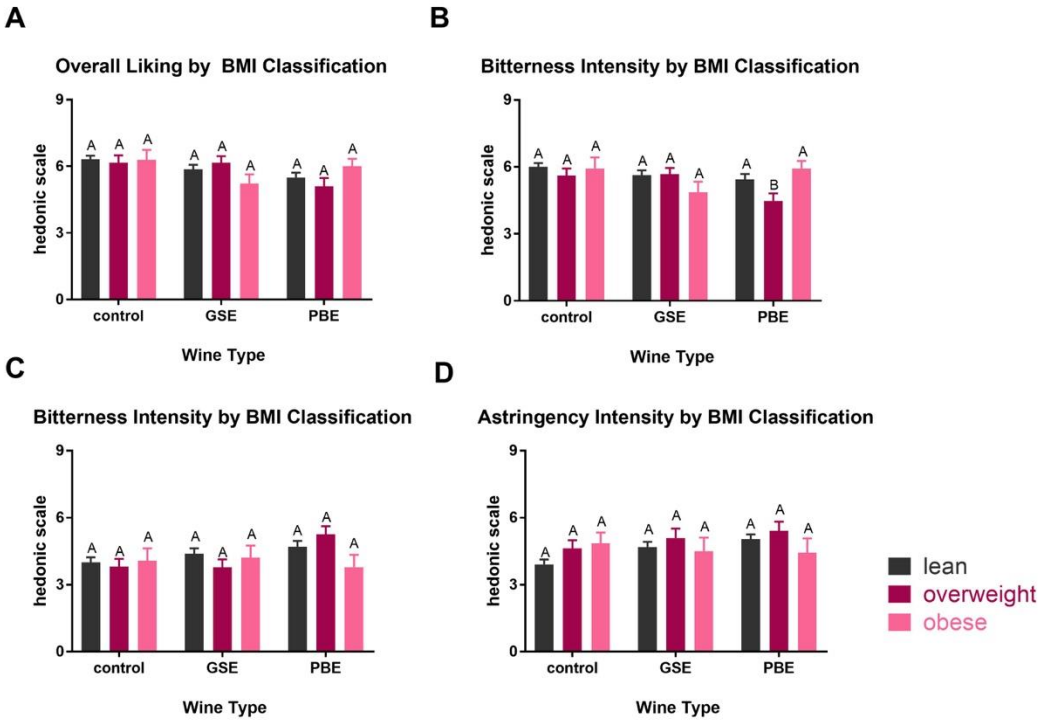

Figure B4. *Sensory panel results segmented based on panelist BMI classification and wine type for overall product liking (A), bitterness liking (B), bitterness intensity (C), and astringency intensity (D). Values are expressed as means ± SEM based on hedonic scale values that range from 1 to 9, with 1 indicating "dislike*

extremely" and 9 indicating "like extremely". Significantly different values are indicated by different letters at the 95% confidence level based on 1-way ANOVA. Panelist BMI classifications ***within each wine category*** *were determined based on ACSM guidelines.*

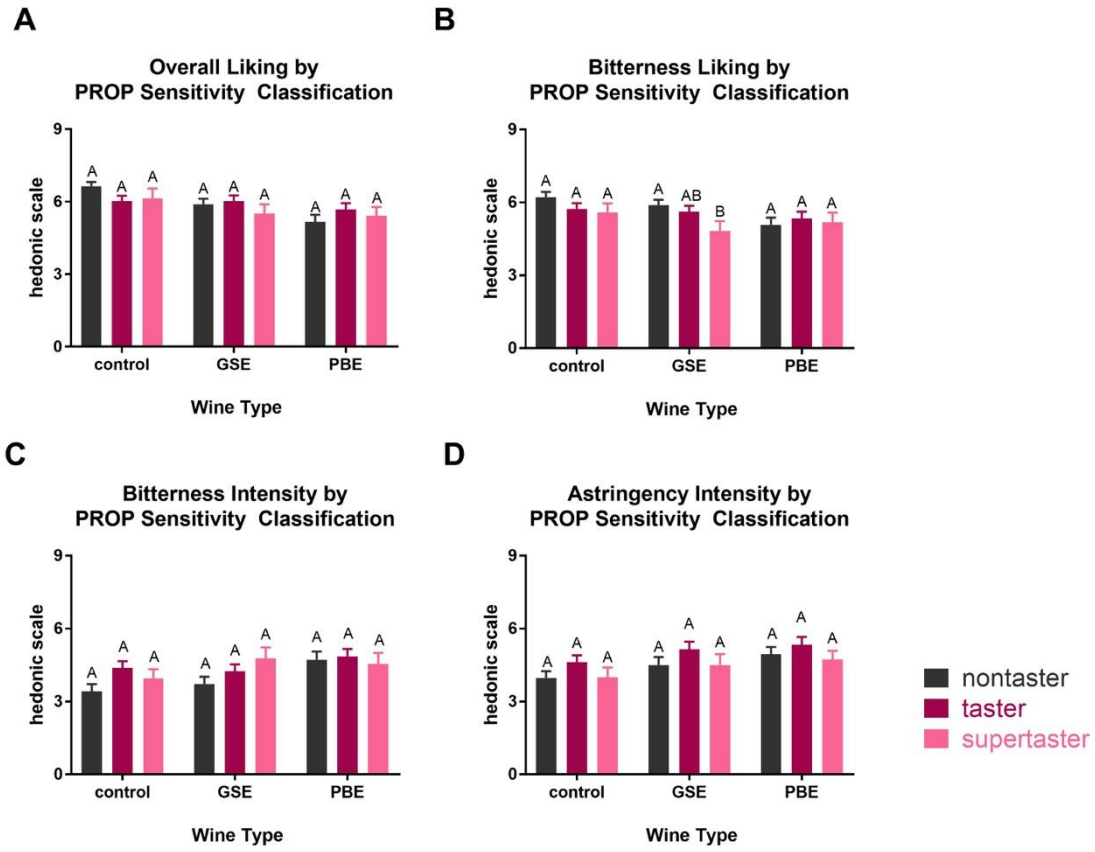

Figure B5. *Sensory panel results segmented based on panelist bitterness sensitivity classification and wine type for overall product liking (A), bitterness liking (B), bitterness intensity (C), and astringency intensity (D). Values are expressed as means ± SEM based on hedonic scale values that range from 1 to 9, with 1 indicating "dislike extremely" and 9 indicating "like extremely". Significantly different values* ***within each wine category*** *are indicated by different letters at the 95% confidence level based on 1-way ANOVA. PROP sensitivity classifications were determined based on LMS intensity ratings of a PROP solution against a sodium chloride solution. Non-tasters rated PROP lower than sodium chloride, tasters rated PROP equal to sodium chloride, and supertasters rated PROP higher than sodium chloride.*

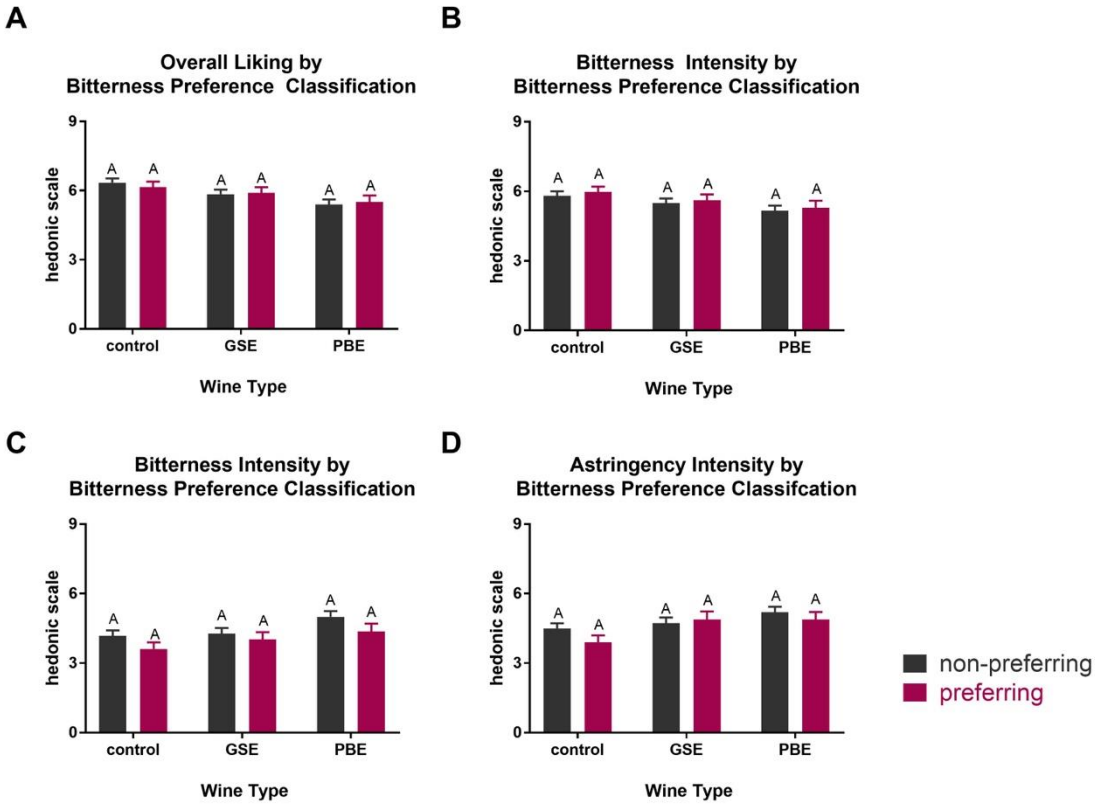

Figure B6. *Sensory panel results segmented based on panelist bitterness preference classification and wine type for overall product liking (A), bitterness liking (B), bitterness intensity (C), and astringency intensity (D). Values are expressed as means ± SEM based on hedonic scale values that range from 1 to 9, with 1 indicating "dislike extremely" and 9 indicating "like extremely". Significantly different values* ***within each wine category*** *are indicated by different letters at the 95% confidence level based on 1-way ANOVA. Bitter preference was determined on an individual level based on panelist hedonic scores for 5 bitter foods. To be classified as bitter preferring, panelists had to score 3 out of the 5 bitter foods at an 8 (like very much) or higher.*

REFERENCES

American College of Sports Medicine. (2013). *ACSM's health-related physical fitness assessment manual*. Lippincott Williams & Wilkins.

Burns, J., Gardner, P. T., O'Neil, J., Crawford, S., Morecroft, I., McPhail, D. B., … Lean, M. E. (2000). Relationship among antioxidant activity, vasodilation capacity, and phenolic content of red wines. *Journal of Agricultural and Food Chemistry*, *48*(2), 220–230.

Lesschaeve, I., & Noble, A. C. (2005). Polyphenols: factors influencing their sensory properties and their effects on food and beverage preferences. *The American Journal of Clinical Nutrition*, *81*(1), 330S–335S.

Sato, M., Ramarathnam, N., Suzuki, Y., Ohkubo, T., Takeuchi, M., & Ochi, H. (1996). Varietal differences in the phenolic content and superoxide radical scavenging potential of wines from different sources. *Journal of Agricultural and Food Chemistry*, *44*(1), 37–41.

Simonetti, P., Pietta, P., & Testolin, G. (1997). Polyphenol content and total antioxidant potential of selected Italian wines. *Journal of Agricultural and Food Chemistry*, *45*(4), 1152–1155.

APPENDIX C: SUPPLEMENTARY FILE FOR CHAPTER 5

C1 SUPPLEMENTARY METHODOLOGY

Full Methodology for Estimating Wine mDP. As described above, freeze-dried and resolubilized wine extracts (n = 5) were derivatized using 100 µL benzyl mercaptan (5% w/v in MeOH), 50 µL HCl (3.3% w/v), and 50 µL of wine extract (1 mg/mL MeOH). Controls, used to determine the quantity of unthiolyzed monomers in the wine extracts, were prepared using 50 µL of extract and 150 µL of MeOH. Samples and controls were thiolyzed in a 90°C water bath for 1 min, cooled on ice for 10 minutes, and transferred to HPLC vials in a 1:9 ratio with 95:5 01.% formic acid in water/0.1% formic acid in acetonitrile. Benzylthioether derivitives and corresponding monomers were quantified using UPLC-MS/MS. Samples were analyzed using a Waters Acuity H-class separations module with a Waters Acquity UPLC HSS T3 column (2.1 mm x 100 mm, 1.8 µm particle size) set at 40°C. A binary elution gradient consisting of 0.1% formic acid in water (Phase A) and 0.1% formic acid in acetonitrile (Phase B) was used. Solvent flow rate was 0.6 mL/min and sample injection volume was 20 µL. A linear elution gradient program described in Table A1 was used.

Table C1. Linear elution gradient program for thiolysis.

Time (min)	% Phase A
0.0-0.5	95.0
6.5	65.0
7.5-8.6	20.0
8.7-10.5	95.0

Sample temperature was maintained at 10°C for the duration of the program. The UPLC effluent was analyzed on a Waters Acuity triple quadrupole (TQD) MS using (-)-electrospray ionization (ESI) coupled with tandem mass spectrometry (MS/MS). (-) mode was employed for ionization, with capillary voltage set at -4.25 kV, cone voltage set at 30.0 V, extractor voltage set at 3.0 V, source temperature set at 150°C, and desolvation temperature set at 400°C. N_2 served as the cone and desolvation gas. Respective flow rates of 75 and 900 L/h were used. Argon served as the collision gas for MS/MS with the flow rate set at 0.25 mL/min in the collision cell. The Waters MassLynx program was used to set multi-reaction monitoring (MRM) parameters for parent ions $[M-H]^-$ and collision-induced dissociated daughter ions (Table A2).

Table C2. MRM detection settings for quantification of flavanols and respective thiolyzed derivatives by UPLC-MS/MS

Analyte	Retention Time (min)	MW (g/mol)	[M-H]- (m/z)	Daughter Ion (m/z)	Cone Voltage (V)	Collision Energy (eV)
C/EC	3.45-4.09	290.142	288.98	245.05	40	14
ECG	4.92-5.10	441.952	440.92	169	38	16

C/EC benzylthioether derivative	7.60-8.31	412.031	410.94	124.97	30	18
ECG benzylthioether derivative	7.68-8.01	563.824	563.05	287.06	38	16

The mDP for each sample was calculated from the concentration data obtained using an external standard curve generated from EC and ECG standards. mDP was calculated based on the equations outlined below that either include or exclude baseline monomers:

$$Equation\ C1: mDP = \frac{\Sigma\ monomers\ (native + thiolyzed) + \Sigma\ derivatives}{\Sigma\ monomers}$$

$$Equation\ C2: mDP = \frac{\text{net}\ monomers + \Sigma\ derivatives}{\text{net}\ monomers}$$

Baseline monomers refer to monomers present in the unthiolyzed wine samples, while net monomers refer to those generated by the thiolytic reaction only.

Qualitative Analysis of PCs by Normal Phase HPLC. As noted above, samples of each wine extract were diluted in methanol to 10 mg/mL and inserted into HPLC vials. Samples were injected in triplicate into an Agilent Technologies 1260 Infinity HPLC (Santa Clara, CA, USA) outfitted with a temperature controlled autosampler, thermostat column compartment, solvent degasser, quaternary pump, and fluorescence detector. Samples were chilled to 5°C in the autosampler and 5 µL aliquots were injected into the Develosil Diol Column (100Å, 250 x 4.6 mm, 5 µm particle size) (Phenomenex, Torrance, CA, USA) set at 35°C with a Luna HILIC guard column (4 x 3.0 mm ID SecurityGuard cartridge and cartridge holder) (Phenomenex). Mobile phase A was comprised of 2% acetic acid (v/v) in ACN and mobile phase B was comprised of 2% acetic acid (v/v), 3% ddH_2O (v/v), and 95% MeOH (v/v). Solvent flow rate was set at 1 mL/min with a binary elution gradient program described in Table A3. The FLC excitation wavelength was 230 nm and the emission wavelength was 321 nm.

Table C3. Normal phase HPLC binary elution gradient program.

Time (min)	%Phase A	%Phase B
0	93.0	7.0
3	93.0	7.0
60	62.4	37.6
63	0	100
70	0	100
76	93.0	7.0

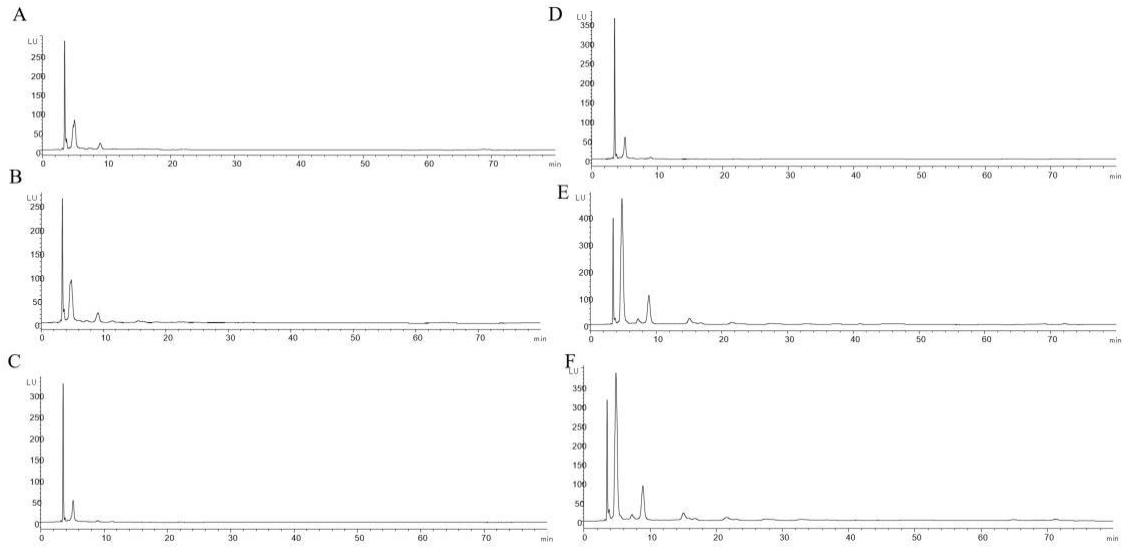

Figure C1. *Qualitative chromatograms from the normal phase HPLC analysis of all wine extracts (n=1) for A) Control red wine B) Cold-Soaked red wine C) Control rosé D) Rosé with added skins E) Rosé with added seeds F) Rosé with added crushed seeds. All chromatograms are expressed in fluorescence units (LU) over time (min).*

APPENDIX D:

Alterations to metabolically active bacteria in the mucosa of the small intestine, predict anti-obesity and anti-diabetic activities of grape seed extract in mice

L.E. Griffin, K.A. Witrick, C. Klotz, M.R. Dorenkott, K.M. Goodrich, G. Fundaro, R.P. McMillan, M.W. Hulver, M.A. Ponder, and A.P. Neilson, Food Func., 2017,8, 3510-3522.

ABSTRACT

Epidemiological and clinical studies suggest that grapes and grape-derived products may reduce the risk for chronic disease. Grape seed extract specifically has been gaining interest due to its reported ability to prevent weight gain, moderate hyperglycemia, and reduce inflammation. The purpose of this study was to examine the long-term effects of two doses of grape seed extract (10 and 100 mg/kg body wt*d in mice) on markers of metabolic syndrome in the context of a moderately high-fat diet. After 12 weeks, the lower dose of grape seed extract was more effective at inhibiting fat gain and improving glucose tolerance and insulin sensitivity. Neither the high fat diet nor grape seed extract altered skeletal muscle substrate metabolism. Most interestingly, when examining the profile of metabolically active microbiota in the mucosa of the small intestine, cecum, and colonic tissue, grape seed extract seemed to have the most dramatic effect on small intestinal tissue, where the population of *Firmicutes* was lower compared to control groups. This effect was not observed in the cecal or colonic tissues, suggesting that the main alterations to gut microbiota due to flavan-3-ol supplementation occur in the small intestine, which has not been reported previously. These findings suggest that grape seed extract can prevent early changes in glucose tolerance and alter small intestinal gut microbiota, prior to the onset of skeletal muscle metabolic derangements, when grape seed extract is consumed at a low dose in the context of a moderately high fat diet.

D1 INTRODUCTION

Grapes and grape products are some of the richest dietary sources of flavan-3-ols, a subclass of polyphenols.[1,2] Polyphenols are secondary plant metabolites which are the most prominent sources of antioxidants found in the human diet.[3–5] They include flavan-3-ols [catechins and procyanidins (PCs)], anthocyanins, and flavonols.[1,4,6] Structures of representative grape flavan-3-ols are shown in **Figure D1**.[1]

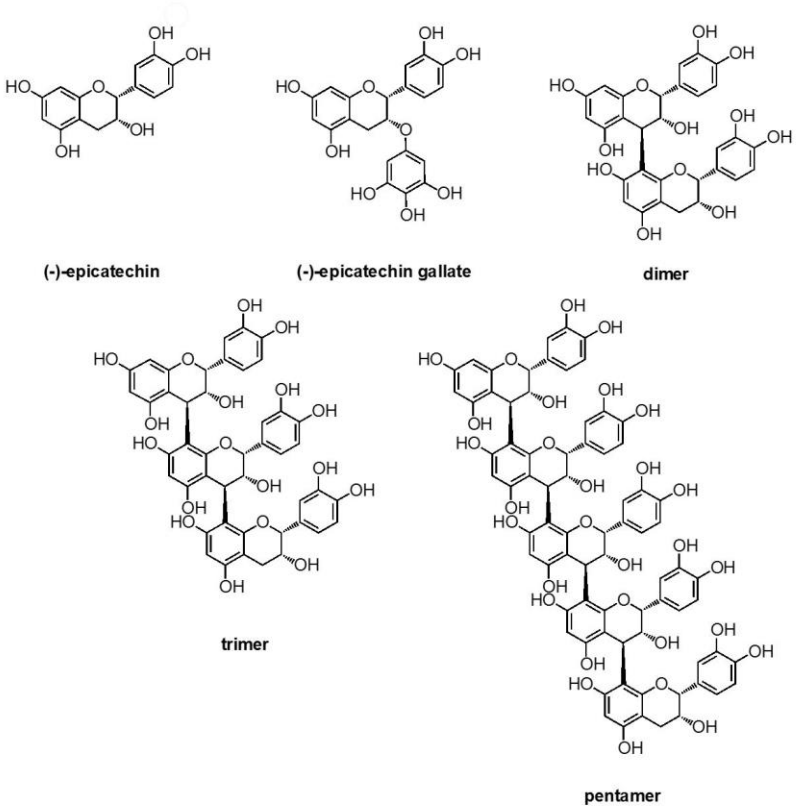

Figure D1 *Structures of (−)-epicatechin, (−)-epicatechin gallate, and a representative (−)-epicatechin dimer, trimer, and pentamer.*

Epidemiological and clinical studies suggest that consumption of grapes and grape products may help reduce the risk of certain diseases, including cardiovascular disease, metabolic syndrome, and cancer.[2,7] The effects of grapes, including improvement of endothelial function, reduction of platelet aggregation, inhibition of weight gain due to high-fat feeding, and increasing insulin sensitivity have all been linked to their flavan-3-ol content.[1,8–10] Feeding studies employing grape seed extract (GSE) rich in flavan-3-ols have demonstrated its ability to reduce weight and fat gain, prevent insulin resistance and improve glucose tolerance, reduce inflammation and increase mitochondrial function.[11] One area that has not been fully explored previously is the subject of skeletal muscle metabolic substrate utilization, which is the ability the muscle to utilize different fuel

sources (glucose, fatty acids, or amino acids) depending on availability.[12] Fatty acid and glucose metabolism dysfunction at the mitochondrial level are characteristic of diet-induced obesity.[13] Several prior studies have suggested an improvement in metabolism due to supplementation with grape compounds, but this was predominantly in conjunction with other compounds, such as combined resveratrol and epigallocatechin-gallate supplementation or combined GSE and docosahexaenoic (DHA)-rich oil supplementation.[14–16] Furthermore, most of the studies have examined changes in respiratory quotients due to supplementation, not metabolism rates of different substrates by skeletal muscle samples. Additional studies are needed in order to elucidate the impact of grape PCs on diet-induced metabolic derangements in skeletal muscle.

GSE has also been gaining attention for its ability to modulate the gut microbiome.[6,17] Many GSE flavan-3-ols (particularly the larger PCs) are poorly absorbed in the small intestine and enter the colon intact.[7] It has been estimated that 90-95% of ingested PCs enter the colon unaltered along with some unabsorbed monomers.[18] The gut microbiota metabolize unabsorbed PCs to smaller phenolic compounds which are highly bioavailable.[19] Unabsorbed polyphenol compounds including flavonoids and non-flavonoids appear to modulate the balance between *Bacteriodes* and *Firmicutes*, which has been linked to obesity trends in humans.[20,21] Grape compounds have also demonstrated the ability to increase the abundance of beneficial bacterial species, including *Bifidobacterium* and *Lactobacillus* spp. in animal models.[18] These changes could alter overall host health status, but the impacts of dose and compound structure also must be considered.[6,18] Despite these findings, the majority of studies have examined total fecal (i.e. excreted microbial DNA) microbial populations; the impact of grape flavan-3-ols on profiles of metabolically active mucosal populations *in situ* in various regions of the colon remains poorly understood.

The objectives of this study were to examine the impact of GSE supplementation in the context of moderate high-fat (HF) feeding on 1) markers of metabolic syndrome and 2) changes to the gut microbiome using an animal model. This was performed by supplementation of incremental doses of GSE and a moderate HF diet (45%) to induce mild metabolic changes, recapitulating slow onset of metabolic changes during progression from normoglycemia to hyperglycemia, with accompanying changes in substrate metabolism by skeletal muscle. Evaluation of the ability of each treatment to prevent weight and fat gain, insulin resistance and impaired glucose tolerance, and changes to the bacterial populations of the small intestine, cecum, and colon was performed. It was hypothesized that GSE would protect against the onset of disease, which would be accompanied by promoting skeletal muscle metabolic adaptation to HF feeding and alteration of the gut microbiota.

D2 MATERIALS AND METHODS

D2.1 Chemical Standards and Reagents. Vitaflavan® GSE was purchased from DRT Nutraceutics (Dax, France). Manufacturer specifications can be found in **Table D1**. Chemical standards included (±)-catechin (C), (−)- epicatechin (EC), (−)-epigallocatechin (EGC), (−)- epicatechin gallate (ECG), and (−)- epigallocatetchin gallate from Sigma (St. Louis, MO, USA), PC dimer B_2 and trimer C_1 from ChromaDex (Irvine, CA, USA), and PC dimer B_1, dimer B_2-gallate, dimer B_5, trimer T_2, tetramer A_2, and pentamers through

decamers (DP5-DP10) from Planta Analytica (Danbury, CT, USA). Solvents for assays were of ACS grade or better (Fisher, Pittsburgh, PA, USA; or VWR Radnor, PA, USA).

Table D1 GSE composition as reported by DRT

Value	%
Procyanidin Content (%)	75.6
Monomeric catechins (%)	24.4
Dimeric + Trimeric Procyanidins (%)	43.6
Procyanidin-Porter content (%)	66.0

D2.2 Animals. The Institutional Animal Care and Use Committee at Virginia Polytechnic Institute and State University approved all animal procedures (protocol #12-049-FST). Male C57BL/6J mice (9 weeks old, ~25 g, *N*=32) were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were housed 4/cage under standard laboratory conditions (12 h light/dark cycle, 30-70% relative humidity, 20-26ºC). Food and water were provided *ad libitum* throughout the study. Upon receipt, mice were acclimatized to laboratory conditions on standard rodent chow diet for 2 weeks prior to the start of the experiment.

D2.3 Diets. Following the acclimatization period, mice were randomized into 4 groups of *n*=8 animals/group with each group fed a distinct diet/GSE combination. Diets were from the standard Diet-Induced Obesity (DIO) models from Research Diets, Inc. (New Brunswick, NJ). One group of mice was fed a control low-fat diet (D12450B) contributing 10% kcal from fat (4.3% fat by weight, 3.85 kcal/g) to serve as a negative control. The second group of mice were fed a high-fat (HF0) diet (D12451) contributing 45% kcal from fat (24% fat by weight, 4.74 kcal/g) to serve as a positive control. A third group of mice was fed the HF diet supplemented with 0.07 g GSE/4057 kcal (HF10). The fourth group of mice was fed the HF diet supplemented with 0.70 g GSE/4057 kcal (HF100). These doses provided a daily GSE intake of ~10 and 100 mg GSE/kg body weight (BW)/d, respectively (based on estimated daily intake of 0.1225 g food/g mouse, or 3.675 g/30 g mouse) (**Figure D2**). These correspond to human equivalent doses of ~0.811 and 8.11 mg/kg BW/d for a 60 kg adult.[22] Full composition information for the diets can be found in **Table D2**. Diets were in pelted form, with GSE added by Research Diets prior to pelleting. Mice were maintained on the dietary treatments for 16 weeks. Food was provided as pelleted diet, available to mice on the wire grating above their cages. Food was replaced once (control diet) or twice (all HF diets) per week to maintain freshness and avoid rancidity due to fat content. Food consumption was determined by measuring the difference between the weight of food added and the subsequent weight of food remaining for each food replacement.There is typically very little loss due to spillage, as mice also eat any small pieces that fall to the cage floor. Diets were stored at −20°C for the duration of the study to prevent lipid degradation.

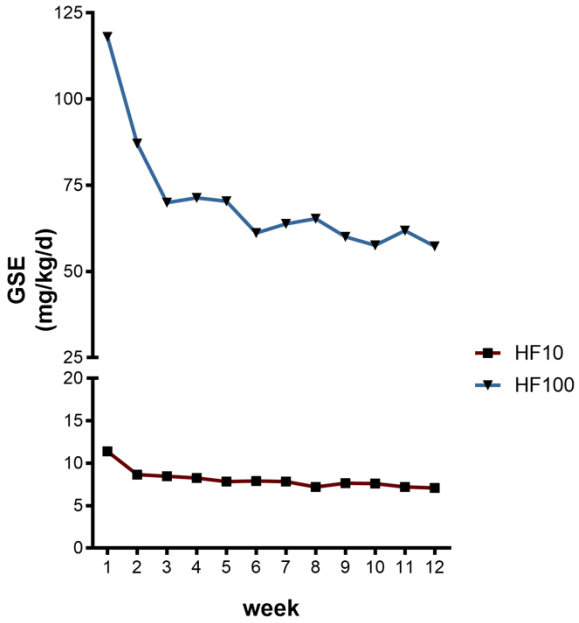

Figure D2 *Weekly GSE intake of animals on the 10 and 100 mg/kg BW/d diets for the first 12 weeks of the study.*

D2.4 Body Weight and Composition. Mice were weighed weekly throughout the study up to week 13 (at which point fasting for glucose and insulin tolerance tests interfered with accurate body weight determinations). Mouse body composition (% lean, fat and fluid mass) was measured in duplicate every 4 weeks using a Bruker LF90 NMR analyzer (Billerica, MA, USA).

D2.5 Glucose and Insulin Tolerance Testing. Glucose tolerance was measured in week 14. For the glucose tolerance test (GTT), mice were fasted for 12 h and then received an intraperitoneal (IP) injection of 16.7% (w/v) glucose solution (Hospira, Inc., Lake Forest, IL, pharmaceutical grade) in saline (volume adjusted for each mouse to provide 1 g/kg body weight). Blood was collected from the tail after removal of the tip using surgical scissors. Blood glucose was measured at 0 (baseline), 30, 60, 90 and 120 min post-injection using One Touch Ultra Blue test strips (LifeScan, Inc., Milpitas, CA) and a standard glucometer. Insulin tolerance was measured during week 15. For the insulin tolerance test (ITT), mice were fasted for 4 h before receiving an IP injection of 0.65 U insulin/kg body weight (Novolin, Novo Nordisk, Denmark, pharmaceutical grade) in saline. Blood was expressed from the tail tip at 0 (baseline), 15, 30, 45 and 60 min post-injection. Blood glucose was measured as described for GTT. For both GTT and ITT, the area under the curve (AUC) of the blood glucose plot for each test was determined by the trapezoidal method using pharmacokinetic (PK) plug-in functions for Microsoft Excel software.

Table D2 Animal diet composition as reported by Research Diets Inc.

	D12450B 10 kcal% fat (C0)		D12451 45 kcal% fat (HF0)		D12031502 45 kcal% fat (HF10)		D12031504 45 kcal% fat (HF100)	
%	**gm**	**kcal**	**gm**	**kcal**	**gm**	**kcal**	**gm**	**kcal**
Protein	19	20	24	20	24	20	24	20
Carbohydrate	67	70	41	35	41	35	41	35
Fat	4	10	24	45	24	45	24	45
Total		100		100		100		100
kcal/gm	3.8		4.7		4.7		4.7	
Ingredient	**gm**	**kcal**	**gm**	**kcal**	**gm**	**kcal**	**gm**	**kcal**
Casein	200	800	200	800	200	800	200	800
L-Cystine	3	12	3	12	3	12	3	12
Corn Starch	315	1260	72.8	291	72.8	291	72.8	291
Maltodextrin 10	35	140	100	400	100	400	100	400
Sucrose	350	1400	172.8	691	172.8	691	172.8	691
Cellulose, BW200	50	0	50	0	50	0	50	0
Soybean Oil	25	225	25	225	25	225	25	225
Lard	20	180	177.5	1598	177.5	1598	177.5	1598
Mineral Mix S10026	10	0	10	0	10	0	10	0
DiCalcum Phosphate	13	0	13	0	13	0	13	0
Calcium Carbonate	5.5	0	5.5	0	5.5	0	5.5	0
Potassium Citrate 1H20	16.5	0	16.5	0	16.5	0	16.5	0
Vitamin Mix V10001	10	40	10	40	10	40	10	40
Choline Bitartrate	2	0	2	0	2	0	2	0
GSE	0	0	0	0	0.07012	0	0.7012	0
FD&C Yellow Dye #5	0.05	0	0	0	0.025	0	0.025	0
FD&C Red Dye 340	0	0	0.05	0	0.025	0	0	0
FD&C Blue Dye #1	0	0	0	0	0	0	0.025	0
Total	1055.05	4057	858.15	4057	858.22	4057	858.851	4057
GSE, gm/4057 kcal	0		0		0.07012		0.7012	

D2.6 Euthanasia and Necropsy. Animals were euthanized with CO_2 following AVMA guidelines. Animals were maintained under CO_2 for 1 min following apparent clinical death (cessation of respiration). Following euthanasia, the abdominal cavity was opened ventrally from the rectum to the neck, and bilateral pneumothorax was performed to ensure death. Gastrocnemius and quadriceps muscles were harvested and manually separated into red and white muscle portions. The red portions of each were combined and used for metabolism studies. The muscle was then placed in mammalian cell lysis buffer for protein analyses and frozen in liquid N_2. Small intestine (SI), cecum, and colonic tissue were also collected from each animal. Segments were flushed with ice-cold PBS to remove luminal contents and associated microbiota, leaving only mucosal adherent microbiota. Tissue segments were placed into microcentrifuge tubes containing RNAlater (Thermo Fischer), snap frozen in liquid N_2, and stored at −80°C for later microbial analysis.

D2.7 Tissue Substrate Oxidation. Skeletal muscle metabolism analyses were conducted as described in Zhang et al.[12] and Gao et al.[13] Approximately 50 mg fresh quadriceps muscle samples were immediately placed into 0.2 mL of a modified sucrose EDTA medium (SET) on ice containing 250 mM sucrose, 1 mM EDTA, 10 mM tris-HCl, and 10 mM tris-HCl, pH 7.4. Muscle samples were then minced thoroughly with scissors and then SET buffer was added to a 20-fold diluted (wt/vol) suspension. The minced samples were homogenized in a Potter-Elvehjem glass homogenizer at 10 passes across 30 sec at 1,200 rpm with a motor-driven Teflon pestle.

Palmitate and glucose oxidation rates were determined in the fresh muscle homogenates. The oxidation rate in muscle homogenates were measured by counting the $^{14}CO_2$ produced from [1-^{14}C] palmitic acid (1 μCi/mL) or [U-^{14}C] glucose (1 μCi/mL). Isotopes were obtained from American Radiolabeled Chemicals, St. Louis, MO. Eighty μL of a 20-fold (wt/vol) diluted muscle homogenates were incubated with 320 μL of reaction media (pH 7.4). Final concentrations of the reaction media were in mmol per liter: sucrose, 100; Tris-HCl, 10; potassium phosphate, 5; potassium chloride, 80; magnesium chloride, 1; L-carnitine, 2; malate, 0.1; ATP, 2; coenzyme A, 0.05; dithiothreitol, 1; EDTA, 0.2; and bovine serum albumin, 0.3%. Reagents were obtained from Sigma. After 60 min of incubation at 37°C, 200 μL of 70% perchloric acid was injected to stop the reaction and evolve CO_2 from the reaction media. CO_2 produced during the 60 min incubation was trapped with 400 μL of 1M sodium hydroxide. Trapped $^{14}CO_2$ was determined by liquid scintillation counting by use of 5 mL EcoLite liquid scintillation cocktail (MP Biomedicals, Santa Ana, CA, USA).

D2.8 RNA Extraction and conversion to cDNA. Tissue samples (small intestine, cecum, colon) preserved in RNALater were extracted using the same method as described by Goodrich et al.[11] with minor modification. Total RNA was converted to cDNA using the USB First-Strand cDNA synthesis kit per manufacturer specification (Affymetrix, Cleveland, OH). Briefly, each 20 uL PCR reaction was composed of 2 uL random primers, 1000 ng of RNA, 2 uL of 10X RT Buffer, 1 uL 10mM dNTPs, 1 uL of RNase Inhibitor, 1 uL of M-MLV RT, and nuclease. The reverse transcription-PCR protocol called for 44ºC for 60 min, followed by 92ºC for 10 min. Samples were standardized based on amount of 16S rDNA.

D2.9 Amplification, Sequencing of 16S rRNA genes and Analysis. PCR and sequencing were performed using a modified version of the protocol presented in Caporaso et al.[23], adapted for the MiSeq platform (Illumina, San Diego, CA, USA). Briefly, the V4 region of the 16S rRNA gene was amplified using barcoded primers allowing the pooling of up to 26 different samples in each sequencing run. Sequencing was performed by an Illumina MiSeq DNA sequencer at the Virginia Bioinformatics Institute with a depth of ~10,000 reads per sample. Sequences were then trimmed and classified with the Quantitative Insight Into Microbial Ecology (QIIME, http://www.qiime.org) toolkit. Using the QIIME wrappers, operational taxonomic units (OTUs) were picked at 97% sequence identity using cdhit, and a representative sequence was then chosen for each OTU by selecting the most abundant sequence in that OTU. These representative sequences were aligned using PyNAST, and taxonomy was assigned to them using the RDP Classifier. The PyNAST-aligned sequences were also used to build a phylogenetic tree with FastTree and unweighted UniFrac distances and then computed between all samples for additional ecological analyses, including principal coordinates analysis.

D2.10 Statistical Analysis. Mouse phenotype and metabolism data were analyzed using GraphPad Prism v6.0f (GraphPad, La Jolla, CA, USA). Analysis by 1-way ANOVA followed by Tukey's HSD post-hoc test was used to determine significant differences between means. Microbiota data was analyzed using the Wilcoxon signed rank test using JMP Pro 12 (SAS, Cary, NC, USA). Outliers were removed from datasets on the basis of Dixon's Q test. Significance was defined *a priori* as $P < 0.05$.

D3 RESULTS AND DISCUSSION

D3.1 Food Intake, Weight Gain, and Body Composition. Food intake increased slightly, but non-significantly, for all treatment groups after the first 3 weeks (**Figure D3A**). The decreases in the first 3 weeks are likely due to the adjustment from standard lab mouse chow (on which mice are were maintained prior to the experiment, (2018 Teklad Global 18% Protein Rodent Diet, Envigo, Indianapolis, IN, USA, l/g3.1 kca) to the more energy-dense semi-purified diets (3.8 and 4.7 cal/g for the LF and HF diets, respectively, **Table D2**). After the first 3 weeks, the HF0 and HF10 groups exhibited higher food intake than the lean diet group and HF 100 group. Still, due to the higher fat content, the HF diet groups had higher energy intake than the lean control group (**Figure D3B**). This suggests that GSE supplementation does not decrease intake (by mechanisms such as increasing satiety or by altering the palatability of the diets) at the low doses tested. This also suggests that physiological changes induced by GSE were not simply due to altered caloric or macronutrient intake. All groups fed the HF diet gained significantly more weight compared to lean control (**Figure D3C,D**). As such, GSE supplementation did not inhibit weight gain relative to either control group. Similarly, mice fed the HF0, HF10, and HF100 diets gained significantly more fat over time than the lean control (**Figure D3E,F**). More interestingly though, overall fat gain was significantly lower in the mice supplemented with the low dose of GSE compared to the high dose (**Figure D3G**). Furthermore, the fat gain of the low dose group was not statistically different from the lean control group (**Figure D3G**). Lean body mass gains were not significantly different for any group (**Figure D3H**). A similarly designed study using 300 mg/kg body weight*d GSE supplementation in mice saw similar results of no significant weight gain improvement but some improvement in fat gain.[17] Overall, these data suggest that the lower dose of GSE is more

effective at inhibiting fat gain associated with moderate HF feeding, but total weight gain is not inhibited by GSE in these early stages of metabolic changes associated with a moderate HF diet.

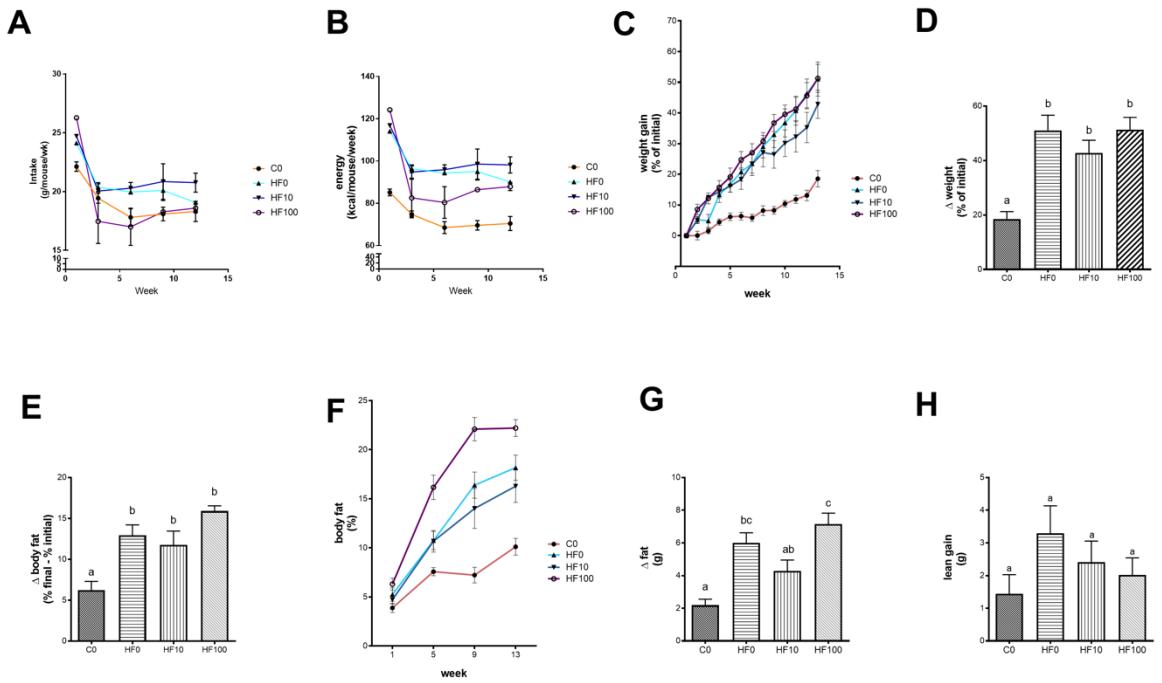

Figure D3 *Mouse food intake (A), energy intake (B), weight gain over time (C), and total body weight gain (D), change in body fat percentage (E), change in body fat percentage over time (F), total body fat gain (G), and total lean mass gain (H) during the feeding study. Food intake was measured up to week 12, after which fasting of animals in groups made accurate food intake determinations impractical. Values are presented as means ± SD (A-B, n=2 cages per treatment) and means ± SEM (all others) for n=8 replicates (n=7 for HF10). Treatments with different superscripts are significantly different (P < 0.05) based on one-way ANOVA followed by Tukey's HSD post-hoc test.*

D3.2 Glucose and Insulin Tolerance. GTTs and ITTs were performed on the animals at the end of the study. A 12 h fast preceded GTTs to significantly reduce blood glucose and to allow observation of true fasting blood glucose (reflecting gluconeogenesis in the liver) and glucose excursions. A 4 h fast preluded ITTs to represent glucose clearance ability by glucose-utilizing tissues. HF feeding alone resulted in significantly elevated 12 h fasting blood glucose levels compared to the control diet (**Figure D4A**). Supplementation of the low GSE dose significantly protected against HF-induced elevation of fasting blood glucose levels compared to HF0 diet alone (**Figure D4A**), which has been observed previously.[8,24] The high dose of GSE, however, did not prevent increases in fasting glucose levels as effectively as the low dose (**Figure D4A**). Mice fed the HF0 diet also exhibited significantly impaired glucose clearance (as measured by the blood glucose AUC during GTT) compared to mice fed the lean control diet (Figure 4B). GSE supplementation at both doses slightly improved glucose clearance (**Figure D4B,C**). The low dose GSE group had significantly improved glucose clearance compared to the HF0 group, and was not statistically different from the lean control. Glucose clearance was significantly poorer in the HF100 group compared to the lean control, and was not significantly different from the HF0 or HF10 groups for the GTTs.

No significant differences were observed in insulin tolerance for the 4 h fasting blood glucose levels (**Figure D4D**). However, the HF100 group had significantly impaired insulin

sensitivity compared to lean control (**D4E,F**). The HF0 and HF10 groups had insulin sensitivities that were not significantly different from the lean control or the HF100, suggesting again that the lower GSE dose is slightly more effective at preventing insulin resistance than the higher dose during the initial stages of diabetes onset.

Based on the reduction in 12 h fasting glucose produced by low dose GSE and subsequent improved GTT clearance, but overall lack of effect on ITT, our data suggest that this HF feeding model did not yet result in insulin intolerance in major insulin-utilizing tissues such as skeletal muscle but did potentially induce mild fasting hyperglycemia (potentially by slight dysregulation of hepatic gluconeogenesis).

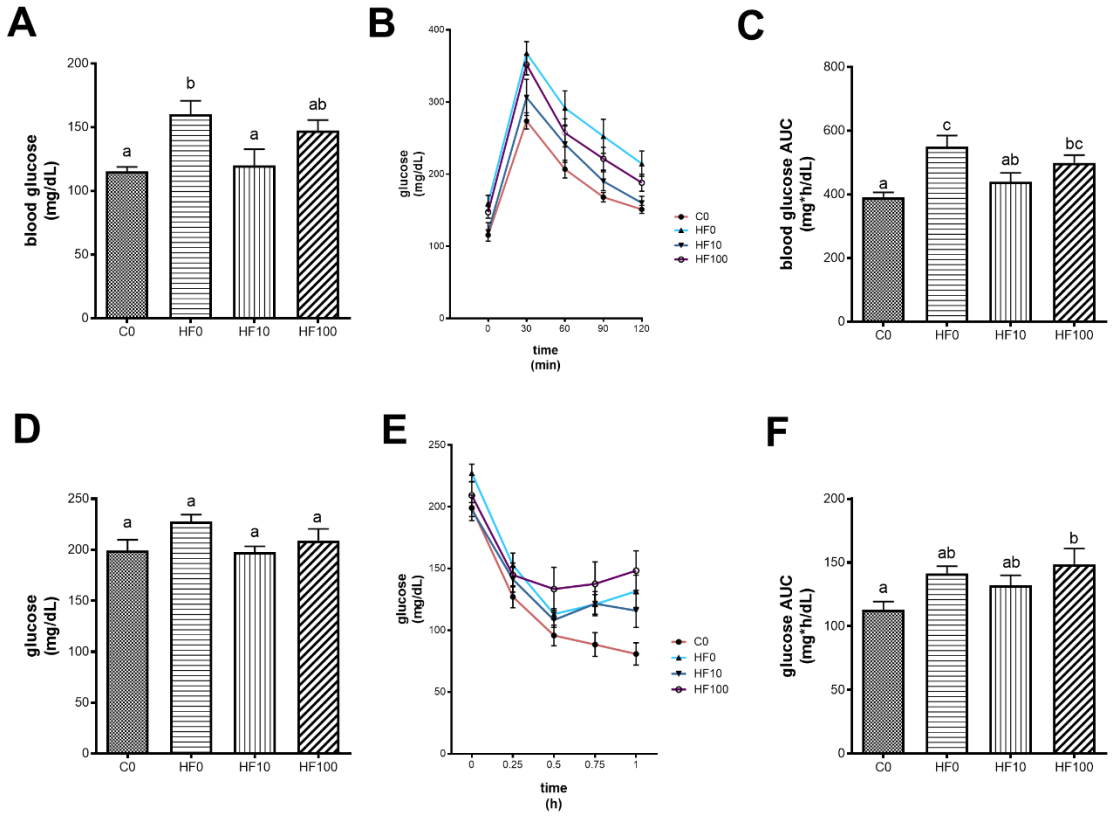

Figure D4 *Twelve h fasting blood glucose (A), blood glucose clearance over time for GTT (B), glucose tolerance test AUC (C), 4 h fasting blood glucose (D), blood glucose clearance over time for ITT (E), and insulin tolerance test AUC (F). Values are presented as means ± SEM for n=8 replicates (n=7 for HF10). Treatments with different superscripts are significantly different (P < 0.05) based on one-way ANOVA followed by Tukey's HSD post-hoc test.*

D3.3 Skeletal Muscle Metabolism. Fatty acid and glucose oxidation experiments were performed in harvested mouse skeletal muscle homogenates (**Figure D5**). As mentioned previously, dysfunction of fatty acid and glucose metabolism is a key feature of diet-induced obesity and subsequent onset of metabolic inflexibility. Typically, prolonged HF diets cause an increase in the abundance of fatty acid supply to the mitochondria. Fatty acid metabolism occurs at a higher rate compared to control groups while glucose oxidation is reduced, contributing to insulin resistance and glucose intolerance.[13] In this study, no significant differences were observed between any treatment or control groups in terms of fatty acid or glucose oxidation. The observation that the HF0 control diet did not significantly alter glucose and fatty acid metabolism compared to lean

control indicates that the HF0 diet was not sufficient in inducing significant derangement in the capacity of skeletal muscle to oxidize major energy substrates. Therefore, the GSE treatments would not have yet been able to demonstrate their ability to improve high fat diet induced muscle metabolic dysfunction. This suggests that these early effects of GSE supplementation possibly occur more in other tissues, including the pancreas and the gut, and the protective effects of GSE on glucose tolerance at this early state of metabolic syndrome are likely predominantly operating in tissues other than skeletal muscle. Metabolic flexibility, or the ability to switch between fuel substrates, was also measured in this study. No significant effects were observed due to lack of metabolic derangements and as such, the data are not presented here.

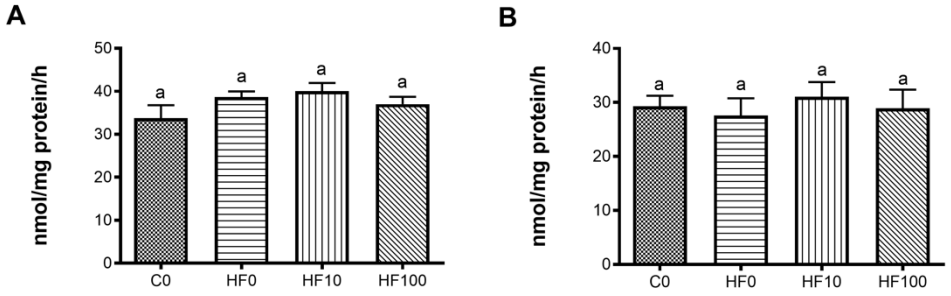

Figure D5 *Total fatty acid oxidation (A) and glucose oxidation (B) of skeletal muscle tissue. Values are expressed as means ± SEM for n=8 replicates (n=7 for HF10). Treatments with different superscripts are significantly different (P < 0.05) based on one-way ANOVA followed by Tukey's HSD post-hoc test.*

D3.4 Gut Microbiome. Due to the costly nature of 16S rRNA gene sequencing, it was decided to only proceed with the lean control, HF0 control, and HF10 treatments for this analysis, as the low GSE dose appeared to be more effective than the high dose at improving markers of metabolic syndrome measured in this study. It must be mentioned that in this experiment, the results from 16S rRNA sequencing were converted to 16S cDNA values. Thus, the data presented here generally represent the living, metabolically active bacteria only. This presents a different picture of the influence of GSE on gut microbiota than simply measuring total abundance, and potentially offers additional insights into the influence of GSE on the function of the gut microbiome.

To our knowledge, this is the first study to analyze mucosal-adherent microbial populations of the gut in different sections in response to GSE feeding in mice. Typically, excreted fecal pellets are used for analysis of gut microbiota composition, which give a representation of the gut microbiota as a whole, not individual regions.[25,26] This design allows us to examine specific changes in populations directly interacting with the gut mucosa in various regions of the gut, as opposed to total excreted, which represents the sum of gut profiles but does not provide location specificity. *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Deferribacteres* and *Actinobacteria* represented >90% of the total taxonomic phyla observed in the mucosa of various regions of mouse gut (**Figure D6, Table D3**). *Firmicutes* (relative abundance 41%-73%) and *Bacteroidetes* (10%-37%) were the most abundant phylum across the three regions of the gut for all treatments. The small intestine had a higher percentage of *Proteobacteria* compared to the cecum and colon. *Bifidobacterium* and *Mucispirillum* were the only genera detected (above 0.1% abundance) within the *Actinobacteria* and *Deferribacteres* phyla respectively across treatment groups.

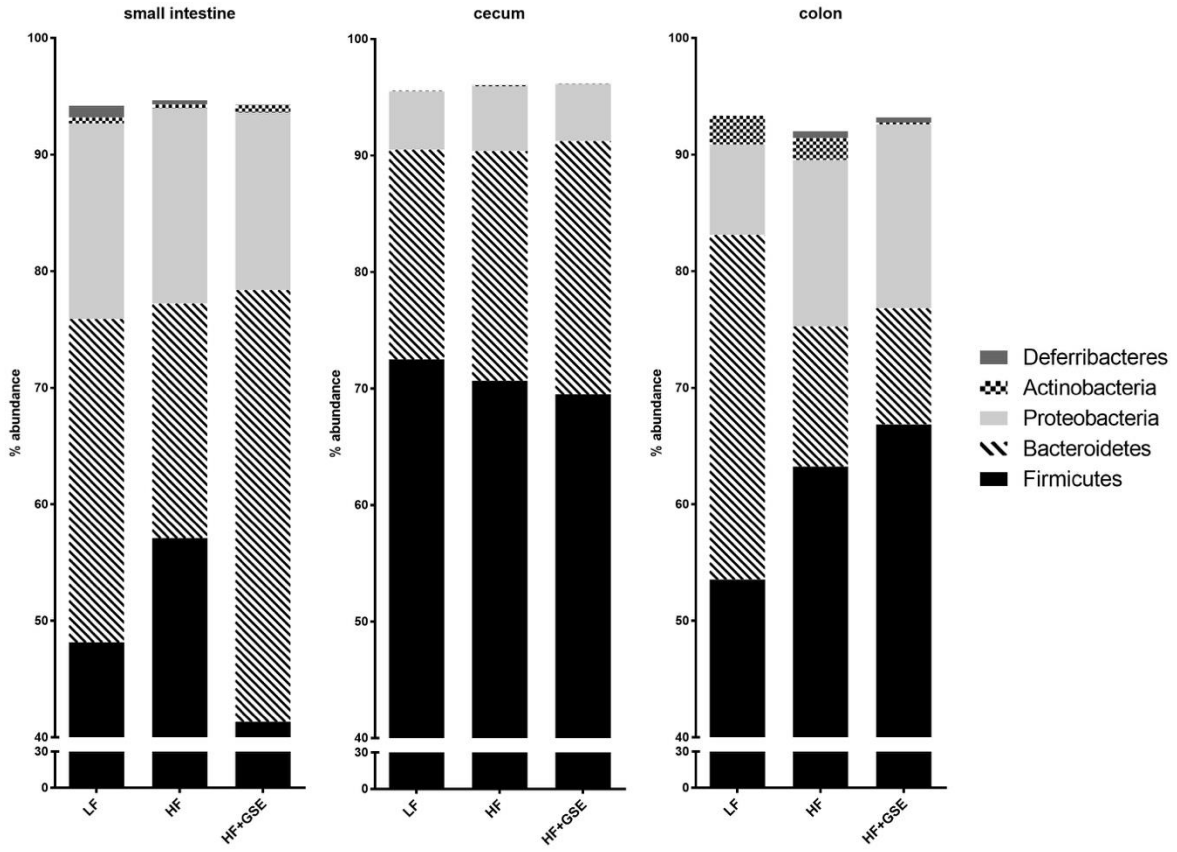

Figure D6 *Mean relative abundance (%) of the top five phyla present in the small intestine, cecum, and colon of mice fed lean control, HF0, and HF + GSE10 diets.*

Table D3 Abundance* of the five most prominent bacterial phyla (as a % of total bacterial 16S rDNA sequences) in the cecum, SI, and colon of mice

	small intestine			cecum			colon		
phylum	LF	HF0	HF+GSE	LF	HF0	HF+GSE	LF	HF0	HF+GSE
Firmicutes	$48.2^a \pm 6.7$	$57.1^a \pm 10.4$	$41.3^a \pm 10.1$	$72.5^a \pm 4.5$	$70.7^a \pm 5.3$	$69.5^a \pm 11.2$	$53.6^a \pm 9.8$	$63.2^a \pm 12.3$	$66.8^a \pm 7.5$
Bacteroidetes	$27.8^a \pm 6.6$	$20.^a1 \pm 6.1$	$37.1^a \pm 10.3$	$18.0^a \pm 4.0$	$19.7^a \pm 5.3$	$21.7^a \pm 9.0$	$29.6^a \pm 9.4$	$12.0^a \pm 5.1$	$9.98^a \pm 3.7$
Proteobacteria	$16.8^a \pm 6.4$	$16.8^a \pm 9.7$	$15.2^a \pm 5.7$	$5.0^a \pm 1.5$	$5.55^a \pm 2.7$	$4.9^a \pm 2.7$	$7.8^a \pm 4.7$	$14.2^a \pm 6.6$	$15.8^a \pm 4.7$
Deferribacteres	$1.0^a \pm 0.88$	$0.37^a \pm 0.31$	$0.029^a \pm 0.02$	$0.0014^a \pm 0.001$	$0.031^a \pm 0.01$	$0.0057^a \pm 0.003$	$0.02^a \pm 0.01$	$0.61^a \pm 0.48$	$0.44^a \pm 0.32$
Actinobacteria	$0.49^a \pm 0.2$	$0.31^a \pm 0.19$	$0.63^a \pm 0.041$	$0.037^a \pm 0.018$	$0.11^a \pm 0.046$	$0.019^a \pm 0.0071$	$2.5^a \pm 1.7$	$1.9^a \pm 1.6$	$0.15^a \pm 0.04$

*Abundance depicts the percentage of total bacteria belonging to the corresponding phyla and gut region, expressed as means ± SEM. Treatments with different superscripts are significantly different ($P < 0.05$) based on one-way ANOVA followed by Tukey's HSD post-hoc test.

In many animals, including mice, the dominant gut bacteria belong to the *Firmicutes* and *Bacteroidetes* phyla. The role of these bacteria in obesity remains unclear. Some studies report that the balance between *Firmicutes* and *Bacteroidetes* is a key biomarker of obesity[27], while other studies show no difference in proportions of *Bacteroides* and *Firmicutes* in the feces of lean and obese subjects.[28,29] In mice and humans, a greater population of *Firmicutes* has been linked to obesity.[30,31] The predominant product of the phylum *Firmicutes* is butyrate, which serves as an energy source for colonic enterocytes. Butyrate has been shown to increase insulin sensitivity in mice, enhance leptin gene expression, and may enhance GPR41 expression, which can lead to improved intestinal mucosal barrier function.[32,33] Interestingly, the GSE treated group exhibited decreased abundance of *Firmicutes* compared to lean control in the small intestine, but increased abundance compared to both lean and HF0 control groups in the colon (**Figure D6**). This may be important because the by-products of *Firmicutes* metabolism can more readily diffuse or be transported within the small intestine compared to the large intestine.[32] Furthermore, when examining the *Firmicutes*:*Bacteroidetes* ratios for the treatments in different portions of the gut (**Figure D7**), no significant differences were observed between different groups, but the ratios were different in distinct portions of the gut. It has been speculated that this ratio is a key indicator of an obesity associated microbiome when using fecal samples to determine gut microbiota populations.[21,27] The results from this study suggest that a ratio calculated based on fecal samples may not accurately represent the *Firmicutes*:*Bacteroidetes* ratio along the entire gut.

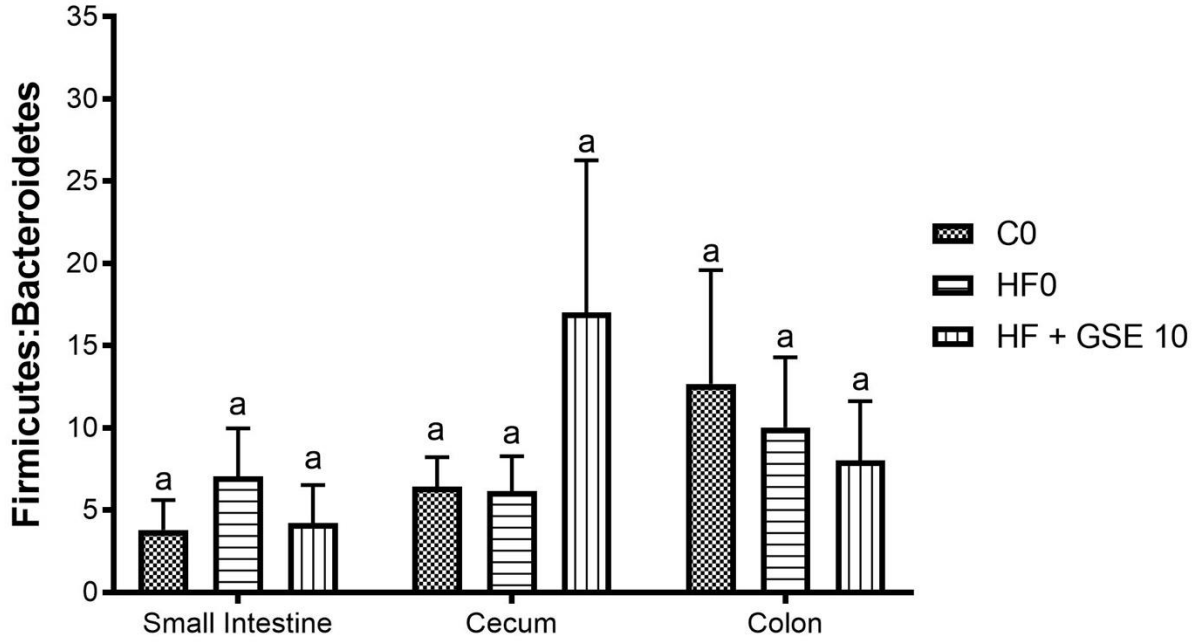

Figure D7 *Ratio of Firmicutes to Bacteroidetes phyla in the small intestine, cecum, and colon of mice fed lean control, HF0, and HF + GSE10 diets. Values are expressed as means ± SEM.*

The trend toward increased *Firmicutes* population in the small intestine was ameliorated by the addition of GSE to the diet, featuring an increase in Bacteroidetes. Still, the HF0 control diet had a higher abundance of *Firmicutes* and reduced *Bacteroidetes* abundance compared to lean control. GSE supplementation, however, appeared to decrease the abundance of *Firmicutes* and increase the abundance of *Bacteroidetes* compared to both lean and HF0 control groups suggesting that GSE supplementation could suppress obesity promoting phyla in the small intestine.[21,27,34,35]

This effect was not observed in other tissue samples from the cecum or colon. One possible explanation for this observation is the fact that the small intestine has more contact time with the non-metabolized native flavan-3-ol compounds.[36,37] Unabsorbed dietary flavan-3-ols enter the colon and undergo numerous biotransformations by the microbiota, some of which are incomplete, according to bioavailability studies.[38] Flavan-3-ol metabolites could interact with the colonic microbiota differently than the parent compounds interact with the small intestine tissues, as it has also been observed that metabolism and associated compound profiles are different in various regions of the colon.[18,39] Another possible explanation is related to the observation that the smaller monomer compounds, which are absorbed in the small intestine, are the compounds responsible for changing the gut microbiota composition. It has been observed *in vitro* that monomer flavan-3-ols inhibit several non-beneficial bacterial species.[6] It is possible that monomers in the GSE interacted with the small intestinal microbiota, but were then extensively metabolized later on in the distal parts of the large intestine and were thus present at much lower levels in the cecum and colon, reducing alterations to the microbiota there. GSE supplementation also increased the relative abundance of *Bifidobacterium* spp. with concomitant decrease in *Bacteroides - Prevotella* spp. and *Parabacteroides* spp. in the small intestine. It has been previously observed that certain strains of *Lactobacillus* and *Bifidobacteria* are able to metabolize PCs, while others are resistant. The GSE supplemented diet used here could have provided an energy source for similar strains of *Lactobacillus* and *Bifidobacteria* to flourish.[40]

In cecum tissue samples, distinguishable differences were not observed at the phyla level across treatments (**Table D3**), but some significant changes were determined at the genera level (**Figure D8**). The *Bacteroidetes* population was much lower than in the small intestine and the *Firmicutes* population was highest of all tissues for all treatments. This result was somewhat unexpected, as other studies report a significantly higher population of *Firmicutes* in the cecum due to HF feeding.[41] It is possible that changes to the cecal microbiota occur slowly, which would coincide with the other markers of disease assessed in this moderate HF feeding study. At lower taxonomic levels, GSE supplementation reduced the abundance of *Clostridiaceae* ($p=0.10$) *Coprobacillaceae* ($p<0.05$) and *Erysipelotrichaceae* ($p<0.05$) families. At the genera level, GSE treatment appeared to significantly reduce the abundance of *Allobaculum*, while the HF0 diet significantly increased the abundance of *Lactococcus* (**Figure D8**). As this is one of the few studies to examine various portions of the active gut bacteria in response to GSE treatment and HF feeding, very few other reports regarding cecal gut microbiota changes due to flavanol supplementation have been documented. One such study involving pomegranate peel supplementation in Balb/c mice indicated that three weeks of 0.2% peel supplementation, as part of a HF diet, was correlated with an increase in cecal *Bifidobacterium* spp. and *Bacteroides-Prevotella* spp.[42] It would not be wise to compare results to this study however, as a completely different experimental model was used.

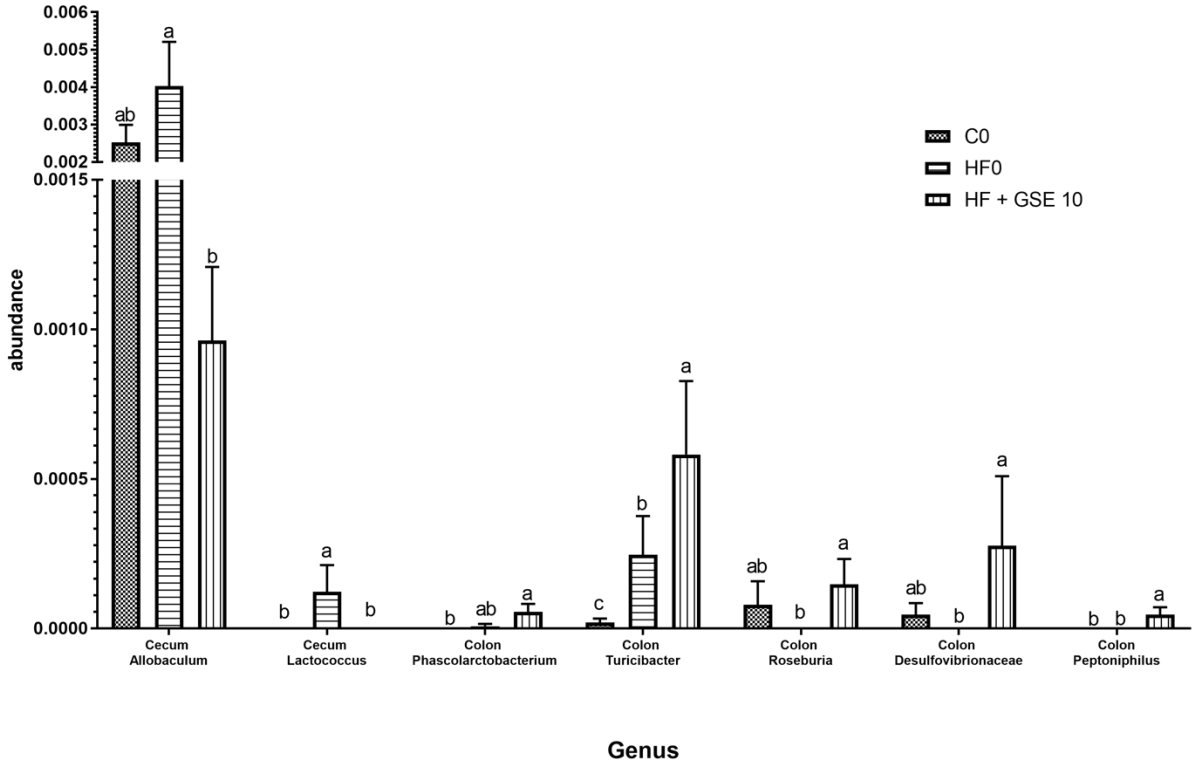

Figure D8 *Mean relative abundance (%) of select gut microbiota genera exhibiting significant differences between lean control, HF0, and HF + GSE10 treatment groups in cecum and colonic tissues. Values are expressed as means ± SEM. Treatments with an asterisk are significantly different (P < 0.05) based on Wilcoxon's signed rank test.*

In colon tissue samples, HF0 and GSE diets increased the relative abundance of *Firmicutes* and *Proteobacteria* with related reductions in *Bacteroidetes* compared to lean control (**Figure D6**). The major contributors to the increase in abundance of *Firmicutes* were Turicibacteraceae ($p<0.05$), Dehalobacterriaeae ($p=0.06$), Veillonellaceae ($p<0.05$), while non-significant changes in *Lachnospiraceae*, and *Ruminococcaceae* occurred with GSE supplementation. At the genera level, the HF0 diet increased the *Turicibacter* spp. abundance, while the GSE diet increased the abundance of *Turicibacter*, *Phascolarctobacterium*, *Roseburia*, *Peptoniphilus* and *Desulfovibrionaceae* spp. (**Figure D8**). It would appear from this data, that GSE supplementation was not able to prevent the obesity-induced changes to the abundance of *Firmicutes* to *Bacteroidetes* in the colon as it did in the small intestine based on total abundance. However, despite the increase in total *Firmicutes* abundance in the GSE treated colon samples, the *Firmicutes*:*Bacteroidetes* ratio was not significantly higher than the lean control samples (**Figure D7**). The abundance of *Firmicutes* in the colon could possibly explain why GSE supplementation was unable to prevent weight gain compared to control (**Figure D3**), as *Firmicutes* abundance is associated with increased energy harvest capacity.[21] The unchanged ratio provides further evidence that perhaps total abundance of the two key phyla is more important than the ratio in determining health status. Overall, it seems possible that while this low-dose GSE treatment was sufficient to combat some of the other parameters of metabolic syndrome, it was not significant enough to prevent the obesity-related shift in colon microbiota in the early stages of metabolic syndrome onset. Flavanols undergo substantial metabolism in the colon, which could impact the

extent of their effects on the microbiota.[18,43] It is possible that a higher dose of GSE would result in greater concentrations of compounds reaching different areas of the colon and causing a favorable shift in the abundance of *Firmicutes* to *Bacteroidetes*.[39] In agreement with our study, a different porcine study found similar trends in colonic microbiota due to GSE supplementation. In this study, female pigs were given a diet containing 1% (w/w) GSE daily for 6 days and feces were analyzed for flavanols and their metabolites. The primary microbial phenolic metabolites from the GSE consumption were phenolic acids and valerolactones, which are much more readily absorbed than intact flavanols of large DP. Similar to our study, increases in *Ruminococcaea* and *Lachnospiraceae* were observed in the fecal samples. However, in this study, these increases were postulated to be a positive outcome, as these bacterial spp. produce butyrate, and butyrate is thought by some to modulate inflammation and cancer development in colonic cells.[43] Together with our study, these findings highlight the notion that GSE flavanols may have different effects depending on the disease model in question. In any case, it is necessary to further examine the findings of these studies as they are so fundamentally different in design. The porcine study was performed using fecal samples representing the entire microbial population throughout the gut over a very short timeframe, while ours looks at specific regions of the gut and only metabolically active microbes associated with the intestinal mucosa after a 12-week supplementation period.

As mentioned previously, it is difficult to compare the results of this study to others due to differences in measures (mucosal-adherent metabolically active bacteria, whereas most studies measure all bacterial DNA in fecal samples or the colonic lumen). Therefore, many differences could be accounted for by the differences in what was measured; we believe that the present measures give additional insights into the impacts of GSE on active bacteria interacting directly with the gut mucosa. Furthermore, the majority of the studies of the gut microbiome involving flavanol supplementation have been either performed *in vitro* or using excreted fecal pellets, not tissue samples from various regions of the intestine. From studies of this design, it has been determined that grape-derived compounds generally have a positive effect on select gut microbiota. Oral gavage (300 mg/kg body weight*d) of C57BL/J6 mice fed a HF diet had a decreased presence of *Firmicutes* after 7 weeks when examining fecal pellet microbial populations. While the dosage in this study was much higher than those used here, the results also suggest that alterations to the gut microbiota are related to obesity-associated changes in metabolism due to HF feeding. Moreover, GSE may change microbiota populations and subsequently metabolism, but based on the results presented here, a dose of 300 mg/kg body weight*d may not be necessary.[17] Human GSE supplementation (0.5 g/d) for two weeks caused an increase in *Bifidobacterium* in fecal samples.[6] Supplementation of wine-polyphenols in F344 rats (50 mg/kg) for 15 weeks produced an increase in fecal abundance of *Bacteroides, Lactobacillus,* and *Bifidobacterium* spp. compared to control groups.[20] Promotion of *Bifidobacterium* and *Bacteroidetes* were observed in the small intestine region of this study, which coincide with the findings of other studies, but at a much lower dosage. It is impossible to know where the effects were taking place in the previous studies since fecal pellets were used to generalize the gut microbiota, but it is possible that the effects were also occurring in the small intestine as observed here. It has been observed in a piglet study that improvements to the microbial diversity and gut barrier function in the ileum and colon were due to GSE treatment, which also suggest that GSE behaves differently in different regions of the gut, but as this study was looking at using GSE as an antibiotic alternative to weaning stress induced diarrhea, the study design is not comparable to ours.[33] More work is needed to verify that GSE is primarily altering the small intestinal microbiota, not the colon.

This study demonstrated that low-dose GSE supplementation can reduce fat gain, improve whole-body glucose clearance and alter the gut microbial composition in the context of moderate HF feeding. Interestingly, the lower GSE dose appeared to be more effective than the high dose at reducing the markers of metabolic syndrome measured here including fat gain, 12 h fasting glucose levels, and glucose tolerance. The effectiveness of low GSE dosage has been observed in other studies which suggests that the flavanols in GSE behave in a hormetic manner.[44] Other studies have indicated that dietary antioxidants including resveratrol behave in a U-shaped manner *in vitro* (i.e. hormesis), with low doses being highly potent and higher doses leading to toxic side effects.[45] Since the lower GSE dose was more effective at inhibiting the onset of metabolic syndrome, this study provides *in vivo* evidence that GSE flavanols exhibit hormesis. Another possible explanation for these results is related to PC transport and absorption in the gut. In any case, the results of this study imply that a positive health affect could be achieved with a very small GSE supplement in pre-diabetic individuals. Efficacy at lower doses increases the feasibility for translation to humans, where grape compounds would be ingested primarily from foods. The lowest dose employed here (10 mg/kg BW/d) is equivalent to roughly 0.811 mg/kg BW/d in a 60 g human, or 48.7 mg total per day, which can reasonably be obtained from common dietary flavan-3-ol sources.

In examining the hypotheses from this study, based on these data, we were unable to test our hypothesis that GSE prevents disease by preventing derangements to skeletal muscle induced by HF feeding. Given that this study used a moderate HF diet to simulate the slow onset of metabolic syndrome, derangements to skeletal muscle metabolism were not observed in the HF0 control group after the 16-week feeding study. Without derangement of the positive control group, we were unable to identify a positive effect of GSE supplementation during this pre-diabetic state. Improvements to skeletal muscle metabolism with GSE supplementation has been observed in a genetically obese rat model, so it is possible that GSE could also be effective in a diet-induced obesity model with a longer study duration or higher fat diet than used here.[44] However, the present results suggest that GSE can exert protective effects even before metabolic derangements occur in skeletal muscle. This suggests that GSE may have preventive benefits at the early stages of metabolic syndrome. Still, improvements to insulin signaling and glucose uptake were observed, suggesting overall that GSE impacts these ramifications of metabolic syndrome.

This study supports the conclusion that GSE induces changes to the active gut microbiota, but surprisingly found that the greatest changes to the microbiota occurred in the adherent populations in the small intestine. GSE supplementation reduced the population of *Firmicutes* and increased the population of *Bacteroidetes* to levels lower than the lean control group in the small intestine only. In the cecum, the *Firmicutes* and *Bacteroidetes* populations were similar in all treatment groups and in the colon, the ratio of *Bacteriodetes* to *Firmicutes* increased compared to lean control in both the HF0 control and the HF10 group. Possible explanations for these results included that differences in flavanol metabolism in various regions of the colon yielded different interactions with the colon microbiota[39] and monomer flavanols, which are reportedly more potent at positively altering the gut microbiota, interacted with the small intestinal microbiota and were absorbed there.[6]

D4 CONCLUSION

It appears that the mechanisms behind the health promoting effects of GSE in the early stages of metabolic disease include its ability to promote glucose clearance, and alterations to the gut microbiota in the small intestine. This study is one of the first to examine the mucosal adherent,

metabolically active bacteria in specific regions of the gut. While significant differences among species at the phyla level were not observed due to GSE treatment, the results indicated that microbiota population in specific regions of the gut is highly variable depending on diet. Moreover, the effects on total bacteria are not necessarily reflective of the effects on metabolically active bacteria, while gut region matters when elucidating the effects of diet on microbiota. It is possible that GSE stimulates the liver and pancreatic β-cells to secrete insulin, which has also been observed in other models. This finding was not, however, confirmed by genetic sequencing of the target organs and is a consideration for future studies. Blood samples were not collected during glucose and insulin tolerance tests serum insulin analysis (only a small drop was obtained for blood glucose readings), which would indicate whether GSE itself stimulates glucose uptake or whether it promotes insulin secretion from the β-cells of the pancreas. Another limitation was that target organs were not used to determine if GSE altered genetic expression of key pathways related to metabolism. Future work should be directed towards verifying the findings of the gut microbiota results and determining if increasing dosage causes different changes in various areas of the gut. Still, this study suggests that GSE, at a low dose, may protect against the slow onset of diet-induced metabolic syndrome.

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Conflict of Interest. There are no conflicts of interest to declare.

REFERENCES

(1) Rasmussen, S. E.; Frederiksen, H.; Struntze Krogholm, K.; Poulsen, L. *Mol. Nutr. Food Res.* **2005**, *49* (2), 159–174.
(2) Xu, Y.; Simon, J. E.; Welch, C.; Wightman, J. D.; Ferruzzi, M. G.; Ho, L.; Passinetti, G. M.; Wu, Q. *J. Agric. Food Chem.* **2011**, *59* (19), 10586–10593.
(3) Han, X.; Shen, T.; Lou, H. *Int. J. Mol. Sci.* **2007**, *8* (9), 950–988.
(4) Dorenkott, M. R.; Griffin, L. E.; Goodrich, K. M.; Thompson-Witrick, K. A.; Fundaro, G.; Ye, L.; Stevens, J. R.; Ali, M.; O'Keefe, S. F.; Hulver, M. W.; Neilson, A. P. *J. Agric. Food Chem.* **2014**, *62* (10), 2216–2227.
(5) Crozier, A.; Del Rio, D.; Clifford, M. N. *Mol. Aspects Med.* **2010**, *31* (6), 446–467.
(6) Requena, T.; Monagas, M.; Pozo-Bayón, M. A.; Martín-Álvarez, P. J.; Bartolomé, B.; del Campo, R.; Ávila, M.; Martínez-Cuesta, M. C.; Peláez, C.; Moreno-Arribas, M. V. *Trends Food Sci. Technol.* **2010**, *21* (7), 332–344.
(7) Neilson, A. P.; O'Keefe, S. F.; Bolling, B. W. *Annu. Rev. Food Sci. Technol.* **2016**, *7* (1), 43–64.
(8) Suwannaphet, W.; Meeprom, A.; Yibchok-Anun, S.; Adisakwattana, S. *Food Chem. Toxicol.* **2010**, *48* (7), 1853–1857.

(9) Gourineni, V.; Shay, N. F.; Chung, S.; Sandhu, A. K.; Gu, L. *J. Agric. Food Chem.* **2012**, *60* (31), 7674–7681.
(10) Meeprom, A.; Sompong, W.; Suwannaphet, W.; Yibchok-anun, S.; Adisakwattana, S. *Br. J. Nutr.* **2011**, *106* (08), 1173–1181.
(11) Goodrich, K. M.; Fundaro, G.; Griffin, L. E.; Hulver, M. W.; Ponder, M. A.; Neilson, A. P. *Nutr. Res.* **2012**, *32* (10), 787–794.
(12) Zhang, S.; Mcmillan, R. P.; Hulver, M. W.; Siegel, P. B.; Sumners, L. H.; Zhang, W.; Cline, M. A.; Gilbert, E. R. *Int. J. Obes.* **2014**, *38* (10), 1374–1382.
(13) Gao, S.; McMillan, R. P.; Zhu, Q.; Lopaschuk, G. D.; Hulver, M. W.; Butler, A. A. *Mol. Metab.* **2015**, *4* (4), 310–324.
(14) Casanova, E.; Baselga-Escudero, L.; Ribas-Latre, A.; Cedó, L.; Arola-Arnal, A.; Pinent, M.; Bladé, C.; Arola, L.; Salvadó, M. J. *J. Nutr. Biochem.* **2014**, *25* (10), 1003–1010.
(15) Most, J.; Goossens, G. H.; Jocken, J. W. E.; Blaak, E. E. *Int. J. Obes.* **2014**, *38* (5), 698–706.
(16) Timmers, S.; Konings, E.; Bilet, L.; Houtkooper, R. H.; van de Weijer, T.; Goossens, G. H.; Hoeks, J.; van der Krieken, S.; Ryu, D.; Kersten, S.; Moonen-Kornips, E.; Hesselink, M. K. C.; Kunz, I.; Schrauwen-Hinderling, V. B.; Blaak, E. E.; Auwerx, J.; Schrauwen, P. *Cell Metab.* **2011**, *14* (5), 612–622.
(17) Liu, W.; Zhao, S.; Wang, J.; Shi, J.; Sun, Y.; Wang, W.; Ning, G.; Hong, J.; Liu, R. *Mol. Nutr. Food Res.* **2017**.
(18) Cardona, F.; Andrés-Lacueva, C.; Tulipani, S.; Tinahones, F. J.; Queipo-Ortuño, M. I. *J. Nutr. Biochem.* **2013**, *24* (8), 1415–1422.
(19) Selma, M. V.; Espín, J. C.; Tomás-Barberán, F. A. *J. Agric. Food Chem.* **2009**, *57* (15), 6485–6501.
(20) Etxeberria, U.; Fernández-Quintela, A.; Milagro, F. I.; Aguirre, L.; Martínez, J. A.; Portillo, M. P. *J. Agric. Food Chem.* **2013**, *61* (40), 9517–9533.
(21) Turnbaugh, P. J.; Ley, R. E.; Mahowald, M. A.; Magrini, V.; Mardis, E. R.; Gordon, J. I. *Nature* **2006**, *444* (7122), 1027–1031.
(22) Reagan-Shaw, S.; Nihal, M.; Ahmad, N. *FASEB J.* **2008**, *22* (3), 659–661.
(23) Caporaso, J. G.; Lauber, C. L.; Walters, W. A.; Berg-Lyons, D.; Lozupone, C. A.; Turnbaugh, P. J.; Fierer, N.; Knight, R. *Proc. Natl. Acad. Sci.* **2011**, *108* (Supplement 1), 4516–4522.
(24) Chis, I. C.; Ungureanu, M. I.; Marton, A.; Simedrea, R.; Muresan, A.; Postescu, I.-D.; Decea, N. *Diab. Vasc. Dis. Res.* **2009**, *6* (3), 200–204.
(25) Hayashi, H.; Sakamoto, M.; Benno, Y. *Microbiol. Immunol.* **2002**, *46* (8), 535–548.
(26) Andersson, A. F.; Lindberg, M.; Jakobsson, H.; Bäckhed, F.; Nyrén, P.; Engstrand, L. *PLOS ONE* **2008**, *3* (7), e2836.
(27) Ley, R. E.; Bäckhed, F.; Turnbaugh, P.; Lozupone, C. A.; Knight, R. D.; Gordon, J. I. *Proc. Natl. Acad. Sci. U. S. A.* **2005**, *102* (31), 11070–11075.
(28) Duncan, S. H.; Lobley, G. E.; Holtrop, G.; Ince, J.; Johnstone, A. M.; Louis, P.; Flint, H. J. *Int. J. Obes.* **2008**, *32* (11), 1720–1724.
(29) Ismail, N. A.; Ragab, S. H.; ElBaky, A. A.; Shoeib, A. R.; Alhosary, Y.; Fekry, D. *Arch. Med. Sci. AMS* **2011**, *7* (3), 501.
(30) Arora, T.; Sharma, R. *Nutr. Rev.* **2011**, *69* (2), 99–106.
(31) Spor, A.; Koren, O.; Ley, R. *Nat. Rev. Microbiol.* **2011**, *9* (4), 279–290.
(32) Chakraborti, C. K. *World J. Gastrointest. Pathophysiol.* **2015**, *6* (4), 110–119.

(33) Han, M.; Song, P.; Huang, C.; Rezaei, A.; Farrar, S.; Brown, M. A.; Ma, X. *Oncotarget* **2016**, *7* (49), 80313–80326.
(34) Frank, D. N.; Amand, A. L. S.; Feldman, R. A.; Boedeker, E. C.; Harpaz, N.; Pace, N. R. *Proc. Natl. Acad. Sci.* **2007**, *104* (34), 13780–13785.
(35) Booijink, C. C.; Zoetendal, E. G.; Kleerebezem, M.; De Vos, W. M. *Future Microbiol. Lond.* **2007**, *2* (3), 285–295.
(36) Goodrich, K. M.; Neilson, A. P. *J. Chromatogr. B* **2014**, *958*, 63–74.
(37) Goodrich, K. M.; Smithson, A. T.; Ickes, A. K.; Neilson, A. P. *J. Nutr. Biochem.* **2015**, *26* (10), 1007–1014.
(38) Choy, Y. Y.; Jaggers, G. K.; Oteiza, P. I.; Waterhouse, A. L. *J. Agric. Food Chem.* **2013**, *61* (1), 121–127.
(39) van Dorsten, F. A.; Peters, S.; Gross, G.; Gomez-Roldan, V.; Klinkenberg, M.; de Vos, R. C.; Vaughan, E. E.; van Duynhoven, J. P.; Possemiers, S.; van de Wiele, T.; Jacobs, D. M. *J. Agric. Food Chem.* **2012**, *60* (45), 11331–11342.
(40) Tabasco, R.; Sánchez-Patán, F.; Monagas, M.; Bartolomé, B.; Moreno-Arribas, M. V.; Peláez, C.; Requena, T. *Food Microbiol.* **2011**, *28* (7), 1345–1352.
(41) Harris, K.; Kassis, A.; Major, G.; Chou, C. J. *J. Obes.* **2012**, *2012*.
(42) Neyrinck, A. M.; Van Hée, V. F.; Bindels, L. B.; De Backer, F.; Cani, P. D.; Delzenne, N. M. *Br. J. Nutr.* **2013**, *109* (05), 802–809.
(43) Choy, Y. Y.; Quifer-Rada, P.; Holstege, D. M.; Frese, S. A.; Calvert, C. C.; Mills, D. A.; Lamuela-Raventos, R. M.; Waterhouse, A. L. *Food Funct.* **2014**, *5* (9), 2298–2308.
(44) Pajuelo, D.; Fernández-Iglesias, A.; Díaz, S.; Quesada, H.; Arola-Arnal, A.; Bladé, C.; Salvadó, J.; Arola, L. *J. Agric. Food Chem.* **2011**, *59* (15), 8491–8498.
(45) Calabrese, V.; Cornelius, C.; Trovato-Salinaro, A.; Cambria, M. T.; Locascio, M. S.; Rienzo, L. D.; Condorelli, D. F.; Mancuso, C.; De Lorenzo, A.; Calabrese, E. J. *Curr. Pharm. Des.* **2010**, *16* (7), 877–883.

Appendix E: Supplementary Data for Appendix D

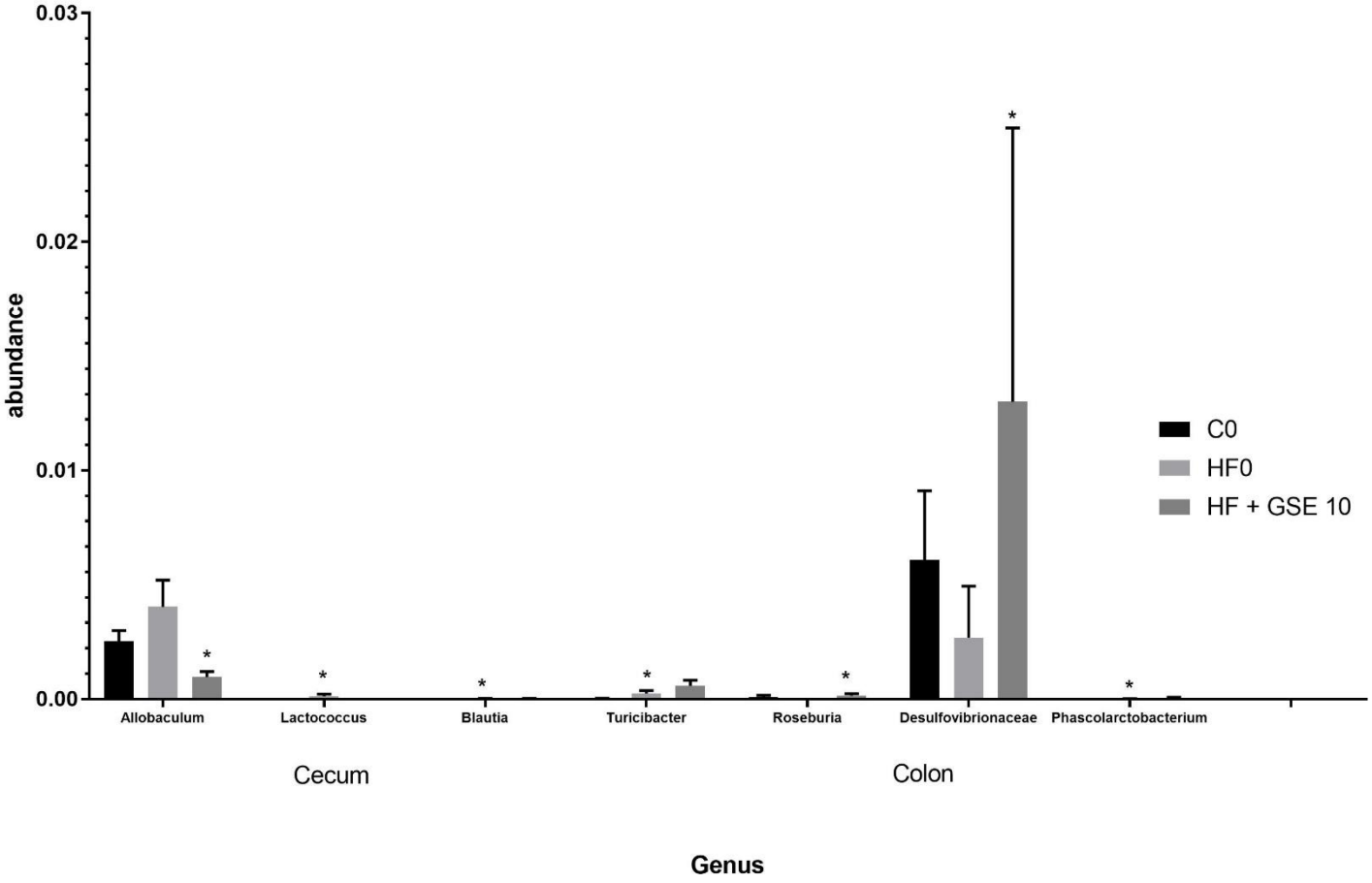

Figure E1. *Mean relative abundance (%) of select gut microbiota genera exhibiting significant differences between lean control, HF0, and HF + GSE10 treatment groups in cecum and colonic tissues. Values are expressed as means ± SEM. Treatments with an asterisk are significantly different ($P < 0.05$) based on Wilcoxon's signed rank test. Outliers were not removed from the dataset.*