Unlocking the health enhancing potential of grape marc through chemical and microbial analysis of its oligosaccharides and phenolic compounds

By

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ABSTRACT

Grape marc, or pomace, is the largest waste product of wine production with over 1.2 tons produced in the 2019 crush alone. The marc is the combination of grape skins, seeds, and stems removed from the juice with pressing. Although grape marc is currently underutilized primarily as compost and animal feed, initial studies identified its potential to act as a functional food ingredient to improve the gut microbiome and overall health. This thesis compiles the work of a multi-lab approach to identify the potentially bioactive oligosaccharides and phenolics in chardonnay marc and its individual components, how to isolate chardonnay marc oligosaccharides and phenolic compounds for individual analysis, and the potential bioactivity and health benefits of chardonnay marc.

Chapter 1 describes the chemical characterization of chardonnay marc, its seed and seedless fractions, a seed extract, and unripe chardonnay grapes. This included determining their gross compositions (i.e., protein, lignin, fat, carbohydrates, polysaccharides), phenolic contents, and oligosaccharide profiles. The gross compositions were as expected with the seeds containing more protein, fat, and polysaccharides than the seedless marc which had more carbohydrates and sugars. Phenolic compounds were abundant throughout the samples with the seed extract possessing the highest concentration. The individual phenolics varied with each fraction with most abundant phenolics being (-)-gallocatechin in the marc and seedless marc and (-)-epicatechin in the seeds and seed extract. Thirty-six distinct oligosaccharides with three to nine

degrees of polymerization and eleven distinct monosaccharide subunits were discovered within the chardonnay marc samples. Each fraction had a unique oligosaccharide profile however overlap occurred such as with some hexose and hexose-pentose oligosaccharides present in all samples.

Chardonnay marc oligosaccharide and polyphenol separation and purification is essential to assess their individual potential health benefits. Chapter 2 evaluates six separation methodologies for chardonnay marc oligosaccharide and phenolics purification: C18 and PGC SPE, C18 and PGC SPE with dialysis, C18 and DPA-6S SPE, C18 and HLB SPE, PVPP absorption, and PVPP absorption and C18 SPE. Oligosaccharide purification with PVPP and C18 SPE produced the most distinct confirmable oligosaccharides through NanoChip QToF mass spectrometry and had minimal phenolic contamination. Applying C18 and HLB SPE enabled the confirmation of the largest oligosaccharides (DP 8) and had the lowest phenolic content. Chardonnay marc phenolics were best isolated by washing loaded C18 with 40% methanol and PVPP with 70% acetone. Using C18 with 40% methanol generated the highest yields of (-)-epigallocatechin gallate, vanillic acid, (-)-gallocatechin gallate, and (-)-epicatechin but almost no gallic acid. Desorbing phenolic compounds from PVPP with 70% acetone produced high yields of gallic acid, (-)-epigallocatechin, (-)-gallocatechin, and (+)-catechin. Further improvement to chardonnay marc oligosaccharide identification was made through pairing a Dionex HPIC system with Q Exactive HF-X hybrid quadrupole-Orbitrap MS. This new analysis identified three new oligosaccharides, increased isomer separation, and corrected three oligosaccharides confirmed using LC NanoChip QToF MS.

Several published studies have begun analyzing the potential beneficial health effects of consuming grape marc. Chapter 3 performs a literature review of these *in vitro*, GI tract

simulation, small animal, livestock, and human studies in addition to investigating the potential functionalities of isolated chardonnay marc oligosaccharides and phenolics. I*n vitro* studies illustrate grape marc's prebiotic potential as it increased the growth of several commensal *Bifidobacterium* and *Lactobacillus* strains and prevented the growth of pathogenic strains including some in the *Enterobacteriaceae* family. When livestock consumed grape marc, they experienced increased growth of commensal bacteria and decreased populations of pathogenic bacteria in their intestines. Additionally, the livestock had improved health and meat quality through decreased lipid and protein oxidation. Furthermore, mice and rats who consumed grape marc had increased gut microbiome complexity and decreased obesity related illnesses. A human clinical trial, however, did not find chardonnay seed phenolics to have cardioprotective effects. We performed initial *in vitro* analysis of isolated chardonnay marc oligosaccharides and phenolics and found the oligosaccharides acted as a carbon source for commensal bacteria but not pathogenic bacteria and that the phenolics suppressed Gram positive pathogen growth but not Gram negative pathogen growth.

LIST OF ABBREVIAITONS

1. Low-density lipoprotein: LDL
2. Very low-density lipoprotein: VLDL
3. Acetonitrile: ACN
4. Ethanol: EtOH
5. Methanol: MeOH
6. Trifluoracetic acid: TFA
7. Polymethylpentene: PMP

8. Ultra-performance liquid chromatography: UPLC
9. Diode array detector: DAD
10. High-performance liquid chromatography: HPLC
11. Electrospray ionization: ESI
12. Mass spectrometry: MS
13. Quadrupole time of flight mass spectrometry: QTOF-MS
14. Tandem mass spectrometry: MS/MS
15. Degree of polymerization: DP
16. Concept map: Cmap
17. Triple quadrupole mass spectrometry: QqQ-MS
18. Solid phase extraction: SPE
19. Porous graphitized carbon: PGC
20. Hydrophilic-lipophilic balance: HLB
21. Poly(vinylpolypyrrolidone): PVPP
22. Hexose: Hex
23. N-acetylhexosamine: HexNAc
24. Pentose: Pent
25. Hexuronic acid: HexA
26. Theoretical mass or mass to charge ratio: m/z
27. Lipopolysaccharide: LPS

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CHAPTER 1: A second life for wine grapes: discovering potentially bioactive oligosaccharides and phenolics in chardonnay marc and its processing fractions

*A reduced version of this chapter was published in *LWT- Journal of Food Science and Technology*[1]

Abstract

Chardonnay marc (pomace), an agricultural waste product, has demonstrated significant potential health benefits in previous studies. This study represents the first comprehensive chemical characterization of chardonnay marc, its seed and seedless fractions, a seed extract, and unripe chardonnay grapes to uncover the bioactive compounds inducing their observed health benefits. Chardonnay marc and its processing fractions' gross composition (i.e., protein, lignin, fat, carbohydrates, polysaccharides), phenolic contents, and oligosaccharide profiles were determined. The chardonnay seeds contained higher quantities of protein, fat, and polysaccharides than the seedless marc while the seedless marc contained more total carbohydrates and sugars. All samples had abundant phenolics with the seed extract being the most concentrated (34.72±0.13 mg/g). (-)-Gallocatechin was the most abundant phenolic in the marc (1.4905±0.0393 mg/g) and seedless marc (0.94±0.04 mg/g), and (-)-epicatechin was the most concentrated phenolic in the seeds (9.4093±0.1018 mg/g) and seed extract (14.22±0.09 mg/g). Thirty-six distinct oligosaccharides were discovered between the four samples with three to nine degrees of polymerization and eleven distinct monosaccharide subunits. Overlap existed between the samples' oligosaccharides with four of the same hexose and hexose-pentose

1 Sinrod, A. J. G., Li, X., Bhattacharya, M., Paviani, B., Wang, S. C., & Barile, D. (2021). A second life for wine grapes: discovering potentially bioactive oligosaccharides and phenolics in chardonnay marc and its processing fractions. LWT, 111192. https://doi.org/10.1016/j.lwt.2021.111192.

oligosaccharides present in all. Each sample, however, had a distinct oligosaccharide profile such as with eight oligosaccharides unique to the seed extract.

1. Introduction

Grapes are a leading agricultural commodity largely grown for wine production. The four largest wine producing countries are Italy, France, Spain, and the United States with 258 million hectoliters of wine produced worldwide in 2020 (International Organisation of Vine and Wine, 2020). In California alone, 4,115,000 tons of grapes were crushed in 2019 (California Department of Food and Agriculture, 2020). Wine grapes undergo two primary phases of maturation before harvest and wine production. The first growing phase is berry formation which ends when the berries enter veraison, the transition from the berry formation phase to the berry ripening phase. At veraison the grapes change color and begin expanding. The grapes continue to ripen, increasing their sugar content until they reach the desired sweetness and Brix. The grapes are then harvested and sent through a specific set of steps to produce wine. Broadly, grapes are harvested, destemmed, crushed, and macerated to release the juice. For white wines, the grapes are immediately pressed to separate the solids from the juice, which is then fermented. For red wines, the skins and seeds are fermented with the juice and pressed at a later stage. After pressing, both white and red wines are further fermented, clarified, matured, stabilized, and filtered before bottling.

Regrettably, wine production generates massive quantities of agricultural waste. Grape marc, also called grape pomace, is the primary coproduct of wine production, consisting of approximately 30% of the grapes' original weight (Boussetta, Lanoisellé, Bedel-Cloutour, & Vorobiev, 2009). Grape marc is composed of the skins, flesh, and seeds separated from the juice during pressing. Currently grape marc is largely underutilized through composting. Grape marc

is also used to feed livestock, produce grape seed oil, and fermented and distilled into alcoholic beverages like grappa. The marc, however, holds great potential for valorization. Both white and red grape marc contain large amounts of indigestible fiber, including polysaccharides and oligosaccharides, as well as phenolics, a class of antioxidants. Recent studies have investigated these compounds, including catechins and gallic acid, within chardonnay marc for their potential health benefits (Alvarez-Casas, Pájaro, Lores, & Garcia-Jares, 2016). Most of these studies center on extracting phenolic and antioxidant compounds, whereas relatively few investigated chardonnay marc's potential valorization and health benefits (Table 1).

Table 1. Consolidation of published literature on the analysis and valorization of chardonnay marc and its components.

Study Scope	Materials	References
Valorizing through dietary fiber or specific isolated polysaccharides	Whole marc, skins, and stems	González-Centeno et al., 2010; P. Lu & Hsieh, 2012; Moncalvo et al., 2016; Zietsman, Moore, Fangel, Willats, & Vivier, 2017
Analyzing polyphenols and antioxidants	Whole marc, skins, seeds, and seed extract	Alvarez-Casas, Pájaro, Lores, & Garcia-Jares, 2016; Y. Lu & Yeap Foo, 1999; Rodríguez Montealegre, Romero Peces, Chacón Vozmediano, Martínez Gascueña, & García Romero, 2006; Washington State University & Mj, 2015; Yeap Foo, Lu, & Wong, 1998
Developing novel methods for polyphenol extraction	Whole marc	Boussetta, Lanoisellé, et al., 2009; Boussetta, Lebovka, et al., 2009; Garrido et al., 2019
In vitro and *in vivo* small animal studies of potential health benefits	Whole marc, skin, seeds, stems, and leaves	Hogan et al., 2010; Kamiyama, Karasawa, Kishimoto, Tani, & Kondo, 2019; H. Kim et al., 2014; Parry et al., 2011; Seo et al., 2016, 2017, 2015
Incorporating marc into food products	Whole marc and skins	Marchiani, Bertolino, Belviso, et al., 2016; Marchiani, Bertolino, Ghirardello, McSweeney, & Zeppa, 2016

While a few grape marc products on the market incorporate whole grape marc into foods like chocolate bars, the majority are supplements featuring grape marc or seed extracts. Grape marc and seeds can be extracted in a myriad of ways to concentrate their abundant phenolic compounds. These extraction methods include solvent extraction with mixtures of ethanol or methanol in water, enzymatic extraction using xylanase, and ultrasound assisted extraction

(Chacar et al., 2018; Costa et al., 2019; Hervert-Hernández, Pintado, Rotger, & Goñi, 2009; Karamati Jabehdar, Mirzaei Aghjehgheshlagh, Navidshad, Mahdavi, & Staji, 2018; F. Lu, Liu, Zhou, Hu, & Zhang, 2019). Another possible extraction method is subcritical water extraction. Subcritical water extraction of phenolic compounds is a safe and environmentally friendly strategy for potential chardonnay marc valorization. During extraction, water is held at 100–374 °C and kept under pressure to keep it in the liquid state. These conditions decrease the water's dielectric constant to those of organic solvents traditionally used in phenolic compound extraction (Duba, Casazza, Mohamed, Perego, & Fiori, 2015), enabling phenolics extraction from chardonnay seeds with water. This could be potentially advantageous as the market for grape marc, seeds, and extracts as health supplements has expanded with growing research that shows their potential health benefits.

Much of the research investigating grape marc's health effects highlight the potential effects of grape marc on the gut microbiome. The gut microbiome consists of a complex collection of trillions of bacteria, both commensal and pathogenic, which colonize the intestines (Rodrigues Hoffmann, Proctor, Surette, & Suchodolski, 2016). These bacteria break down food components that are indigestible to humans into smaller metabolites which can often be absorbed by the intestines or otherwise utilized. Commensal bacteria also help prevent intestinal infection from pathogens, thus reducing the burden of severe disease and concomitant inflammation. Red grape marc phenolics and chardonnay seeds beneficially altered rodent gut microbiomes by promoting commensal bacteria growth, including *Bifidobacterium*, while inhibiting pathogenic bacteria such as *Clostridium sensu stricto* and *Enterococcus*, some of whose abundances were correlated with the anti-obesity effects of chardonnay seeds (Chacar et al., 2018; Seo, Kim, Jeong, Yokoyama, & Kim, 2017). Grape phenolic compounds also provide significant

cardioprotective effects in humans by decreasing oxidative stress, lipoprotein metabolism, and inflammatory markers (Zern et al., 2005). Studies also indicate that grape phenolics could be anti-inflammatory and thus help mitigate obesity and diabetes (Chacón et al., 2009). In hamster and mouse studies, chardonnay seed consumption prevented body weight and adipose tissue gain and lowered plasma low-density lipoprotein (LDL), very low-density lipoprotein (VLDL), and total cholesterol levels when fed high-fat diets (H. Kim et al., 2014, 2015; Seo et al., 2016).

Many studies on the health benefits of grape marc and seeds attribute their positive results to high phenolic concentrations, however, other bioactive compounds could have also potentially contributed. Oligosaccharides are indigestible carbohydrates that were recently identified in red grape seeds as well as in white and red wines (Bordiga, Montella, Travaglia, Arlorio, & Coïsson, 2019; Bordiga et al., 2012; Ducasse, Williams, Meudec, Cheynier, & Doco, 2010). In addition to having well documented prebiotic activity, oligosaccharides have anticancer, anti-obesity, anti-diabetes, and cardioprotective effects (Kapoor & Dharmesh, 2017; Kumar et al., 2009; Zhang, Cai, & Ma, 2015). Unfortunately, little is known about the presence, structure, or biological activity of oligosaccharides in grapes and wine coproducts.

Importantly, oligosaccharides and phenolic compounds dissolve under the same conditions during sample preparation as is evident when comparing Bordiga et al. (2019) and Johansen et al. (1996). Literature analysis revealed that many phenolics studies such as Chacar et al. (2018) used techniques that would have extracted oligosaccharides in addition to phenolics, hence the health effects thus far attributed solely to grape phenolics could have been confounded by the presence of oligosaccharides. Similarly, other studies including Zern et al. (2005) used grape marc or seeds to study the cardioprotective activities and other health benefits of phenolic compounds–yet such whole products certainly also contained the naturally occurring

oligosaccharides. Previous studies likely neglected oligosaccharides as a potential source of these health benefits because of the analytical challenges associated with oligosaccharide characterization in complex matrices such as grapes, the lack of commercial oligosaccharide standards, and the conspicuous absence of bioinformatics databases, none of which are hurdles for phenolic analysis.

Chardonnay represents the largest single variety of grape crushed in California (15.6% of total crush) resulting in approximately 192,600 tons of chardonnay marc generated in California alone for a single harvest (California Department of Food and Agriculture, 2020). The present study leveraged a dedicated analytical platform expressly assembled to study bioactive food components to comprehensively characterize the composition of unripe chardonnay grapes, chardonnay marc, and chardonnay marc's processing fractions with a focus on phenolics and oligosaccharides–to elucidate the presence of compounds that could benefit human health.

2. Materials and Methods

2.1. Materials

HPLC grade hexane, methanol, acetonitrile (ACN), ethanol (EtOH), and formic acid were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Nanopure water was generated from a Milli-Q system (Millipore, Bedford, MA, USA). Trifluoracetic acid (TFA), polymethylpentene (PMP), ammonia hydroxide (NH_4OH), ammonium acetate, and D-(+)-glucose were ordered from Sigma-Aldrich (St. Louis, MO, USA). Sulfuric acid and analytical grade gallic acid, (-)-gallocatechin, (-)-epigallocatechin, (+)-catechin, (-)-epigallocatechin gallate, (-)-epicatechin, (-)-gallocatechin gallate, (-)-epicatechin gallate, and (-)-catechin gallate

were obtained from Millipore Sigma (Burlington, MA, USA). Anthrone reagent was purchased from Alfa Aesar (Haverhill, MA, USA).

2.2. Sample processing

Sonomaceuticals, LLC (Santa Rosa, CA, USA) provided 0.5 kg of 3 commercial samples: chardonnay marc powder (marc), partially defatted seed powder (seeds), and seed extract powder obtained through subcritical water extraction and spray drying (seed extract). The marc underwent infrared and convection drying and was subsequently milled using a standard grain mill. The seeds were convection dried in a Bühler Aeroglide dryer (Bühler, Uzwil, Switzerland), cold pressed to remove oil, and milled at a copacker. These commercial samples were stored at ambient temperatures and protected from light and oxygen before analysis. To analyze the seed and seedless marc fractions, Sonomaceuticals, LLC provided 2 gallons of fresh chardonnay marc from which the seeds and seedless fractions were manually separated (dubbed seeds and seedless marc, respectively). Sonomaceuticals, LLC also supplied several bunches of unripe chardonnay grapes harvested at veraison which were pureed. The seeds were then manually removed from the puree. The fresh marc and unripe grapes were frozen prior to transport to the laboratory for analysis (Davis, CA, USA). The unripe grape pulp, seeds, and seedless marc fractions were freeze-dried in a VirTis Ultra 25EL freeze dryer (SP Scientific, Gardiner, NY, USA) and milled with a Bodum 11160-294US-3 coffee grinder (New York City, NY, USA). Fat removal was performed as described in subsection 2.3. Unripe grapes, chardonnay marc, chardonnay marc fractions, and seed extract were prepared for analysis as illustrated in Figure 1.

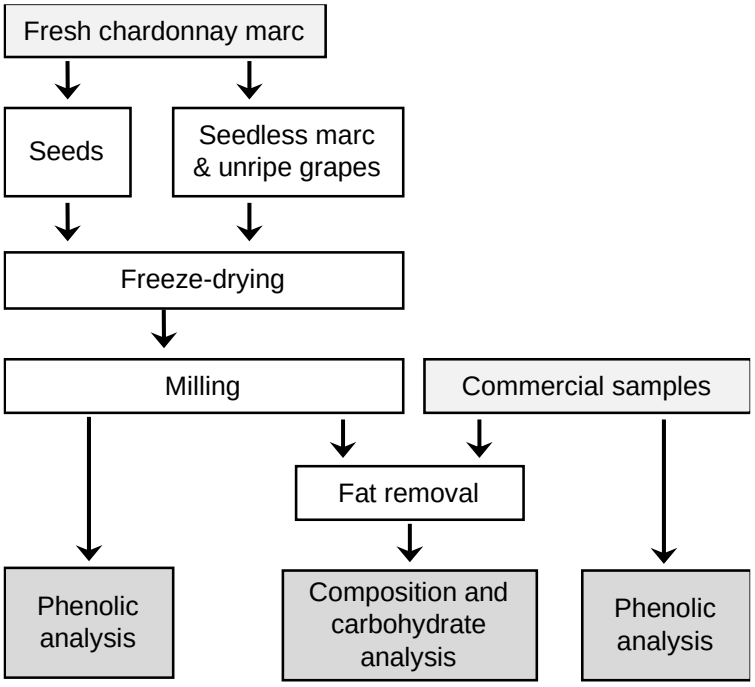

Figure 1. Sample processing flow diagram. The commercial samples were chardonnay marc, partially defatted chardonnay seeds, and chardonnay seed extract.

Either the commercial partially defatted seeds or the freeze-dried seeds were used to represent the seed fraction in each analysis. The results were mathematically adjusted for the partially defatted seeds to have the initial fat content of the freeze-dried seeds and to be by dry weight. Thus, the partially defatted seeds and freeze-dried seeds were deemed equal representations of chardonnay seeds.

2.3. Compositional analyses

To determine the marc's seed content, 3 aliquots were removed from the well-mixed fresh marc provided. The marc was thawed, and the seeds, skins, and debris were manually separated. The debris included all non-skin or seed components (stems, insects, etc.). The

percentages of the components were determined (w/w) and adjusted for their moisture contents. The debris was discarded.

For ash content, the unripe grapes, marc, marc fractions, and seed extract were heated overnight in a 550 °C Thermolyne™ Benchtop Muffle Furnace (ThermoFisher Scientific, Waltham, MA, USA) (Yan et al., 2016) and subsequently cooled to 100 °C in the oven before coming to room temperature in a desiccator. Ash content was calculated gravimetrically in triplicate.

Moisture content was assessed by drying the unripe grapes, chardonnay marc, marc components, and seed extract in triplicate at 80 °C for 5 days in a muffle oven (Thakur, Saharan, & Gupta, 2010) before cooling to room temperature in a desiccator. Moisture content was determined gravimetrically and was used to calculate composition values by dry weight.

The unripe chardonnay grapes, marc, partially defatted seeds, seedless and seed fractions, and seed extract were defatted using a Soxhlet device with Spectrum Chemical cellulose extraction thimbles (New Brunswick, NJ, USA) and hexane for 4 h. Residual hexane was evaporated from the thimbles at room temperature overnight and then in a 40 °C muffle oven for 2 h. Fat content was measured once and calculated gravimetrically due to limited sample quantities.

Total Kjeldahl nitrogen was determined via diffusion-conductivity of samples oxidized with sulfuric acid and a digestion catalyst as described by Horneck (1998) and Isaac et al. (1976). Lignin and cellulose were found using AOAC 973.18 where they were isolated through hot acidified detergent washing. The cellulose was hydrolyzed with sulfuric acid and the remaining lignin was quantified by ashing. The cellulose was calculated by weight difference (AOAC, 1997). Hemicellulose isolated with hot neutral detergent washes and enzymes was quantified

using the methods described above for cellulose according to AOAC 2002-04 (AOAC, 2006). Starch was hydrolyzed with amyloglcosidase and the resulting glucose was measured with a Perkin Elmer Series 200 Quaternary HPLC (Waltham, WA, USA) fitted with a Sciex API 2000 mass spectrometer (Redwood City, CA, USA) with APCI- negative ion settings and an 8:1 split ratio post column. A Phenomenex Luna NH2 (250 mm x 4.6mm) HPLC column was used at 35 °C with a mobile phase of 78% ACN:H_2O at 2.75 mL/min (Smith, 1969). Glucose, fructose, and sucrose were extracted with hot deionized water and quantified using the HPLC method described for starch (Johansen et al., 1996). The partially defatted seeds represented the seed fraction for protein, starch, glucose, fructose, and sucrose analyses. Protein and lignin were determined in duplicate for all samples except the seeds. The protein and lignin contents for the seeds were measured once due to limited sample quantities. Cellulose and hemicellulose were analyzed once, while starch, glucose, fructose, and sucrose were determined in duplicate for the seedless marc and seed extract and once for the seeds due to the limited amounts of material available. The seed extract was unable to be analyzed for cellulose and hemicellulose as it was water soluble and therefore incompatible with the analysis methods.

Total carbohydrate content was measured using an Anthrone reagent reaction based on principles described in Ludwig et al. (1956) and Yemm et al. (1954). Unripe grapes, marc, partially defatted seeds, seedless marc, and seed extract were homogenized in nanopure water and diluted. These mixtures were hydrolyzed in a microplate using 98% sulfuric acid at 100 °C for 15 min with intermittent mixing. Anthrone reagent in 98% sulfuric acid (4 mg/mL) was added to the microplate wells to obtain a ratio of 1:16 sample to Anthrone reagent (w/w) and a final concentration of 88.6% sulfuric acid. The mixture was shaken and incubated at 100 °C for 10 min. The absorbances were measured at 620 nm with a SpectroMax M5 UV/Vis

spectrophotometer (Molecular Devices, San Jose, CA, USA). Values were calculated using a glucose standard curve. Three replicates were prepared, and each replicate was run in duplicate.

2.4. Quantification of phenolic compounds

Phenolic compounds were extracted by sonicating unripe grapes, chardonnay marc, seedless and seed marc components, and seed extract in 50% MeOH:H_2O in a 1:50 ratio by weight for 1 h at room temperature in a Lyman Turbo Sonic 6000 (Middletown, CT, USA) at 35 kHz. An aliquot of the supernatant was filtered through a 0.45 μm Fisher Brand Nylon membrane (Waltham, MA, USA) prior to ultra-performance liquid chromatography (UPLC) injection (5 μL). Phenolic compound separation and quantification was achieved on an Agilent Zorbax Eclipse Plus C18 Rapid Resolution HD (3 x 100 mm, 1.8 micron) installed on an Agilent 1290 UPLC coupled with a diode array detector (DAD) (Santa Clara, CA, USA) based on a method modified from Ji, Li, and Li (2015). The mobile phase consisted of solvent A (0.2% formic acid in nanopure water) and solvent B (50% ACN:MeOH), with the gradient 95% to 65% A at 0-30 min; 65% to 60% A at 30-40 min; and 60% to 95% at A 40-42 min. The flow rate was 0.5 mL/min. Each phenolic's concentration was determined by peak area at 280 nm and individual calibration curves. The individual phenolics were summed to obtain total phenolic content.

2.5. Oligosaccharide analysis

To extract oligosaccharides, 15 mg defatted unripe grapes, chardonnay marc, partially defatted seeds, seedless marc, and seed extract were each combined with 500 μL 85% EtOH:H_2O (v/v), incubated at 90 °C with constant mixing for 10 min, and centrifuged. The extraction was

performed three times, combining the supernatants. The extract was dried in a Genevac™ miVac Centrifugal Concentrator (Ipswich, UK).

The extract was reconstituted with nanopure water and purified through a Supelco Discovery® DSC-18 SPE Tube (Bellefonte, PA, USA) conditioned with ACN and nanopure water. The oligosaccharides were eluted with nanopure water and further purified with a Supelclean™ ENVI-Carb™ SPE Tube (Bellefonte, PA, USA) conditioned with nanopure water and 80% ACN:H_2O with 0.1% TFA (v/v). The cartridges were washed with nanopure water, and the oligosaccharides were eluted with 40% ACN:H_2O with 0.1% TFA (v/v) and dried with vacuum centrifugation. The oligosaccharides were reconstituted with nanopure water and filtered through a 0.22 μm membrane prior to compositional analysis with an Agilent 6520 NanoChip LC-QToF mass spectrometer (Santa Clara, CA, USA).

A microfluidic high-performance liquid chromatography (HPLC)-Chip with enrichment (4 mm, 40 nL) and analytical (75 μL X 43 mm) columns packed with 5 μm 250 Å porous graphitized carbon and a nanoelectrospray tip separated the oligosaccharides by molecular weight using binary solvent gradients of solvent A (3% ACN:H_2O with 0.1% formic acid (v/v)) and solvent B (90% ACN:H_2O with 0.1% formic acid (v/v)). The gradient was 0 to16% B at 2.5–20 min, 16–40% B at 20–30 min, 40–100% B at 30–40 min, 100% B at 40–50 min, and 100–0% B from 50–55 min, followed by a 10 min re-equilibration of 100% A (Bhattacharya, Salcedo, Robinson, Henrick, & Barile, 2019).

Data were acquired in positive ionization mode with a 450–2500 mass/charge (m/z) range. The electrospray capillary voltage was 1800–1900 V. Continuous internal calibration was performed using *m/z* 922.009 and 1221.991 reference masses (ESI-TOF Tuning Mix G1969–85000, Agilent Technologies, Santa Clara, CA, USA). Spectra were manually examined, and

molecular formulas were confirmed using Agilent MassHunter Qualitative Analysis B.07.00 software with the molecular feature extraction and an error of 20 ppm. All samples were prepared and analyzed in duplicate, and oligosaccharide compositions were confirmed with tandem mass spectrometry (MS). The tandem fragmented peaks were selected with the automated precursor selection setting with a threshold of 200 ion counts and 5 ion counts for MS and MS/MS, respectively. A ramped collision energy of slope 1.3 and an offset of -3.6 V were used. The isolation width was medium for tandem MS and the acquisition rate was set to 1 spectra/s (Bhattacharya et al., 2019).

Following extraction and purification methods outlined in subsection 2.5, seedless chardonnay marc oligosaccharides' monosaccharide subunits were identified and quantified by Triple Quadrupole mass spectrometry according to methods in Amicucci et al. (2018). Briefly, the oligosaccharides underwent complete acid hydrolysis with TFA, which was quenched with cold water. The resulting monosaccharides were combined with NH_4OH and PMP in methanol, incubated at 70 °C, and dried by vacuum centrifugation. The monosaccharides were reconstituted with water and excess PMP was removed with two extractions of chloroform-water. Monosaccharides were separated on an Agilent C18 column and analyzed using an Agilent 1290 Infinity II UHPLC coupled with an Agilent 6495 QqQ mass spectrometer (Santa Clara, CA, USA). Solvent A was 25 mM ammonium acetate in 5% ACN:H_2O (v/v) adjusted with NH_4OH to pH 8.2. Solvent B was 95% ACN:H_2O (v/v). The mass spectrometer was in positive ion mode and the dynamic multiple reaction monitoring mode enabled data acquisition. Additional settings used are described in Xu et al. (2017). Data were analyzed with Agilent Mass Hunter B.08.

2.7. Statistical analysis

One-way analysis (ANOVA) and Tukey's multiple comparison test (95% confidence) was used with Minitab® software version 19.2020.10 (Minitab, LLC, State College, PA, USA).

3. Results and Discussion

3.1. Composition of chardonnay unripe grapes, marc, marc fractions, and seed extract

Chardonnay seeds contained higher amounts of protein, fat, and polysaccharides than seedless chardonnay marc (Tables 2-3). These macromolecules support the growth of the seed's embryo into a new vine to fulfill the seed's biological function. The seeds also had more lignin than the seedless marc. As grapes mature, the seed coat undergoes lignification to increase its hardness and strength for protection (Cadot, Miñana-Castelló, & Chevalier, 2006). Lignin gives seeds their hard, woody texture, which greatly contrasts with the soft physical properties of grape skin and flesh that contain little lignin. Additionally, the seedless chardonnay marc had a significantly (P<0.05) higher total carbohydrate content than the seeds (Table 4). This correlates

Table 7. Composition of unripe chardonnay grapes, chardonnay marc, its seed and seedless fractions, and seed extract by dry weight. The marc and seed extract's lignin contents were not measured as their particle size was too small for successful analysis.

	Fat (mg/g dw)	**Ash** (mg/g dw)	**Protein** (mg/g dw)	**Lignin** (mg/g dw)
Unripe grapes	27.42	51.3 ± 6.7	8.8 ± 0.3	75.0 ± 14.3
Marc	86.78	46.5 ± 0.3	16.0 ± 1.4	
Seedless marc	24.90	37.8 ± 4.2	10.8 ± 0.1	51.7 ± 0.8
Seeds	148.16	44.5 ± 3.7	23.4	364
Seed extract	9.06	40.7 ± 2.7	12.2 ± 0.1	

Table 17. Total carbohydrates content of chardonnay unripe grapes, marc, seedless marc, seeds, and seed extract by dry weight. The marc consisted of 50±5% (dw) seeds and 5±1% (dw) debris that was removed from each individual seed and seedless fraction, lowering the marc's total carbohydrates content. Superscripts indicate statistical differences ($P<0.05$).

	Total Carbohydrates (mg/g dw)
Unripe grapes	460 ± 27^{c}
Marc	456 ± 34^{c}
Seedless marc	695 ± 54^{a}
Seeds	538 ± 17^{b}
Seed extract*	705 ± 24^{a}

Table 16. Non-oligosaccharide carbohydrate composition of the unripe grapes, seedless marc, seeds, and seed extract by dry weight.

	Cellulose (mg/g dw)	**Hemicellulose** (mg/g dw)	**Starch** (mg/g dw)	**Free Glucose** (mg/g dw)	**Free Fructose** (mg/g dw)	**Sucrose** (mg/g dw)
Unripe grapes	80.6 ± 1.6	38.6±0.8	23.0 ± 5.5	105.3 ± 3.2	64.9 ± 4.8	<2.2 ± 0.0
Seedless marc	86.0	26.1	8.71 ± 4.62	276 ± 6	298 ± 7	<2.2 ± 0.0
Seeds	60.6	78.6	13.0	23.1	29.6	30.6
Seed extract			18.0 ± 0.0	61.5 ± 0.0	76.3 ± 1.5	13.8 ± 0.0

with the seedless marc's higher concentrations of glucose, fructose, and sucrose (Table 3). Chardonnay flesh produces the berry's sugars. Although most of these sugars are removed with the juice during pressing, some remain in and on the flesh and skin. Additional carbohydrates like pectin are also present in grape skins (Deytieux-Belleau, Vallet, Donèche, & Geny, 2008). These sugars and additional carbohydrates contributed to the seedless marc's high total carbohydrate content despite having less cellulose and hemicellulose than the seeds (Tables 3-4).

The degree of berry ripening greatly affects the gross composition of the chardonnay grape as can be seen in comparing the seedless unripe chardonnay berries and the seedless marc. The unripe grapes had higher fat, ash, and lignin contents and a lower protein content than the seedless marc (Table 2). The most interesting differences, however, were in the carbohydrate compositions of the samples. The unripe grapes contained more hemicellulose and almost three times more starch than the seedless marc (Table 3). Meanwhile the seedless marc had about

twice as much glucose and over four times as much fructose than the unripe grapes (Table 3). The unripe berries were harvested at veraison which is when the grapes enter the ripening phase and begin accumulating sugars. Therefore, the sugars had not yet developed in the unripe grapes as they had in the fully ripened chardonnay grapes which were used to create the seedless marc. The unripe grapes had produced so little glucose and fructose that despite containing the pulp and juice along with the skins, the unripe grapes still only had a fraction of the sugar content of the seedless marc which did not include the sugar rich grape juice that was removed during pressing. Instead, the unripe grapes have more polysaccharides like hemicellulose and starch which are important to the structure of the developing grapes.

Unfortunately, it was not possible to obtain an accurate measurement of the seed extract's total carbohydrate content (Table 4). When combined with the seed extract, the Anthrone reagent used in the assay generated a dark brown color instead of the intended blue green that was observed in all other samples, causing increased absorbance readings and therefore an overestimated total carbohydrate content. Anthrone reagent assays can experience interference from non-carbohydrate compounds. For instance, furfural, a non-carbohydrate pentose degradation product that forms a brown precipitate in Anthrone reagent assays (Dreywood, 1946), is produced during subcritical water extraction of grape seeds (Prado et al., 2014). Furfural is likely the cause of the assay's brown discoloration for the seed extract.

3.2. Phenolic analysis of chardonnay marc, its fractions, and seed extract

Phenolics were extracted with 50% MeOH, separated with C18, and individually quantified (Table 5) with UPLC-DAD and summed to obtain the total phenolics content (Figure 2). The majority of chardonnay marc's phenolics stem from its seed fraction. While the seeds made up 50±5% dw of the marc, they contained more than seven times the phenolics concentration of the seedless marc (Figure 2). The marc's seedless component counteracted the seeds' high phenolics concentration to decrease the marc's total phenolics.

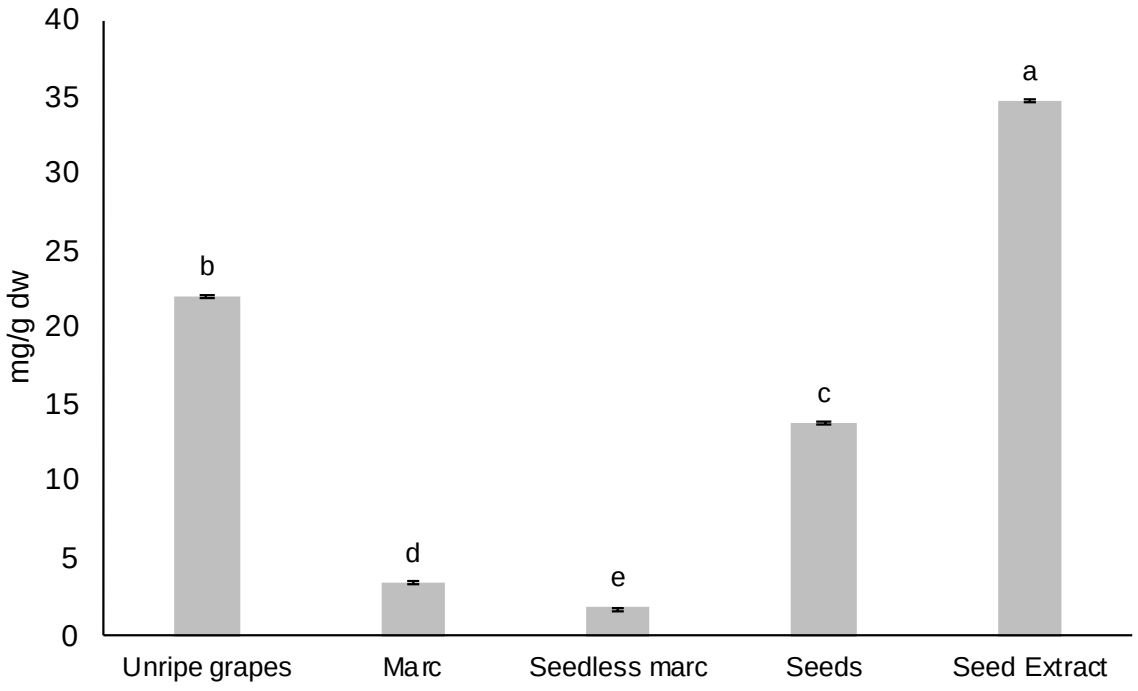

Figure 6. Total phenolics in unripe chardonnay grapes, chardonnay marc, seedless marc, seeds, and seed extract obtained from the sum of their individual phenolic contents. All samples were analyzed with an Agilent Zorbax Eclipse Plus C18 Rapid Resolution HD column (3 x 100 mm, 1.8 micron) and an Agilent 1290 UPLC-DAD in triplicate. Statistical differences (P<0.05) are indicated by different letters over the bars.

Table 19. Concentrations of individual phenolic compounds in unripe chardonnay grapes, chardonnay marc, seedless marc, seeds, and seed extract. The individual phenolics are classified as follows: gallic acid and vanillic acid are phenolic acids; (+)-catechin, (-)-epicatechin, (-)-epigallocatechin gallate, (-)-gallocatechin gallate, (-)-epicatechin gallate, (-)-epigallocatechin, and (-)-gallocatechin are flavan-3-ols; trans-resveratrol and trans-polydatin are stilbenes. All samples were analyzed in triplicate with an Agilent Zorbax Eclipse Plus C18 Rapid Resolution HD column (3 x 100 mm, 1.8 micron) and an Agilent 1290 UPLC. Superscripts indicate statistical differences (P<0.05) between the samples within each phenolic compound.

	Unripe grapes (mg/g dw)	**Marc** (mg/g dw)	**Seedless marc** (mg/g dw)	**Seeds** (mg/g dw)	**Seed extract** (mg/g dw)
Gallic acid	0.1010 ± 0.0025	0.1160 ± 0.0018^{b}	0.0690 ± 0.0005^{b}	0.1236 ± 0.0019^{b}	3.6444 ± 0.0566^{a}
Vanillic acid	0.1548 ± 0.0017	0.0154 ± 0.0017^{b}	0.0418 ± 0.0014^{b}	0.1545 ± 0.0023^{a}	0.1651 ± 0.0020^{a}
(-)-Epigallocatechin	0.994 ± 0.021	0.2497 ± 0.0042^{c}	0.3848 ± 0.0040^{b}	0.4263 ± 0.0088^{b}	2.2585 ± 0.0227^{a}
(+)-Catechin	6.405 ± 0.050	0.6712 ± 0.0116^{c}	0.2854 ± 0.0011^{d}	3.3851 ± 0.0450^{b}	12.5858 ± 0.1103^{a}
(-)-Epigallocatechin gallate	0.1666 ± 0.016	0.0242 ± 0.0002^{a}	0.02233 ± 0.00002^{a}	0.0558 ± 0.0010^{a}	0.1189 ± 0.0022^{a}
(-)-Gallocatechin	2.126 ± 0.037	1.4905 ± 0.0393^{a}	0.9413 ± 0.0399^{b}	ND	ND
(-)-Epicatechin	11.147 ± 0.041	0.8878 ± 0.0287^{c}	ND	9.4093 ± 0.1018^{b}	14.2227 ± 0.0937^{a}
(-)-Epicatechin gallate	0.6484 ± 0.0030	0.0277 ± 0.0012^{b}	ND	0.0569 ± 0.0017^{b}	0.6125 ± 0.0057^{a}
(-)-Gallocatechin gallate	0.1386 ± 0.0022	ND	ND	0.0994 ± 0.0026^{a}	0.1018 ± 0.0030^{a}
(-)-Catechin gallate	0.0819 ± 0.0032	ND	ND	0.0832 ± 0.0030^{b}	0.9590 ± 0.0087^{a}
Trans-polydatin	ND	ND	ND	0.0064 ± 0.0004^{a}	0.0390 ± 0.0009^{a}
Trans-resveratrol	ND	0.0263 ± 0.0005^{a}	0.0984 ± 0.0008^{a}	0.0063 ± 0.0003^{a}	0.0118 ± 0.0002^{a}

The bulk of the seeds' phenolics are cross-linked with carbohydrates and proteins in the seed coat as a defense mechanism (Cadot et al., 2006). It is possible that the subcritical water extraction released these phenolics. Chardonnay seed extract had over 2.5 and 9.9 times the total phenolics content of the seeds and marc, respectively. (Figure 2). The subcritical water extraction dissolved the phenolics into the water phase, separating them from the seed solids. The extract was removed from the solids and dried, giving the extract its high total phenolic content as it does not contain the insoluble seed components. Chardonnay seed extract had more than 90% and approximately 40% the phenolics content of cocoa powder and green tea by dry weight, respectively, two food products marketed for their high phenolic concentrations and correlated health benefits (Genovese & Lannes, 2009). Chardonnay seed extract is therefore a remarkably rich source of dietary phenolic compounds.

Grape marc and its fractions contained a wide array of phenolic compounds with high levels of flavan-3-ols, a subclass of flavonoids (Table 5). Both the seedless marc and seeds contained gallic acid, trans-resveratrol, vanillic acid, (-)-epigallocatechin, (+)-catechin, and (-)-epigallocatechin gallate. Similar to previous findings (González-Manzano, Rivas-Gonzalo, & Santos-Buelga, 2004; Pantelić et al., 2016), (-)-gallocatechin was only present in the seedless marc and marc and was their most prominent phenolic compound. (-)-Gallocatechin has been shown to increase the expression of the TPH1, DDC, AANAT, and ASMTL genes, improving natural melatonin levels and mitigating sleep disorders (US10646466B2, 2017).

(-)-Epicatechin, (-)-epicatechin gallate, (-)-gallocatechin gallate, (-)-catechin gallate, and trans-polydatin were present in the seeds but not in the seedless marc. Moderate concentrations of (-)-epigallocatechin gallate were found in all samples (Table 5). (-)-Epicatechin was the most concentrated phenolic compound in the seeds and the second most abundant in the marc. Notably, among the phenols present in the seeds and not in the seedless marc, only (-)-epicatechin and (-)-epicatechin gallate were found in the chardonnay marc. The absence of (-)-gallocatechin gallate, (-)-catechin gallate, and trans-polydatin in the marc could be partially due to their relatively low concentrations within the seeds being reduced by the seedless fraction, thus decreasing these phenolics to non-detectible concentrations.

Catechin concentrations in chardonnay marc measured in this study (Table 5) were much higher than published concentrations of catechins in green tea, a food product celebrated for its high flavan-3-ol content and related health benefits. Chardonnay seeds contain over twice as much (-)-epicatechin as green tea by dry weight (Khokhar & Magnusdottir, 2002). (-)-Epicatechin has numerous health benefits including improving vascular function and preventing some brain disorders (Alañón et al., 2020; Bernatova, 2018). Chardonnay seeds also contained

over 3 mg/g dry weight of (+)-catechin while most brands of green tea analyzed did not detect any (+)-catechin (Khokhar & Magnusdottir, 2002), a catechin with anticancer and neuroprotective effects (J. S. Kim, Kim, O, & Jeon, 2010; Shimizu et al., 2008). Grape marc and its processing fractions, therefore, hold great potential as functional food ingredients to improve human health.

Although chardonnay seeds and seed extract had the same phenolic compounds, their concentrations differed dramatically (Table 5). Similar to results obtained in a previous study (Prieur, Rigaud, Cheynier, & Moutounet, 1994), chardonnay seeds contained highly polymerized as well as monomer, dimer, trimer, and oligomer phenolics. In grape seed extract, the three most abundant phenolics, gallic acid, (+)-catechin, and (-)-epicatechin, were monomers. Subcritical water extraction increased these monomers' concentrations disproportionately to the other phenolic compounds in chardonnay seeds. For example, gallic acid's concentration in the seeds rose over 2800% when measured in the seed extract. Hydrolysis of larger phenolic compounds within the grape seeds induced by the subcritical water extraction likely led to the production of smaller phenolics (García-Marino, Rivas-Gonzalo, Ibáñez, & García-Moreno, 2006). Better phenolics' bioavailability and health benefits can be expected as small phenols are better absorbed through the paracellular route of the human intestine than large phenolics (Deprez, Mila, Huneau, Tome, & Scalbert, 2001).

Several larger phenolic compounds also increased disproportionately with subcritical water extraction. For example, (-)-catechin gallate's concentration surge of over 1050% (Table 5). The high temperatures used in subcritical water extraction and drying may have promoted phenolic polymerization to generate increased quantities of dimer, trimer, and oligomer phenolic

compounds (Ioannone et al., 2015). Extraction of phenolics due to different solubilities was also affected by subcritical water extraction.

In addition to marc fraction and extraction technique, grape maturity also has a large impact on the concentrations of phenolic compounds in chardonnay grapes. The unripe grapes contained more total phenolics than chardonnay seeds which had the highest concentration of phenolics of the marc and marc fractions (Figure 2). In particular, the unripe grapes contained about ten times as much (+)-catechin as the mature seedless marc and similar amounts of (-)-catechin gallate as the seeds while none was detected in the seedless marc (Table 5). The unripe grapes also had higher amounts of (-)-epicatechin than the seeds whereas the seedless marc had no detectable (-)-epicatechin (Table 5). Grapes increase their total phenolics content and particularly their concentrations of catechin, epicatechin, and catechin gallate during berry formation, reaching peak concentrations at veraison. The concentrations of these phenolic compounds then rapidly decline with ripening and reach their lowest concentrations when the grapes are ready to be harvested (Lee & Jaworksi, 1989). The unripe chardonnay berries were harvested at veraison and thus were at a developmental stage with higher phenolic contents than the seedless marc derived from mature chardonnay grapes (Table 5). To produce grape marc with higher phenolic concentrations for functional food products, marc could be collected from the production of wines made from the least ripe grapes possible to produce marc with maximum possible phenolics for that particular varietal.

3.3. Oligosaccharide profiles of chardonnay marc, its fractions, and seed extract

Mass spectrometry analysis enabled the discovery and identification of 36 distinct naturally occurring oligosaccharides between the chardonnay marc, its fractions, and seed

extract. A grape oligosaccharide chromatogram is illustrated in Figure 3. The single stage MS provided the oligosaccharides' accurate molecular weight (Table 6) and tandem MS/MS enabled confirmation of each oligosaccharide's composition, mass, and degree of polymerization (DP) (Tables 6-7, Figure 4). Based on peak intensity, the oligosaccharides were broadly quantified as "abundant" or "trace" (Figure 4). An inherent limitation of studying intact oligosaccharides by

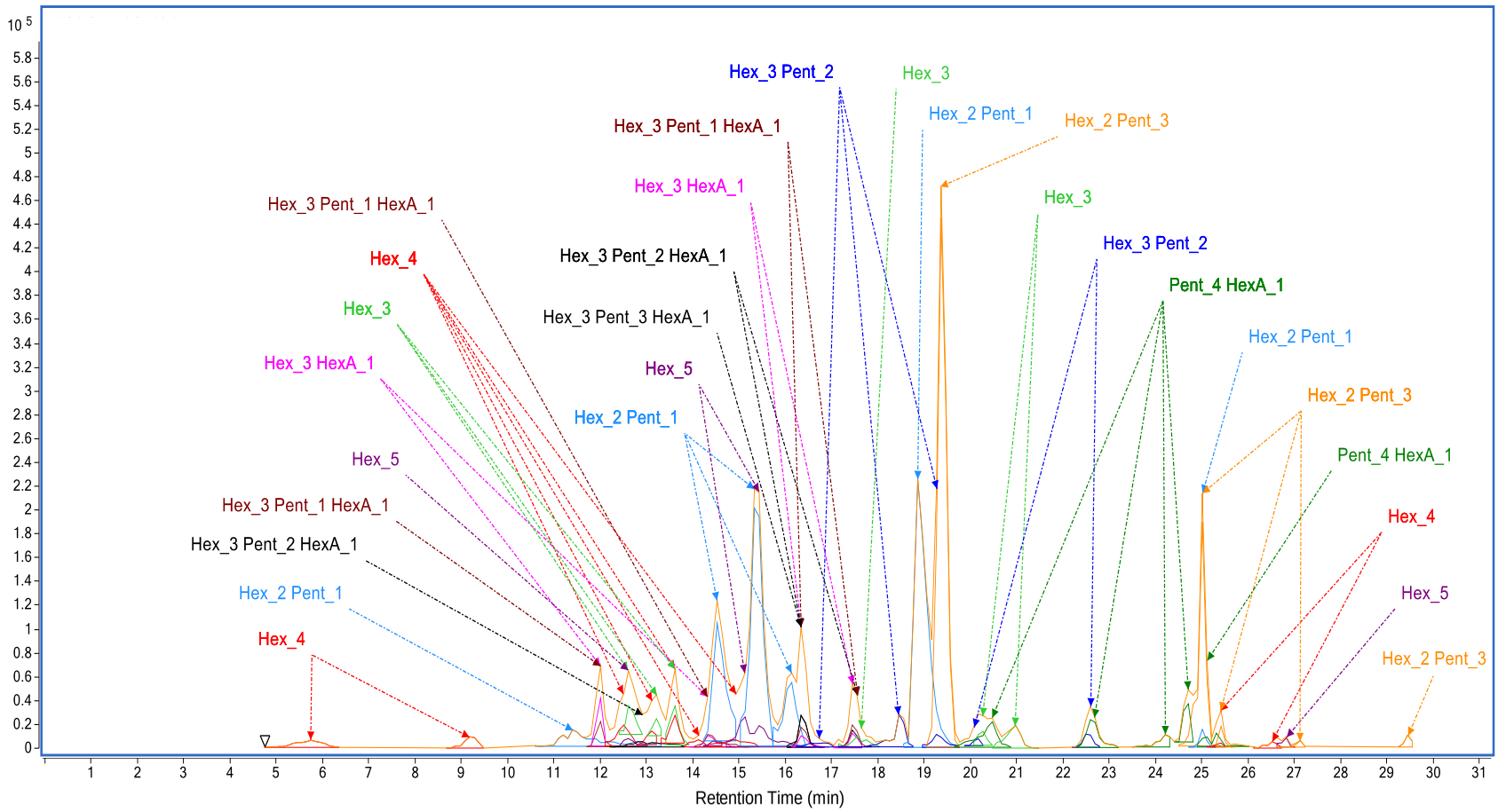

Figure 10. Example of Extracted Compound Chromatogram (ECC) for grape oligosaccharides obtained during MS oligosaccharide analysis with data obtained with an Agilent 6520 NanoChip LC-QToF mass spectrometer and processed with Agilent MassHunter Qualitative Analysis B.07.00 software. This chromatogram was produced from one of the chardonnay marc replicates.

Table 21. Compositions, mass/charge (m/z), charge, and theoretical mass of the 36 oligosaccharides confirmed by tandem MS/MS in at least one of the chardonnay unripe grapes, marc, seed and seedless fractions, and seed extract. Each sample was analyzed with an Agilent 6520 NanoChip LC-QToF mass spectrometer.

	m/z	z	mass	Hex	Pent	HexNAc	HexA
Hex_2 Pent_1	475.1658	1	474.1585	2	1		
Hex_3	505.1763	1	504.1691	3			
Hex_2 HexA_1	519.1556	1	518.1486	2			1
Hex_2 HexNAc_1	546.2029	1	545.1957	2		1	
Hex_1 HexNAc_1 HexA_1	560.1822	1	559.1752	1		1	1
Hex_2 Pent_2	607.2081	1	606.2011	2	2		
Hex_1 Pent_2 HexA_1	621.1874	1	620.1804	1	2		1
HexNAc_3	628.2561	1	627.2488			3	
Hex_3 Pent_1	637.2186	1	636.2116	3	1		
Hex_4	667.2291	1	666.222	4			
Hex_2 HexNAc_1 Pent_1	678.2452	1	677.2382	2	1	1	
Hex_3 HexA_1	681.2084	1	680.2011	3			1
Hex_3 HexNAc_1	708.2557	1	707.2485	3		1	
Pent_4 HexA_1	723.2192	1	722.2119		4		1
Hex_2 Pent_3	739.2504	1	738.2431	2	3		
Hex_1 HexNAc_2 HexA_1	763.2616	1	762.2546	1		2	1
Hex_3 Pent_2	769.2609	1	768.2536	3	2		
Hex_4 Pent_1	799.2714	1	798.2644	4	1		
Hex_3 Pent_1 HexA_1	813.2507	1	812.2434	3	1		1
Hex_5	829.2819	1	828.2748	5			
Hex_3 HexNAc_1 Pent_1	840.298	1	839.291	3	1	1	
Pent_5 HexA_1	855.2615	1	854.2545		5		1
Hex_3 HexA_2	857.2405	1	856.2335	3			2
Hex_4 HexNAc_1	870.3085	1	869.3014	4		1	
Hex_2 Pent_3 HexA_1	915.2825	1	914.2755	2	3		1
Hex_4 Pent_2	931.3137	1	930.3067	4	2		
Hex_3 Pent_2 HexA_1	945.293	1	944.2857	3	2		1
Hex_5 Pent_1	961.3242	1	960.3172	5	1		
Hex_6	991.3347	1	990.3277	6			
Hex_5 HexNAc_1	516.1807	2	1031.354	5		1	
Hex_3 Pent_3 HexA_1	1077.335	1	1076.328	3	3		1
Hex_7	1153.388	1	1152.38	7			
Hex_4 HexA_3	1195.325	1	1194.318	4			3
Hex_8	1315.44	1	1314.433	8			
HexNAc_4 Pent_1 HexA_2	1315.442	1	1314.435		1	4	2
Hex_9	738.7466	2	1476.486	9			

mass spectrometry is that their monosaccharide epimers/anomer building blocks of identical

molecular weights are indistinguishable to the detection method. Therefore, the broader category of monosaccharide subunits is conventionally determined instead of specific monosaccharides. For example, the Hex oligosaccharide subunit in this analysis could correspond to glucose or galactose. A separate approach was applied to unravel the identity of the specific monosaccharides after total oligosaccharide hydrolysis (see subsection 3.4).

Table 28. Number of oligosaccharides identified for each degree of polymerization (DP) and total number of oligosaccharides confirmed in unripe chardonnay grapes, chardonnay marc and each processing fraction (seedless marc, seeds, seed extract). Analysis was performed in duplicate with an Agilent 6520 NanoChip LC-QToF mass spectrometer.

	DP3	DP4	DP5	DP6	DP7	DP8	DP9	Total
Unripe grapes	2	4	4	3				13
Marc	3	3	5	3	1			15
Seedless marc	4	4	4	6	1			19
Seeds	4	4	4	2	1	1		16
Seed extract	3	6	5	1	3	1	1	20

Oligosaccharide composition varied between the chardonnay marc components. We used a free online Concept maps tool (Cmap Tools, Pensacola, FL, USA) developed by The Florida Institute for Human & Machine Cognition to illustrate oligosaccharide composition diversity and overlap between samples (Figure 4). The Cmap in Figure 4 displays how the oligosaccharides in the marc, seedless and seed fractions, and seed extract relate to each other through common and divergent oligosaccharides to facilitate visual interpretation and identification of their oligosaccharide patterns. Eleven oligosaccharides were present in both the seedless marc and seeds, which suggests these oligosaccharides are important in multiple types of grape cells. The most abundant of these were hexose-pentose oligosaccharides, which were potentially generated from arabinogalactan polysaccharides in grape cell walls (Moore, Fangel, Willats, & Vivier, 2014). Bifidobacteria have been found to ferment arabinogalactans, like the hexose-pentose oligosaccharides found, making these oligosaccharides prebiotic (Van Laere, Hartemink,

Bosveld, Schols, & Voragen, 2000). The seedless marc and seed oligosaccharide profiles differentiated with five oligosaccharides present in the seeds but not in the seedless marc, and nine oligosaccharides in the seedless marc but not in the seeds (Figure 4). Distinct grape structures requiring different oligosaccharides for signaling and other functions could cause this variation (Tran Thanh Van et al., 1985). The seedless marc and seeds' distinctive

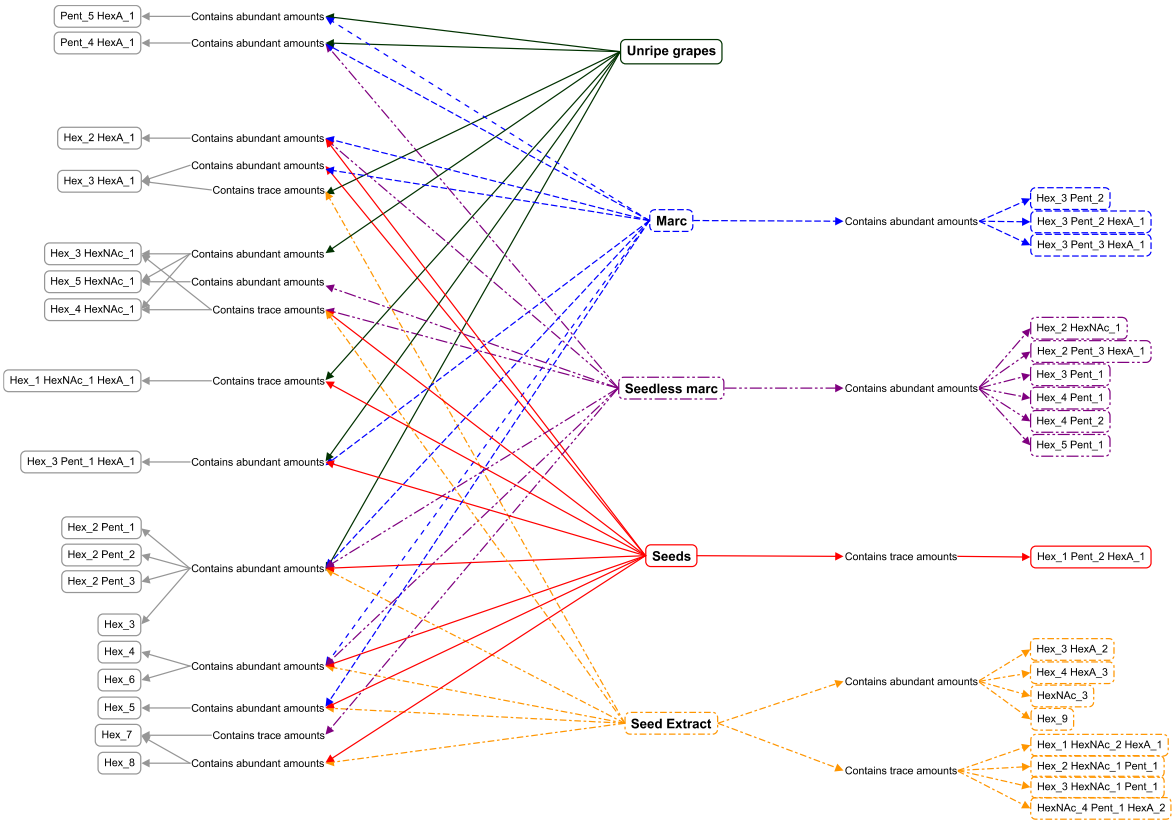

Figure 11. Visualization of oligosaccharides and their abundances in unripe chardonnay grapes, chardonnay marc, seedless marc, seeds, and seed extract to indicate relative oligosaccharide abundance and oligosaccharide overlap between samples. Oligosaccharides' compositions and abundances were obtained from an Agilent 6520 NanoChip LC-QToF mass spectrometer. Abundant oligosaccharides had peak intensities at or above 10,100 cps (counts per second) while trace oligosaccharides had peak intensities below 10,100 cps. The visualization was made using CmapTools software which can be downloaded at https://cmap.ihmc.us/products/.

oligosaccharides could also be from their differing polysaccharide profiles (Table 3). These polysaccharides could have degraded into the unique oligosaccharides in each marc component such as starch into hexose oligosaccharides and pectin into uronic acid oligosaccharides.

Many of the oligosaccharides present in the seedless chardonnay marc and seeds were also found in the marc (Figure 4), thus indicating each component's oligosaccharide contribution. However, some of the seed and seedless marc components' oligosaccharides were not in the marc. These missing oligosaccharides are primarily present in trace amounts and only in the seed or seedless fraction, not both. Potentially, having both the seed and seedless fractions in the marc reduced these oligosaccharides below detectable levels. Additionally, the marc was exposed to heat during drying while the seedless marc was freeze-dried. High temperatures can break oligosaccharides' glycosidic bonds, degrading them into smaller oligosaccharides and monosaccharides. The oligosaccharides present only within the seedless marc could have depolymerized during the chardonnay marc's thermal drying process, thus removing them from the marc. Interestingly, three oligosaccharides were found only in the chardonnay marc. A partial break-down of chardonnay skin and flesh polysaccharides during high temperature drying could have generated these oligosaccharides as food polysaccharides can depolymerize at temperatures as low as 75 °C (Lai, Lii, Hung, & Lu, 2000).

Berry maturity also affects chardonnay oligosaccharides. The unripe chardonnay grapes contained the fewest confirmable oligosaccharides with the smallest DP diversity as the largest unripe grape oligosaccharides had only six monosaccharide subunits (Table 7). While the unripe berries contained the Hex_2 Pent_1, Hex_2 Pent_2, Hex_2 Pent_3, and Hex_3 oligosaccharides that were present in all the other fractions, they did not have the larger hexose oligosaccharides that were common amongst the other fractions (Figure 4). Fruit produce enzymes like β-1,4-D-

glucanase and xyloglucan endotransglycosylase during ripening which degrade their cell wall polysaccharides and produce oligosaccharides (Lahaye, Quemener, Causse, & Seymour, 2012; Redgwell & Fry, 1993). Grapes likely generate similar enzymes which increasingly break down the grape polysaccharides as they ripen. This is likely the cause of the seedless marc having a higher number of confirmable oligosaccharides as well as larger, more diverse oligosaccharides than the seedless unripe chardonnay grapes as it had undergone berry ripening and produced these enzymes whereas the unripe grapes had not.

The chardonnay seed and seed extract's oligosaccharide profiles differed significantly from each other. Ten of the oligosaccharides in the chardonnay seeds were also present in the seed extract, five oligosaccharides in the seeds were not found in the extract, and nine oligosaccharides in the extract were not in the seeds (Figure 4). However, eight of the seed extract's oligosaccharides were not present in any of the other samples. These distinct oligosaccharides were relatively large in size with DPs up to nine and mostly contained multiple classes of monosaccharides. Subcritical water extraction hydrolyzes carbohydrates, breaking them into smaller carbohydrates (Carvalheiro, Esteves, Parajó, Pereira, & Girio, 2003). The extraction therefore likely hydrolyzed some of the seeds' naturally occurring oligosaccharides into smaller oligosaccharides or sugars, eliminating those oligosaccharides' presence. Likewise, the subcritical water extraction probably hydrolyzed the seeds' polysaccharides to generate the unique oligosaccharides identified in the extract.

3.4. Seedless marc oligosaccharides' subunit composition and quantification

The oligosaccharides isolated from seedless chardonnay marc were hydrolyzed into their monosaccharide building blocks and quantified using QqQ mass spectrometry (Figure 5). This analysis is complementary to the compositional oligosaccharide profiles discussed in subsection 3.3 and identified the specific monosaccharides present in each group. The seedless chardonnay marc oligosaccharides were composed of 11 distinct monosaccharide building blocks. This large number of monosaccharide subunits signifies that the oligosaccharides had great structural diversity. For comparison, human milk oligosaccharides are composed of just five discrete monosaccharides and yet are considered the gold standard for prebiotics because of their

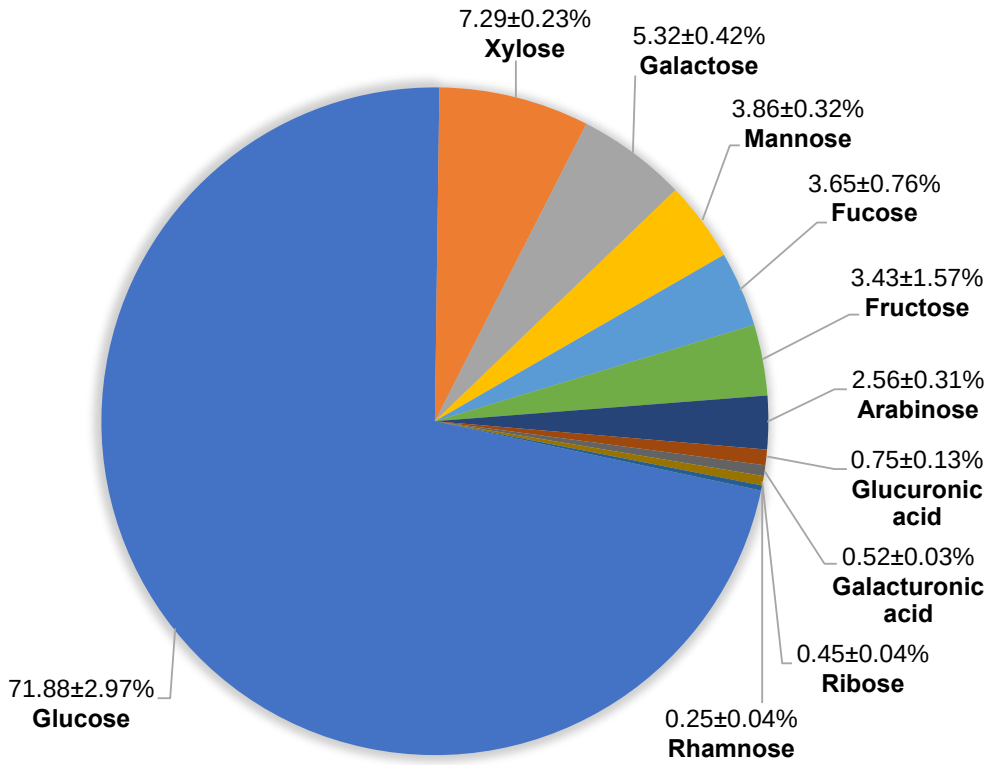

Figure 15. Quantification of the monosaccharide subunits that compose seedless chardonnay marc oligosaccharides. The monosaccharides were analyzed with an Agilent 1290 Infinity II UHPLC coupled with an Agilent 6495 QqQ mass spectrometer.

abundant diversity (Wu, Tao, German, Grimm, & Lebrilla, 2010). It is therefore plausible to infer that chardonnay marc oligosaccharides, likely being even more diverse than human milk oligosaccharides based on their monosaccharide subunits, could potentially deliver unique bacterial selectivity to act as prebiotics. Previous grape marc studies whose materials and extracts could have contained naturally occurring oligosaccharides should be reinterpreted to potentially credit their results and observed health benefits to the oligosaccharides in addition to the marc's phenolic compounds. Furthermore, future studies should fractionate the oligosaccharides and phenolics to separately analyze their individual effects.

The seedless marc oligosaccharides were composed of 81% hexose monosaccharides with glucose making up the majority (Figure 5). These results are in contrast to those of Bordiga et al. (2019) who found that red grape seed oligosaccharides were primarily composed of arabinose followed by galactose and glucose. These opposing results could stem from varietal differences as Bordiga et al. (2019) used red grape marc while chardonnay is a white grape varietal. Another potential cause could be the differences in marc processing for red and white wines. White grape marc is removed with pressing immediately after crushing while red grape marc undergoes maceration and fermentation with the grape juice before pressing. The maceration and fermentation could have altered the oligosaccharide profile of the red grape seeds used in Bordiga et al. (2019), resulting in a different oligosaccharide composition than found in chardonnay seeds.

4. Conclusions

Chardonnay marc and its seedless and seed fractions contained high concentrations of phenolic compounds, mainly gallic acid and flavan-3-ols, as well as many diverse

oligosaccharides. This study showed that individual phenolics and oligosaccharides differed between marc's seedless and seed components with phenolics like (-)-gallocatechin in the seedless marc and not the seeds and (-)-epicatechin in the seeds and not the seedless marc as well as having only nine of their 23 combined oligosaccharides overlap. Furthermore, processing chardonnay seeds with subcritical water extraction generated a potentially more bioactive functional food ingredient as the extract had 1.5 times the total phenolics content of the seeds and nine oligosaccharides that were not found in the seeds. Chardonnay grape ripening also greatly affects the gross composition, phenolic content, and oligosaccharide profiles of the berries. With the discovery of a multitude of oligosaccharides within chardonnay marc, further studies are needed to determine if the oligosaccharides contribute to grape marc's health benefits separately and synergistically with other endogenous bioactive compounds such as phenolics.

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CHAPTER 2: Uncovering analytical and larger scale methodologies to separate and isolate oligosaccharides and phenolics from chardonnay marc

*The research for this chapter was done in collaboration with Bruna Paviani, Mrittika Bhattacharya, PhD, Xueqi Li, MSc, Mara Baller, and Selina Wang, PhD

Abstract

In vitro and small animal studies indicate that consuming grape marc could produce extensive health benefits. However, the specific compounds inducing these observed health benefits are not known. Grape marc contains abundant phenolic compounds and diverse oligosaccharides, both of which could have bioactive properties. The separation and purification of oligosaccharides and phenolics from grape marc is essential for investigating their potential health benefits individually. Six solid phase extraction and polymeric sorbent methodologies were tested to purify chardonnay marc oligosaccharides. Combining PVPP absorption of phenolics with C18 SPE enabled the purification of the highest number of individual oligosaccharides from Chardonnay grape marc, as confirmed through LC NanoChip QToF mass spectrometry (MS), with moderate oligosaccharide yield and minimal remaining phenolic compounds. A combination of C18 followed by HLB SPE resulted in a product containing 22 confirmed oligosaccharides, which included oligosaccharides with the highest degrees of polymerization and the lowest phenolic content. This method also produced the greatest oligosaccharide yield of 60.46±11.05 mg/g. To purify phenolic compounds from chardonnay marc, multiple solvent elution gradients were tested with C18 SPE. Eluting phenolics from C18 with 40% methanol produced the highest yields of vanillic acid, (-)-epigallocatechin gallate, (-)-epicatechin, and (-)-gallocatechin gallate but very little gallic acid. Desorbing phenolics from PVPP with 70% acetone was also successful in purifying chardonnay marc phenolics and generated high yields of gallic acid, (-)-gallocatechin, (-)-epigallocatechin, and (+)-catechin.

1. Introduction

Grape marc, also called grape pomace, is the solid residue remaining after grape crushing and pressing in winemaking and constitutes the largest byproduct of wine production. The marc consists of the grape skins, seeds, and a small amount of stems that are removed from the juice immediately after crushing for white wine and after the initial fermentation for red wine. California vineyards crushed 3.411 million tons of wine grapes during the 2020 harvest, approximately 30% of which was lost as marc (Boussetta, Lanoisellé, Bedel-Cloutour, & Vorobiev, 2009; California Department of Food and Agriculture, 2021). Chardonnay represented 15.2% of the wine grapes in the 2020 California harvest, making it the largest single varietal grown in California and thus the largest producer of marc (California Department of Food and Agriculture, 2021).

While grape marc is largely underutilized in low-value applications such as compost and animal feed, however recent scientific studies are beginning to recognize grape marc's potential to improve human health. Select strains of commensal gut bacteria including *B. bifidum*, *B. breve*, *L. acidophilus*, and *L. plantarum* had increased growth when exposed *in vitro* to grape marc extract and pre-fermented puree, indicating that the marc itself potentially has prebiotic properties (Campanella et al., 2017; Karamati Jabehdar, Mirzaei Aghjehgheshlagh, Navidshad, Mahdavi, & Staji, 2018). Additionally, grape marc decreased Caco-2 intestinal cell inflammation and oxidative damage induced by exposure to lipopolysaccharide and hydrogen peroxide, respectively, when combined with probiotic strains in *in vitro* tests (Campanella et al., 2017; Pistol, Marin, Dragomir, & Taranu, 2018). Small animal studies similarly indicated grape marc's potential prebiotic abilities with increased *Bifidobacterium* growth and decreased *Clostridia* abundance in rat feces with grape marc diet supplementation (Chacar et al., 2018). Furthermore, mice fed high fat diets supplemented with chardonnay seeds demonstrated decreased LDL levels,

body weight, adipose tissue weight, and liver weight (Seo, Kim, Jeong, Yokoyama, & Kim, 2017).

Recent studies demonstrated that grape marc contains oligosaccharides in addition to phenolic compounds (Bordiga, Montella, Travaglia, Arlorio, & Coïsson, 2019; Sinrod et al., 2021), both of which could contribute the observed health benefits indicated in *in vitro* and small animal studies. Phenolics are antioxidant compounds that possess cardioprotective, anti-cancer, and neuroprotective effects (Luo et al., 2017; Narita, Hisamoto, Okuda, & Takeda, 2011; Perdicaro et al., 2017). Chardonnay marc boasts a wide variety of phenolic compounds including gallic acid, (-)-epicatechin, (-)-epicatechin-gallate, (+)-catechin, (-)-gallocatechin, caftaric acid, procyanidins, quercetins, and trans-resveratrol (Alvarez-Casas, Pájaro, Lores, & Garcia-Jares, 2016; Rodríguez Montealegre, Romero Peces, Chacón Vozmediano, Martínez Gascueña, & García Romero, 2006; Sinrod et al., 2021). Meanwhile grape marc also has a diverse array of oligosaccharides, carbohydrates of 3-16 monosaccharide subunits which can have prebiotic properties (Bordiga, Montella, et al., 2019). Oligosaccharides have been found in table grapes, white and red wines, and grape seeds (Blanch, Sanchez-Ballesta, Escribano, & Merodio, 2011; Bordiga, Montella, et al., 2019; Bordiga et al., 2012). In a previous study, we identified 24 distinct oligosaccharide compositions in chardonnay marc, skins, and seeds which were composed of 11 distinct monosaccharide building blocks (Sinrod et al., 2021).

Because the tools to perform a proper characterization of complex oligosaccharide structures were only recently developed, the observed health benefits from grape marc in *in vitro* and small animal studies have been largely attributed to the grape marc's abundant phenolic compounds and neglected the likely significant contribution of the naturally occurring oligosaccharides (Campanella et al., 2017; Chacar et al., 2018; Karamati Jabehdar et al., 2018;

Pistol et al., 2018; Seo et al., 2017). Additional research, such as the evaluation of prebiotic activity on commensal bacteria and kill studies of pathogenic bacteria, is needed for grape marc phenolics and oligosaccharides individually and in conjunction with each other to determine the actual impacts each contributes to the demonstrated potential health benefits of grape marc. However, for this analysis to occur, the grape marc oligosaccharides and phenolic compounds need to be successfully separated.

Currently, no published literature investigates the separation and recovery of oligosaccharides and phenolic compounds. Conventional separation techniques based on solubility and molecular mass cannot be used in separating oligosaccharides and phenolics as they have similar solubilities and molecular weights. Oligosaccharides are commonly purified at the lab scale via solid phase extraction (SPE) to decrease interferences in mass spectrometry (MS) analysis, which includes removing phenolic compounds. One method that is frequently used pairs Octadecylsilane (C18) and porous graphitized carbon (PGC) SPE where the C18 solid phase removes phenolics from the mixture. Another is combining Octylsilane (C8) and PGC SPE (Tian, Freeman, Corey, German, & Barile, 2017). However, the abilities of these purification methods to achieve complete phenolics removal are not detailed. Meanwhile, in addition to purifying oligosaccharides, C18 is also widely used to purify and analyze phenolic compounds (Bajkacz, Baranowska, Buszewski, Kowalski, & Ligor, 2018).

In the present study, multiple oligosaccharide and phenolic separation methods were tested to determine the best technique for generating isolated oligosaccharides and phenolics in high yields from a chardonnay marc extract for further functional testing. The separation matrices under investigation included C18 paired with PGC, hydrophilic-lipophilic balance (HLB), DPA-6S, and poly(vinylpolypyrrolidone) (PVPP).

2. Materials and Methods

2.1 Materials

This study used nanopure water produced by a Milli-Q system (Millipore, Bedford, MA, USDA). HPLC grade hexane, ethanol, acetonitrile (ACN), methanol, hydrochloric acid, and formic acid were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Analytical grade phenolic standards (gallic acid, vanillic acid, (+)-catechin, (-)-epicatechin, (-)-epigallocatechin gallate, (-)-gallocatechin gallate, (-)-epigallocatechin, and (-)-gallocatechin), D-(-)-Fructose, sucrose, poly(vinylpolypyrrolidone) (PVPP), 1 g 6 ml Supelco Discovery® DSC-18 SPE Tubes (C18), 0.5 g 6 ml Supelclean ENVI-Carb™ SPE Tubes (PGC), 0.5 g 6 ml Discovery(R) DPA-6S SPE Tubes (DPA-6S), and sulfuric acid were purchased from Millipore Sigma (Burlington, MA, USA) (Table 8). Acetone, ethyl acetate, D-(+)-glucose, stachyose hydrate, D-(+)-raffinose pentahydrate, and trifluoracetic acid (TFA) were ordered from Sigma-Aldrich (St. Louis, MO, USA). Anthrone reagent was acquired from Alfa Aesar (Haverhill, MA, USA). Xylosyl-Cellobiose was obtained from Megazyme (Bray, Ireland). 60 mg 3 ml Oasis® HLB Extraction Cartridges were purchased from Waters Corporation (Milford, MA, USA) (HLB) (Table 8).

Table 8. Description and cost of each SPE or polymeric sorbent used to separate oligosaccharides and phenolics. Prices are reported in US dollars for the amount needed to purify extract produced from 15 mg defatted chardonnay marc. Prices are accurate as of July 12, 2021.

Purification material	Cost per 15 mg chardonnay marc sample	Supplier
PVPP	$0.02	Millipore Sigma
DSC-18 SPE Tubes (1 g, 6ml)	$5.90	Millipore Sigma
ENVI-Carb SPE Tubes (0.5 g, 6 ml)	$5.73	Millipore Sigma
DPA-6S SPE Tubes (0.5 g, 6 ml)	$5.53	Millipore Sigma
Oasis® HLB Extraction Cartridges (60 mg, 3 ml)	$2.88	Waters

2.2 Extraction and standards preparation

Oligosaccharides and phenolics were extracted from commercial chardonnay marc flour provided by Sonomaceuticals, LLC (Santa Rosa, CA, USA). Fresh 2016 chardonnay marc was dried with infrared and convection drying and then milled with a standard grain mill to produce the chardonnay marc flour. The marc was held at ambient temperatures until its analysis.

A 4 h hexane Soxhlet with cellulose extraction thimbles (Spectrum Chemical, New Brunswick, NJ, USA) was used to defat the marc. The marc was dried overnight at room temperature followed by 2 h in a 40 °C muffle oven (Sinrod et al., 2021). 15 mg defatted chardonnay marc was extracted three times with 0.5 mL 85% ethanol in water (v/v) at 90 °C with constant shaking for 10 min. The mixture was centrifuged between each extraction, combining the supernatants (Sinrod et al., 2021). The extracts were dried in a Genevac™ miVac Centrifugal Concentrator (Ipswich, UK) and frozen until their purification.

A representative mixture of phenolic compounds previously identified in chardonnay marc and plant oligosaccharides were combined to create a standard mixture which was used to determine the phenolic content after each purification methodology. The standard mixture contained gallic acid, vanillic acid, (+)-catechin, (-)-epicatechin, (-)-epigallocatechin gallate, (-)-gallocatechin gallate, (-)-epigallocatechin, and (-)-gallocatechin based on their presence in chardonnay marc (Sinrod et al., 2021). As chardonnay marc oligosaccharide standards are not commercially available, three common plant oligosaccharides (raffinose, xylosyl-cellobiose, and stachyose) were combined in equal quantities in nanopure water to represent the chardonnay marc oligosaccharides. The concentrations of phenolic and oligosaccharide standards applied to each separation technique were chosen to mimic the respective concentrations of chardonnay marc phenolics and oligosaccharides.

2.3 Oligosaccharide and phenolic compound purification

The oligosaccharide and phenolics purification methods applied in this study are depicted in Figure 6. Every purification protocol was run in duplicate.

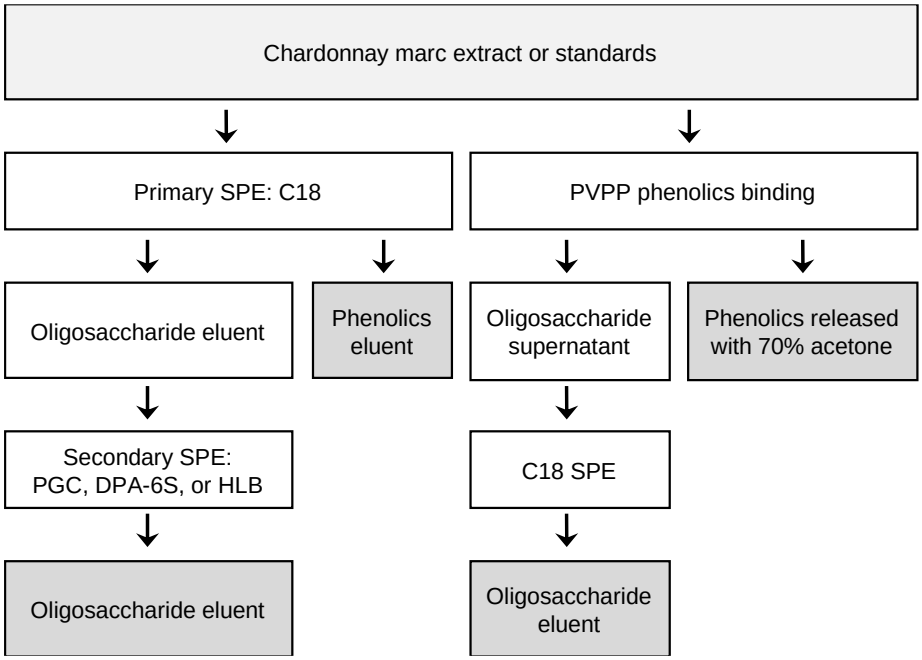

Figure 6. Sample purification flow diagram beginning after oligosaccharide and phenolic compound extraction from defatted chardonnay marc or for the oligosaccharide and phenolic standard mixture.

2.3.1 PVPP Pre-treatment

PVPP, an insoluble polymer that readily absorbs phenolic compounds (Ranatunge, Adikary, Dasanayake, Fernando, & Soysa, 2017), was activated in 12 M HCl at 100 °C for 30 min. The acid was removed, and the PVPP was washed until it reached a neutral pH and was then suspended in nanopure water. The chardonnay marc extract produced from 15 mg marc or an equivalent amount of the standard mixture described in section 2.2 was reconstituted with nanopure water and combined with the PVPP solution to have a total concentration of 5 mg PVPP/ml. The mixture was shaken at room temperature for 15 min, centrifuged, and separated. Additional PVPP was added to the supernatant (5mg PVPP/ml) and the process was repeated to

fully bind the available phenolics (Magalhães et al., 2010). The extract was passed through a 0.2 μm filter. The filtrate was dried in a centrifugal concentrator and frozen until further purification.

To remove phenolic compounds bound to the PVPP polymer, the resulting PVPP-phenolics residue was combined with 70% acetone in water (v/v). The mixture was sonicated for 15 min and then shaken at room temperature for 15 min to release the phenolics (Magalhães et al., 2010). The PVPP was removed via filtration (0.2 μm) and the filtrate was dried in a centrifugal concentrator.

2.3.2 Primary solid phase extraction

The samples produced from extracting 15 mg chardonnay marc were reconstituted with nanopure water and purified with 1 g, 6 ml Supelco Discovery® DSC-18 SPE Tubes (C18) (Burlington, MA, USA). The cartridges were conditioned with acetonitrile (ACN) and nanopure water before loading the sample. The oligosaccharides were eluted with 12 mL nanopure water divided into four washes. The oligosaccharide eluents were dried with a centrifugal concentrator and frozen.

To collect the phenolic compounds held in the DSC-18 cartridges after oligosaccharide elution, the cartridges were washed with a series of solvents: 40% methanol in water (v/v), 0.01 M HCl, ethyl acetate, and 66% acetone in water (v/v) (Pinelo, Laurie, & Waterhouse, 2006). This elution gradient was stopped for one set of samples after each solvent to determine the efficacy of each solvent. Thus, one set of phenolics loaded cartridges were eluted with only 40% methanol. Another set was washed with 40% methanol followed by 0.01 M HCl, and then ethyl acetate. And the final set was eluted with 40% methanol, 0.01 M HCl, ethyl acetate, and 66% acetone. Eluents not containing ethyl acetate or acetone were dried in a centrifugal concentrator while eluents produced with these solvents were dried under a nitrogen gas stream.

2.3.3 Secondary solid phase extraction

After C18, a second round of SPE was applied using PGC, DPA-6S, or HLB solid phases. For PGC, the extracts were reconstituted in nanopure water, and 0.5 g, 6 ml Supelclean ENVI-Carb™ SPE Tubes (PGC) (Burlington, MA, USA) were conditioned with nanopure water and 80% ACN in water with 0.1% TFA (v/v). The extract was loaded onto the cartridge and washed with 30 ml nanopure water. The oligosaccharides were eluted with 12 ml 40% ACN in water with 0.1% TFA (v/v). For DPA-6S, 0.5 g, 6 ml Discovery(R) DPA-6S SPE Tubes (Burlington, MA, USA) were washed with nanopure water, ACN, and 95% ACN in water (v/v). The extracts were dissolved in 95% ACN, loaded onto the cartridges, and washed with 95% ACN. The oligosaccharides were eluted with 24 mL 20% ACN in water (v/v) (Zhang, Li, Feng, Liu, & Liu, 2014). For HLB, the samples were reconstituted with nanopure water and acidified to pH 2 with 1 M HCl (Wang et al., 2014). 60 mg, 3 ml Oasis® HLB Extraction Cartridges (Waters Corporation, Milford, MA, USA) were conditioned with methanol and nanopure water before loading the samples. Oligosaccharides were eluted with 4 mL nanopure water (Yang, Hu, & Zhao, 2011). All SPE eluents were dried in a centrifugal concentrator.

2.4 Oligosaccharide Quantification

Oligosaccharide samples were dialyzed with 0.1-0.5 kD Float-A-Lyzer G2 Dialysis Device (Repligen, Waltham, MA, USA) to remove residual monosaccharides and disaccharides. The dialysis tubes were prepared according to the manufacturer's instructions by soaking them in 15% ethanol in water (v/v) followed by nanopure water (Repligen, 2020). Samples were reconstituted with nanopure water and dialyzed in a stirred water bath at 4 °C for 24 h with three water changes. The dialyzed extracts were dried in a centrifugal concentrator.

An Anthrone reagent (Alfa Aesar, Haverhill, MA, USA) total carbohydrate analysis was used to quantify the isolated oligosaccharides according to reaction principles detailed in Yemm

et al. (1954) and Ludwig et al. (1956). 40 µl oligosaccharides in nanopure water were combined with 100 µl Anthrone reagent in cold 98% sulfuric acid (2 mg/mL) and mixed through aspiration. The microplate was incubated for 3 min at 92 °C in a water bath followed by 5 min in a room temperature water bath and then for 15 min in a 45 °C Thermolyne™ Benchtop muffle furnace (Thermo Fisher Scientific, Waltham, MA, USA). The plate was cooled for 3 min before measuring the absorbance with a SpectroMax M5 UV/Vis spectrophotometer (Molecular Devices, San Jose, CA, USA) at 630 nm (Laurentin & Edwards, 2003). Oligosaccharide quantification calculations were based on a glucose standard curve. Each grape marc sample was prepared in duplicate, and each sample was further analyzed in duplicate.

Simple sugars (glucose, sucrose, and fructose) were quantified by high performance anion exchange chromatography with pulsed amperometric detection (Dionex ICS-5000 HPAE-PAD, Thermo Scientific, Sunnyvale, CA, USA) based on a method used in Lee et al., 2013. Samples were diluted and filtered through a 0.2 mm syringe filter (Acrodisc 13 mm PES, Pall Life Sciences, Port Washington, NY, USA) into 2 mL vials with septa. Calibration curves (coefficient of determination ≥ 0.999) were prepared using glucose, sucrose, and fructose standards (Sigma, St. Louis, MO, USA). The samples (25 µl) were injected into a CarboPac PA200 column (Dionex, Sunnyvale, CA, USA) and were run at a 0.5 mL min^{-1} flow rate. The solvent system consisted of 100% 200 mM sodium hydroxide (NaOH) for the first 15 min followed by a gradient transitioning from 0.6 to 25% 200 mM NaOH over 12.1 min.

2.5 Composition of Oligosaccharides by QToF-MS

Individual oligosaccharide compositions were analyzed with an Agilent 6520 NanoChip LC-QToF mass spectrometer (Santa Clara, CA, USA). Oligosaccharide separation was achieved with a microfluidic high-performance liquid chromatography (HPLC) chip containing 5 µm 250 Å porous graphitized carbon packed enrichment (4 mm, 40 nL) and analytical (75 µL x 43 mm)

columns as well as a nanoelectrospray tip, using a binary solvent gradient of solvent A (3% ACN in nanopure water with 0.1% formic acid (v/v)) and solvent B (90% ACN in nanopure water with 0.1% formic acid (v/v)). A gradient previously optimized in our lab (Bhattacharya et al. 2019) was used and consisted of 0-16% B at 2.5-20 min, 16-40% B at 20-30 min, 40-100% B at 30-40 min, 100% B at 40-50 min, and 100-0% B from 50 to 55 min. Between each run the HPLC chip was re-equilibrated with 100% A for 10 min. The mass spectrometer was operated in positive ionization mode with a mass/charge (*m/z*) range of 450-2500 and an electrospray capillary voltage of 1800-1900 V. Reference masses (*m/z*) of 922.009 and 1221.991 (ESI-TOF Tuning Mix G1969-85000, Agilent Technologies, Santa Clara, CA, USA) provided continuous internal calibration. All samples were analyzed using tandem mass spectrometry (MS/MS) with tandem fragmented peaks selected by the automated precursor selection setting with a threshold of 200 ion counts for MS and 5 ion counts for MS/MS. The QToF MS had a ramped collision energy slope of 1.3 with an offset of -3.6 V, a medium isolation width for MS/MS, and an acquisition rate of 1 spectra/s (Bhattacharya et al., 2019). Each spectrum was manually examined, and molecular masses were confirmed with Agilent MassHunter Qualitative Analysis B.07.00 software using the molecular feature extraction and an error of 20 ppm.

2.6 Phenolics analysis and quantification

Phenolic compounds were analyzed with an Agilent Zorbax Eclipse Plus C18 Rapid Resolution HD (3 x 100 mm, 1.8 μm) on an Agilent 1290 UPLC combined with a diode array detector (DAD) (Santa Clara, CA, USA). The mobile phase gradient was adapted from (Ji, Li, & Li, 2015) and consisted of two solvents. Solvent A was composed of 0.2% formic acid in nanopure water (v/v) and solvent B was 50% ACN in methanol (v/v). The gradient progressed with a 0.5 mL/min flow rate and compositions of 95% to 65% A at 0-30 min, 65% to 60% A at

20-40 min, and 60% to 95% A at 40-42 min. Peak absorbance was measured at 280 nm and phenolic concentration was calculated using individual calibration curves.

3. Results and Discussion

3.1 Oligosaccharide purification

The complexity of the composition of chardonnay marc and the diverse isomeric forms of the oligosaccharides in the marc required the evaluation and comparison of multiple purification strategies to determine their efficacy and feasibility in separating chardonnay marc oligosaccharides and phenolics for use at both analytical and larger scales. An Agilent 6520 NanoChip LC-QToF mass spectrometer was used to identify the oligosaccharides in chardonnay marc extracts following purification with six separation methods. An example chromatogram of the oligosaccharides identified is depicted in Figure 7. The accurate mass-over-charge ratio of each oligosaccharide was measured by single stage MS while tandem MS/MS enabled the identification and confirmation of the composition and hence the degree of polymerization (DP) of each oligosaccharide. Mass spectrometry analysis of oligosaccharides is inherently limited by the fact that monosaccharide epimer subunits composing the oligosaccharides have the same mass and thus appear identically to the mass spectrometer. The oligosaccharide building blocks were therefore identified by their broad monosaccharide subunits as classes of hexose (hex), N-acetylhexosamine (hexNAc), pentose (pent), and hexuronic acid (hexA). Each of these categories included multiple monosaccharides as chardonnay skin oligosaccharides are composed of several hexoses (glucose, mannose, galactose, fructose), pentoses (xylose, arabinose, ribose), hexuronic acids (glucuronic acid, galacturonic acid), and deoxyhexoses (rhamnose, fucose) (Sinrod et al., 2021).

The confirmable oligosaccharides varied for each purification method (Table 9). A freely available online concept maps tool (Cmap Tools Pensacola, FL, USA, Florida Institute for Human and Machine Cognition) was used to aid the visualization of the overlap and differences in the identified oligosaccharides. The Cmap (Figure 8) shows the confirmed oligosaccharides that each purification technique had in common as well as where their identifiable oligosaccharides diverged.

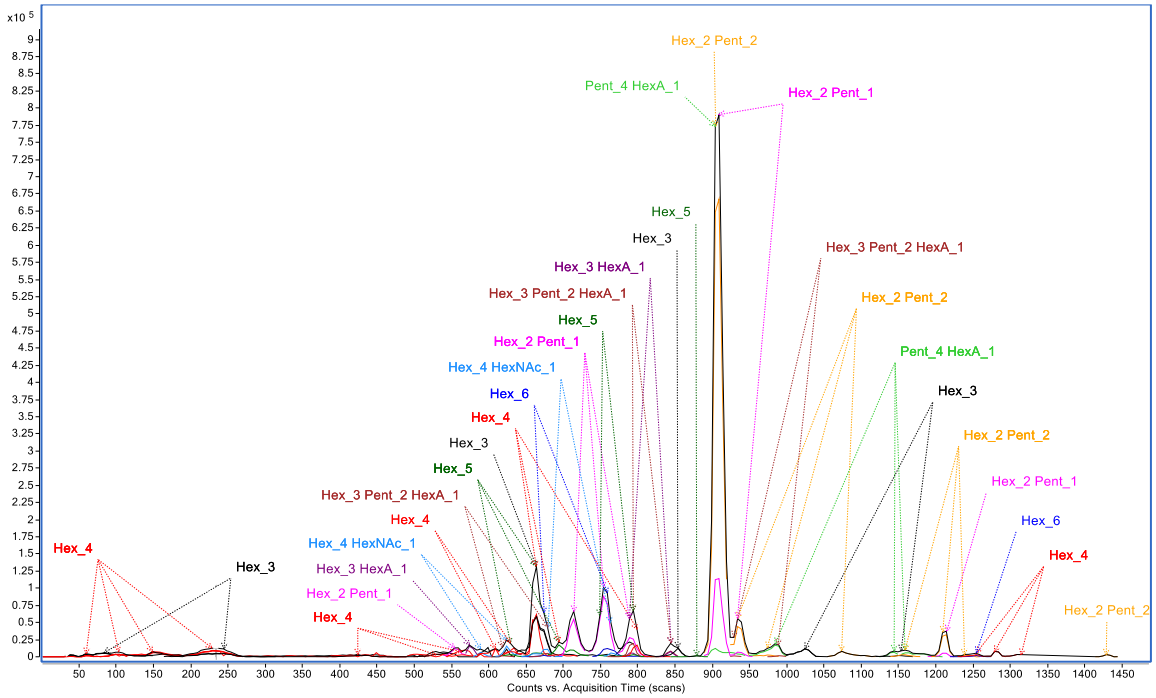

Figure 7. Example Extracted Compound Chromatogram (ECC) for chardonnay marc oligosaccharides from data generated with an Agilent 6520 NanoChip LC-QToF mass spectrometer. Agilent MassHunter Analysis B.07.00 software was used to process the data. This chromatogram was created using data from one of the PVPP, C18 purification replicates.

Table 9. Composition, mass/charge (m/z), charge, number of isomers and retention times for each purification method applied to chardonnay marc. Composition is represented by the number of hexose (hex), N-acetylhexosamine (hexNAc), pentose (pent), and hexuronic acid (hexA) monosaccharides, respectively, in each oligosaccharide confirmed. Samples were analyzed with an Agilent NanoChip LC-QToF mass spectrometer.

			Number of isomers (retention time in min)					
Hex HexNAc Pent HexA	m/z	charge	C18, PGC	C18, PGC, dialysis	C18, DPA-6S	C18, HLB	PVPP	PVPP, C18
2_0_1_0	457.1552, 475.1658	1	1 (15.497)	2 (15.27, 18.396)	2 (11.609, 16.499)	1 (15.436)	3 (14.675, 15.482, 18.658)	3 (11.307, 15.28, 18.305)
0_1_2_0	486.1819	1						1 (14.524)
3_0_0_0	487.1657, 505.1763	1	5 (4.656, 11.497, 13.396, 15.228, 20.942)	2 (4.918, 14.682)	1 (13.373)	3 (4.509, 8.509, 13.836)	3 (4.797, 13.519, 27.497)	2 (0.719, 13.465)
1_0_0_2	515.1243	1					1 (4.069)	
2_0_0_1	519.1556	1			1 (13.239)			
0_0_3_1	573.1663	1	1 (20.034)	2 (19.649, 23.094)		1 (19.415)		
2_0_2_0	589.1975, 607.2081	1		1 (18.573)	1 (19.373)			1 (18.718)
1_0_2_1	603.1768, 621.1874	1		1 (3.792)		2 (3.669, 21.633)		
1_1_2_0	648.2347	1						1 (13.801)
4_0_0_0	649.2185, 667.2291	1	3 (5.043, 13.228, 26.017)	3 (3.473, 12.799, 25.874)	2 (16.298, 26.094)	3 (3.249, 16.259, 25.434)	4 (3.313, 8.864, 13.486, 28.005)	4 (3.013, 5.123, 16.12, 25.884)
2_1_1_0	678.2452	1			1 (14.516)			
3_0_0_1	681.2084	1	1 (12.086)	1 (11.69)	1 (12.332)	2 (11.957, 17.301)		2 (11.6, 17.028)
0_0_4_1	705.2086, 723.2192	1	2 (20.101, 24.117)	1 (19.857)	1 (20.465)	2 (19.989, 23.821)	1 (20.325)	2 (19.834, 23.145)
3_1_0_0	708.2557	1				1 (13.755)		
3_0_1_1	813.2507	1				1 (17.284)	1 (17.737)	
5_0_0_0	829.2819	1		1 (14.523)	1 (14.634)	2 (14.125, 26.843)	1 (14.511)	2 (13.919, 26.892)
3_1_1_0	840.298	1			1 (18.65)			
4_1_0_0	870.3085	1	1 (12.825)		1 (12.97)		1 (12.712)	1 (12.591)
2_2_1_0	881.3246	1						1 (19.229)
3_0_2_1	945.293	1	1 (16.387)		1 (16.751)	1 (16.276)	1 (16.645)	1 (17.011)
6_0_0_0	991.3347	1	1 (15.749)	1 (15.464)	1 (15.894)		1 (15.737)	1 (15.431)
5_1_0_0	1032.3613	1	1 (15.732)					
3_0_3_1	1077.3353	1		1 (16.026)	1 (16.6)	1 (16.175)	1 (16.611)	1 (16.02)
7_0_0_0	1153.3875	1						1 (16.322)
2_0_4_2	602.67315	2				1 (21.297)		
1_2_3_3	746.72105	2				1 (24.271)		

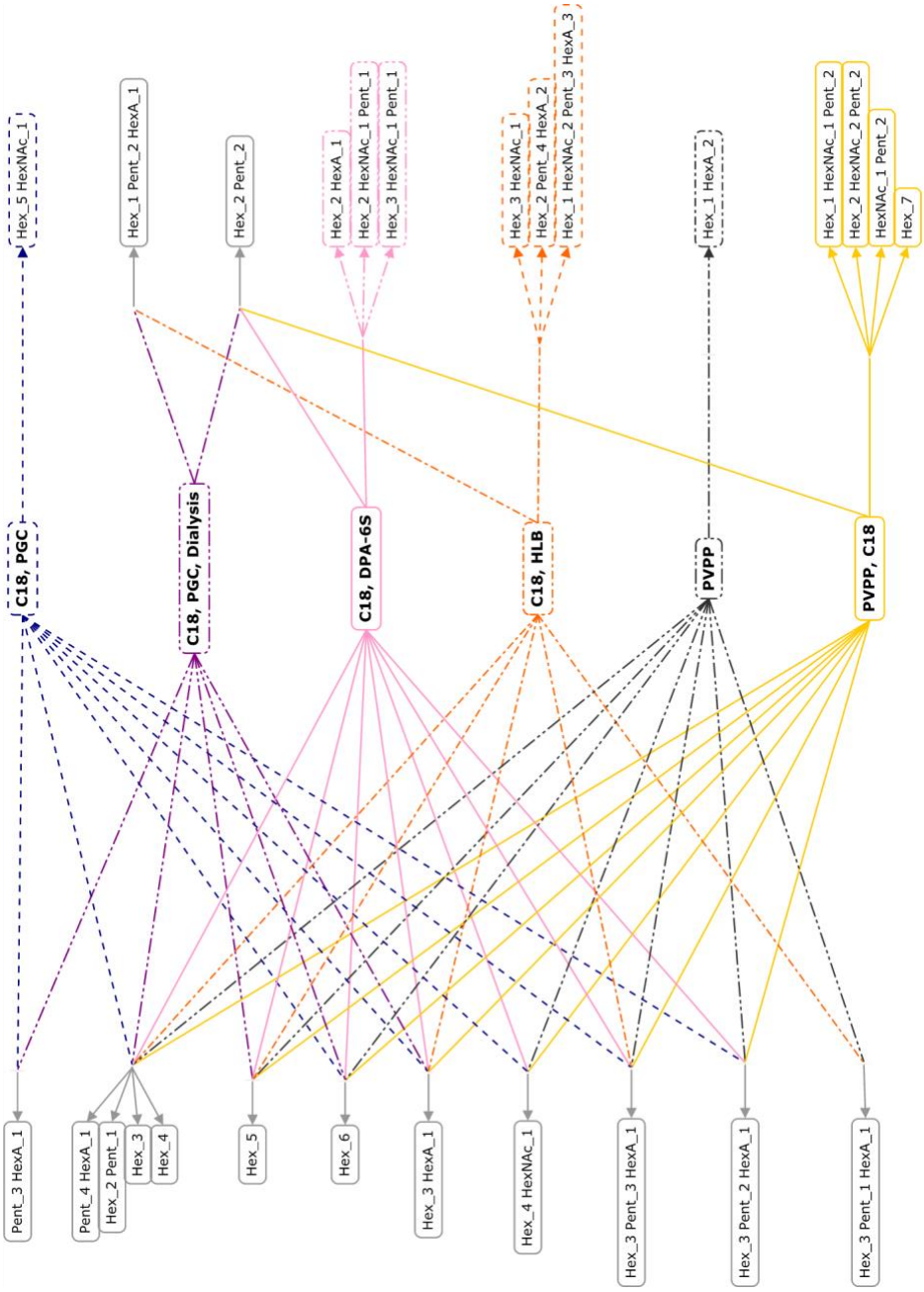

Figure 8. Cmap visualization of oligosaccharides confirmed after six purification methods. Oligosaccharide compositions were found using an Agilent 6520 NanoChip LC-QToF mass spectrometer and analyzed with Agilent MassHunter Analysis B.07.00 software. The Camp was created with CmapTools software which can be obtained for free at http://cmap.ihmc.us/products/.

Oligosaccharide purification is required for successful mass spectrometry analysis. A combination of C18 and PGC SPEs are frequently used to purify oligosaccharides at the analytical scale. This method has been utilized for milk, grape marc and seeds, and hazelnut skin oligosaccharides (Bordiga, Montella, et al., 2019; Montella et al., 2013; Robinson et al., 2019; Sinrod et al., 2021). C18 is a reversed phase silica gel that binds non-polar compounds. The

hydrophobicity of the phenol rings in phenolic compounds interact with the C18 while the hydroxyl groups on the oligosaccharides allow them to flow through the C18 with minimal interaction. Initial trials analyzing the residual phenolic compounds of chardonnay marc oligosaccharides after C18 SPE revealed that the oligosaccharide fraction still contained 0.03±0.0007 mg/g gallic acid, 1.05±0.16 mg/g (+)-catechin, 0.03±0.003 mg/g vanillic acid, 1.98±0.43 mg/g (-)-epicatechin, and 0.05±0.006 mg/g (-)-epicatechin gallate. While greatly reduced from the amount of phenolics present in chardonnay marc (Sinrod et al., 2021), C18 alone did not sufficiently remove the phenolic compounds.

PGC binds the chardonnay marc oligosaccharides while other components like salts, monosaccharides, and disaccharides are washed through the matrix. PGC SPE did remove some of the phenolics remaining after C18 as only gallic acid was present following C18 and PGC SPE (Table 10). However, the oligosaccharides purified with C18 and PGC from the standards mixture had an 87.82±2.57% yield of gallic acid when compared to the original mixture of phenolic and oligosaccharide standards (Table 10). Gallic acid is a small phenolic compound containing only one phenol ring surrounded by a carboxyl group and three hydroxyl groups which make it more polar than the other grape phenolic compounds. Gallic acid thus has a

Table 10. Percent yields of remaining phenolic compounds after the various oligosaccharide purification methods were tested on the mixture of phenolic and oligosaccharide standards. The percent yield was determined by comparing the concentration of each individual phenolic after purification to that of the original standard solution. Samples were analyzed with an Agilent Zorbax Eclipse Plus C18 Rapid Resolution HD column (3 x 100 mm, 1.8 μm) and an Agilent 1290 UPLC in triplicate.

	Gallic acid	Gallocatechin	Epigallocatechin	Catechin	Vanillic acid	Epigallocatechin gallate	Epicatechin	Gallocatechin gallate
C18, PGC	87.82±2.57	0±0	0±0	0±0	0±0	0±0	0±0	0±0
C18, PGC, dialysis	3.97±9.70	5.38±0.77	0±0	0±0	0±0	0±0	0±0	0±0
C18, DPA-6S	17.32±1.69	0±0	0±0	0±0	0±0	0±0	0±0	0±0
C18, HLB	5.32±2.63	0±0	0±0	0±0	0±0	0±0	0±0	0±0
PVPP	5.12±0.84	4.30±0.14	0±0	0±0	35.59±0.81	57.95±0.04	0.16±0.08	0±0
PVPP, C18	5.06±0.74	0±0	0±0	0±0	1.91±0.27	0±0	0±0	0±0

polarity more like that of oligosaccharides than the other phenolic compounds, which allowed it to flow through C18 without binding and to stick to PGC with the oligosaccharides.

Adding a dialysis step after PGC helped remove the remaining gallic acid, thanks to its small size. Gallic acid's mass is 170.12 Da while the smallest oligosaccharide identified in chardonnay marc, Hex_2 Pent_1, weighs 457.1552 Da. Dialysis with a 0.1-0.5 kDa membrane reduced the gallic acid yield from 87.82±2.57% to 3.97±9.70% (Table 10), demonstrating that dialysis is an effective method to remove gallic acid. However, two oligosaccharides found in chardonnay marc after C18 and PGC, Hex_2 Pent_1 and Hex_3, are smaller than 500 Da, which is the upper bound of the pores in the dialysis membrane. Furthermore, it is recommended to use a pore-size much smaller than the target molecules in the sample to prevent unintentional loss (Zumstein, 2001). Thus, there was the possibility of these compounds leaking through the dialysis membrane along with gallic acid. While these oligosaccharides were still identified after dialysis, the number of isomers changed. An additional isomer of Hex_2 Pent_1 was found after dialysis whereas three isomers of Hex_3 that were visible before dialysis were not present after dialysis (Table 9). Unfortunately, the amounts of these small oligosaccharides lost during dialysis cannot be quantified as the exact compositions of these oligosaccharides are not known and therefore commercial standards are not available for the quantification of individual oligosaccharides.

However, the use of PGC to purify oligosaccharides, with or without dialysis, is undesirable due to its high cost (Table 8) and low oligosaccharide yield. While C18 and PGC removed almost all the sugars from the grape marc extract, it also eliminated a high proportion of the oligosaccharides with only 3.70±0.13 mg/g remaining after purification (Table 11). This yield was further lowered with dialysis which decreased the oligosaccharide content of the

extract to 0.93±0.11 mg/g (Table 11). This low yield was likely caused by a large portion of the oligosaccharides eluting from the PGC cartridge during the water washes which targeted the removal of the salts and sugars. A previous study found grape seed oligosaccharides (DP 3-16) loaded onto nonporous graphitized carbon cartridges were eluted when the cartridges were washed with water (Bordiga, Meudec, et al., 2019). This necessary washing likely caused the low oligosaccharide yield in the sample purified with C18 and PGC. The addition of dialysis further decreased the oligosaccharide yield as the dialysis membrane pore size of 0.1-0.5 kD was only slightly below the smallest oligosaccharide found in chardonnay marc, Hex_2 Pent_1. This and other small oligosaccharides could have migrated through the dialysis membrane and been lost from the sample.

Table 11. Quantification (mg/g grape marc) of total carbohydrates, sugars, and oligosaccharides remaining after each purification method. Total carbohydrates were determined via an anthrone sulfuric acid assay and using a SpectroMax M5 UV/Vis spectrophotometer. The sugars were measured with a Thermo Scientific HPAE-PAD with a Dionex ICS-5000 Electrochemical Detector. The oligosaccharides were calculated by subtracting the sugars from the total carbohydrate values. Only one replicate of C18, PGC, dialysis and C18, DPA-6S were analyzed for sugars because of limited remaining sample following total carbohydrate analysis as larger sample portions were required for their total carbohydrates analysis due to their low carbohydrate contents. All negative quantifications were rounded to 0 mg/g.

	Total Carbohydrates	Sucrose	Glucose	Fructose	Oligosaccharides
C18, PGC	6.29 ± 0.10	0.04 ± 0.03	1.44 ± 0.04	1.11 ± 0.07	3.70 ± 0.13
C18, PGC, dialysis	1.78 ± 0.11	0.03	0.47	0.35	0.93 ± 0.11
C18, DPA-6S	0.00 ± 0.12	0.00	0.00	0.00	0.00 ± 0.12
C18, HLB	151.34 ± 0.26	1.23 ± 1.30	42.01 ± 3.23	47.64 ± 10.48	60.46 ± 11.05
PVPP	143.54 ± 0.09	2.16 ± 0.27	36.47 ± 5.30	44.44 ± 5.56	60.47 ± 7.69
PVPP, C18	119.03 ± 0.71	2.39 ± 0.48	39.18 ± 2.02	47.86 ± 3.21	29.60 ± 3.89

One potential solution is substituting PGC with a different solid phase after C18 SPE. DPA-6S and HLB are two solid phases that could be used to follow C18 instead of PGC to further remove remaining phenolic compounds. DPA-6S is a reversed phase polyamide resin marketed for its ability to bind phenolic compounds, specifically gallic acid, and has been previously used to purify oligosaccharides and phenolics individually (Millipore Sigma, 2021).

The hydroxyl groups on the oligosaccharides readily interact with the polar DPA-6S. When applied after C18, DPA-6S removed the residual (+)-catechin, vanillic acid, (-)-epicatechin, and (-)-epicatechin gallate in the chardonnay marc during the acetonitrile wash step as the phenolics were not bound to the DPA-6S as strongly as the oligosaccharides (Dvořáková, Hulín, Karabín, & Dostálek, 2008). The amount of gallic acid remaining after two solid phase extractions was much lower when using DPA-6S (17.32±1.69%) than with PGC (87.82±2.57%) (Table 10). Previous oligosaccharide purification with DPA-6S had a high recovery rate upon elution with 20% ACN (v/v) after washing (Zhang et al., 2014). However, while we detected 16 oligosaccharides that eluted from DPA-6S after C18 (Table 12), these were only present in trace amounts as the sample contained no quantifiable sugars or oligosaccharides (Table 11). Applying DPA-6S after C18 is therefore not an acceptable strategy for oligosaccharide purification.

Table 12. Number of confirmed oligosaccharides of each degree of polymerization (DP) and the total number of oligosaccharides for each method of chardonnay marc oligosaccharide purification. Isomers are each counted as a separate oligosaccharide. Data was obtained with an Agilent 6520 NanoChip LC-QToF mass spectrometer in duplicate.

	DP 3	DP 4	DP 5	DP 6	DP 7	DP 8	Total
C18, PGC	6	5	3	3			17
C18, PGC, dialysis	4	8	2	1	1		16
C18, DPA-6S	4	5	4	2	1		16
C18, HLB	4	9	5	1	1	2	22
PVPP	7	4	4	2	1		18
PVPP, C18	6	8	6	2	2		24

Oasis HLB solid phase consists of microporous poly(divinylbenzene-co-*N*-vinylpyrrolidone) copolymer, is often used instead of C18 to purify phenolic compounds, and has also been used in oligosaccharide isolation (He & Giusti, 2011; Pérez-Magariño, Ortega-Heras, & Cano-Mozo, 2008; Yang et al., 2011). HLB boasts stronger reversed-phase interactions

than C18 during SPE and has a more flexible pH range (He & Giusti, 2011; Masqué, Marcé, & Borrull, 1998). Following C18 with HLB SPE removed all remaining phenolics except 5.32±0.74% of the gallic acid (Table 10). Additionally, this purification method enabled the identification of 22 oligosaccharides which included two oligosaccharides with eight degrees of polymerization (DP), the highest DP found in any of the methods analyzed (Table 12). Chardonnay marc purified with C18 and HLB had the highest number of hex-pent-hexA oligosaccharides (Table 9, Figure 8) and contained three unique oligosaccharides (Hex_3 HexNAc_1, Hex_2 Pent_4 HexA_2, Hex_1 HexNAc_2 Pent_3 HexA_3) (Table 9, Figure 8). Furthermore, C18 paired with HLB produced the highest oligosaccharide yield of the purification methods tested enabling the recovery of 60.46±11.05 mg oligosaccharides per g of marc (Table 11). HLB is thus an excellent option for chardonnay marc oligosaccharide purification when paired with C18 especially for analytical analysis where serial column SPE is easily feasible and the expense is minimal due to the small volumes used.

Another approach to isolate oligosaccharides from chardonnay marc phenolics consists of applying PVPP before C18 SPE, a technique inspired by the wine industry. PVPP is a common fining agent used in wine and beer production to help protect the sensory properties of the beverages. In wines, PVPP binds excess phenolic compounds to prevent color, aroma, and flavor altering chemical reactions that occur with the oxidation of these phenolics (Gil et al., 2019). PVPP is a water insoluble synthetic polymer that is believed to form hydrogen bonds with the phenol groups and CO-N linkages in the phenolic compounds (Laborde et al., 2006; Pierpoint, 2004). This serves to remove the phenolics from solution as the PVPP-phenolic compound complexes remain insoluble in water.

To determine the viability of using PVPP to purify chardonnay marc oligosaccharides, we analyzed the phenolic and oligosaccharide standard mixture for phenolics and the chardonnay marc extract for oligosaccharides after PVPP clean up and again after subsequent C18 SPE. PVPP removed the majority of the phenolics. (-)-Epigallocatechin, (+)-catechin, and (-)-gallocatechin gallate were eliminated, while (-)-epicatechin, (-)-gallocatechin, and gallic acid were reduced to about 5% or less of their original concentrations (Table 10). Vanillic acid and (-)-epigallocatechin gallate had the least interaction with PVPP as 35.59±0.81% and 57.95±0.04% remained with the oligosaccharides after the PVPP treatment, respectively (Table 10). Despite still containing multiple phenolic compounds which could cause MS interference, 18 oligosaccharides were identified with DPs of 3-7 and hex, hexNAc, pent, and hexA monosaccharide building blocks (Tables 2 and 5, Figure 8).

Following PVPP treatment with C18 SPE decreased the residual phenolics and increased the number of identifiable oligosaccharides present in the chardonnay marc. While C18 did not greatly affect the small amount of gallic acid remaining after PVPP, it did completely remove the lingering (-)-gallocatechin, (-)-epigallocatechin gallate, and (-)-epicatechin. C18 also reduced the yield of vanillic acid in the sample to 1.91±0.27% (Table 10). Combining PVPP and C18 allowed for the confirmation of 24 oligosaccharides, the most of any method tested (Table 12). These oligosaccharides included the most diverse hexose oligosaccharides, four unique oligosaccharides (Hex_1 HexNAc_1 Pent_2, Hex_2 HexNAc_2 Pent_2, HexNAc_1 Pent_2, Hex_7), and the most isomers of Hex_4 and Hex_2 Pent_1 (Table 9, Fig, 3). PVPP and C18 purification had a decent oligosaccharide yield of 29.60±3.89 mg/g. These results indicate that the combination of PVPP and C18 is a successful oligosaccharide purification strategy for chardonnay marc and could be followed by dialysis to remove the residual sugars. Although the

oligosaccharide yield with PVPP and C18 is about half of the yield obtained with C18 and HLB, the PVPP needed to purify grape marc is 99.3% less expensive than comparable HLB cartridges and does not require column SPE. Both of these factors make PVPP and C18 SPE a better oligosaccharide purification strategy than C18 and HLB for volumes above the analytical scale.

3.2 Purification of phenolic compounds

C18 is one of the most common solid phases used for phenolics purification and analysis. C18 binds most of the chardonnay marc phenolics during SPE which can then be eluted with various solvents. Previous studies have optimized the phenolics elution gradient from C18. Pinelo et al. (2006) found the best yields for red wine nonpolymeric and polymeric phenolic compounds using a four-step gradient of 10% methanol in water, acidification with hydrochloric acid, ethyl acetate, and then 66% acetone in water. Preliminary trials on chardonnay marc phenolics indicated that an initial wash with 40% methanol in water instead of 10% methanol in water provided increased phenolic yields (data not shown). The gradient steps, with the increased methanol content on the first step, were tested to elute chardonnay marc phenolics from C18 with phenolics analysis at each step.

The phenolic contents varied with the steps in the elution gradient. All the phenolics samples released from C18 had gallic acid yields below 3% as gallic acid does not interact strongly with C18 and thus was removed with the oligosaccharides as discussed in section 3.1

Table 13. Percent yield of phenolic compounds recovered after purification for each method analyzed. A mixture of phenolic compound standards was used for this analysis. Analysis was performed with an Agilent Zorbax Eclipse Plus C18 Rapid Resolution HD column (3 x 100 mm, 1.8 µm) and an Agilent 1290 UPLC in triplicate.

	Gallic acid	Gallocatechin	Epigallocatechin	Catechin	Vanillic acid	Epigallocatechin gallate	Epicatechin	Gallocatechin gallate
C18 eluted with 40% MeOH	1.31±0.08	59.45±0.06	86.05±0.77	87.06±0.41	96.25±0.71	64.53±2.41	90.78±0.67	87.76±0.24
C18 eluted with with 40% MeOH, HCl, ethyl acetate	2.29±0.40	48.82±0.57	64.14±11.66	61.92±12.17	102.24±2.93	0±0.00	68.66±9.95	64.70±10.11
C18 eluted with 40% MeOH, HCl, ethyl acetate, 66% acetone	2.42±0.03	52.59±2.28	50.47±0.76	54.82±0.57	102.23±0.72	0±0.00	58.68±0.43	58.25±0.25
PVPP washed with 70% acetone	88.73±2.34	70.68±2.57	84.31±2.86	91.69±2.87	57.76±1.13	41.09±0.15	47.09±1.60	18.25±1.00

(Table 13). Of the C18 solvent gradients, the elution using only 40% methanol had the best overall phenolics yield with the highest yields of (-)-gallocatechin, (-)-epigallocatechin, (+)-catechin, (-)-epicatechin, (-)-gallocatechin gallate, and (-)-epigallocatechin gallate (Table 13). Adding acidification and ethyl acetate or acidification, ethyl acetate, and 66% acetone caused decreased phenolics yields particularly with no (-)-epigallocatechin gallate detectable in either of these samples (Table 13). The drying of the eluents could have caused this decrease in phenolics. Due to the incompatibility of ethyl acetate and acetone with the centrifugal dryer used in this experiment, the samples eluted with ethyl acetate or ethyl acetate and acetone were dried under a stream of nitrogen gas in the fume hood whereas the eluent generated with only 40% methanol was dried in the centrifugal dryer. The nature of these different drying techniques meant the nitrogen dried eluents were dried slower and at room temperature with some light exposure. These three elements can cause phenolic compound degradation (Volf, Ignat, Neamtu, & Popa, 2014).

Another purification option to obtain chardonnay marc phenolics that is likely more scalable is to release phenolics bound to PVPP with 70% acetone in water (Magalhães et al., 2010). The phenolic extract purified with PVPP contained the highest amounts of (-)-gallocatechin, (+)-catechin, and gallic acid of the methods analyzed and a similar concentration of (-)-epigallocatechin to that of the C18 eluted with 40% methanol (Table 13). Isolating phenolics with PVPP unfortunately yielded much less vanillic acid, (-)-epigallocatechin gallate, (-)-epicatechin, and (-)-gallocatechin gallate than purification with C18 and 40% methanol (Table 13). The key advantage of PVPP phenolic compound purification was the 88.73±2.34% yield of gallic acid which was over 65 times higher than the gallic acid yield obtained with C18 and 40% methanol (Table 13). Gallic acid reversibly binds to PVPP whereas it does not interact

with C18, which allows the gallic acid to be captured and released from PVPP while it was washed through and discarded with C18. PVPP is also much cheaper than C18 SPE cartridges (Table 8).

Ultimately the preference for using C18 and 40% methanol or PVPP to purify phenolics depends on the target phenolic compounds. In the case of chardonnay marc, (-)-gallocatechin and (+)-catechin are two of the three most concentrated phenolic compounds in the marc (Sinrod et al., 2021). Meanwhile gallic acid is the fifth most abundant of the 12 phenolic compounds analyzed in chardonnay marc (Sinrod et al., 2021) and boasts anti-inflammatory and anti-obesity effects (Dludla et al., 2018). Thus, phenolic purification with PVPP is the most promising method for chardonnay marc.

4. Conclusions

Multiple purification strategies were tested to optimize the separation and purification of chardonnay marc oligosaccharides and phenolics. The conventional combination of C18 and PGC SPE did not successfully remove gallic acid and produced low oligosaccharide yields. While the gallic acid was eliminated by a further dialysis step, it greatly decreased the oligosaccharide yield. Pairing C18 with DPA-6S SPE eliminated all quantifiable carbohydrates from the chardonnay marc extract. Following C18 SPE with Oasis HLB SPE removed nearly all phenolic compounds, enabled the identification of the largest oligosaccharides (DP 8), and produced the greatest oligosaccharide yield. Meanwhile using PVPP paired with C18 SPE allowed the confirmation of the highest number of total oligosaccharides and oligosaccharides with DPs of five and seven while generating a moderate quantity of oligosaccharides. For phenolic compound purification, eluting phenolics bound to C18 with 40% methanol and desorbing phenolics from PVPP yielded the best results. C18 washed with 40% methanol

produced the highest yields of vanillic acid, (-)-epigallocatechin gallate, (-)-epicatechin, and (-)-gallocatechin gallate but very little gallic acid. Purifying phenolic compounds with PVPP, however, generated the highest levels of gallic acid, (-)-gallocatechin, (+)-catechin but lower yields of the remaining phenolics. Thus, determining the best phenolics purification method depends on which phenolic compounds are being targeted.

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CHAPTER 3: Evaluating the potential of grape marc as a prebiotic to improve the gut microbiome

*Some of the content in this chapter will be published in an upcoming review paper

1. Abstract

The wine industry produces millions of tons of grape marc each year as their primary coproduct. Grape marc, also known as grape pomace, consists of the seeds and skins removed during pressing and contains various bioactive compounds, including phenolic and oligosaccharides. The vast majority of phenolics and oligosaccharides are known to reach the colon intact where they are able to interact with the gut microbiome. Grape marc phenolics have been shown to positively affect the gut microbiome mainly via suppression of pathogens,

whereas oligosaccharides are known prebiotics. Multiple *in vitro* studies demonstrated that grape marc and its extracts increase the growth and survival of certain strains of *Bifidobacterium* and *Lactobacillus,* important classes of beneficial bacteria in the human gut microbiome, while inhibiting the growth of pathogenic strains such as those in the *Enterobacteriaceae* family. Studies feeding significant amounts of grape marc to livestock indicate that marc increases the growth of beneficial strains and inhibits pathogenic bacteria within their intestines. Furthermore, grape marc supplementation significantly improves the overall health of livestock through multiple pathways including decreased lipid and protein oxidation, which also increases meat quality by commercial standards. Additionally, small animal studies indicate that grape marc could improve human health. Grape marc supplementation in the diet of mice and rats improves the complexity of their gut microbiomes and decreases diet-induced obesity as well as related illnesses such as insulin resistance. A human clinical trial found grape marc, regardless of its phenolic content, had cardioprotective effects suggesting that the dietary fiber in the supplements induced these health benefits.

2. Introduction

The structures of select phenolic compounds and oligosaccharides present in grape marc and other grape products are illustrated in Figure 9. The potential health impact of grape marc has become an increasingly investigated topic, primarily regarding its content of phenolic compounds and overall effect on the gut microbiome. The gut microbiome consists of a complex collection of trillions of bacteria, both commensal and pathogenic, which colonize the intestines (Hoffmann, Proctor, Surette, & Suchodolski, 2016). These bacteria break down food components that are indigestible to humans into smaller metabolites, which can often be absorbed by the

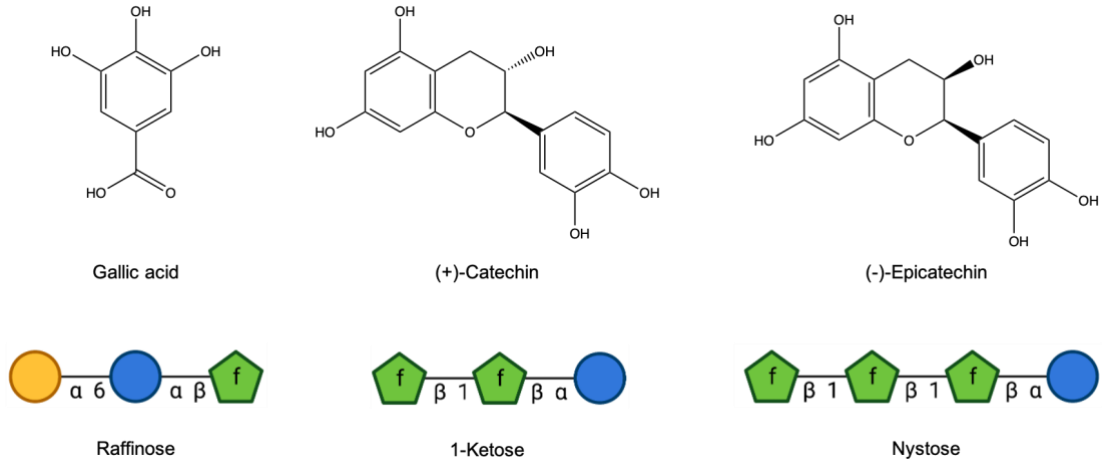

Figure 9. Structures of three example phenolic compounds and oligosaccharides identified in grape marc or grape products. For the oligosaccharides, raffinose is composed of a terminal galactose (orange) with a α1-6 bond to a glucose (blue) with an α1-2 β to a fructose (green). 1-ketose is a terminal fructose (green) bound to another fructose with a β2-1 bond which is then bound to a glucose with a β2- 1α bond. Nystose is composed of three linear fructose monosaccharides connected with β2-1 bonds that is terminated with a glucose bound by a β2-1α bond. The phenolic structures were created with ChemDraw 20.0 and the oligosaccharide structures were made using BioRender.

intestines or otherwise utilized. Commensal bacteria also help prevent intestinal infection from pathogens, thus reducing the burden of severe disease and concomitant inflammation.

The market for grape marc and seeds as health supplements has expanded with growing research that shows its potential health benefits. These supplements are marketed for their antioxidant properties and associated health benefits (Life Extension, 2021; Bulk Supplements, 2021; Pure Formulas, 2021).

Another avenue for grape marc valorization is to identify its capabilities as a prebiotic or

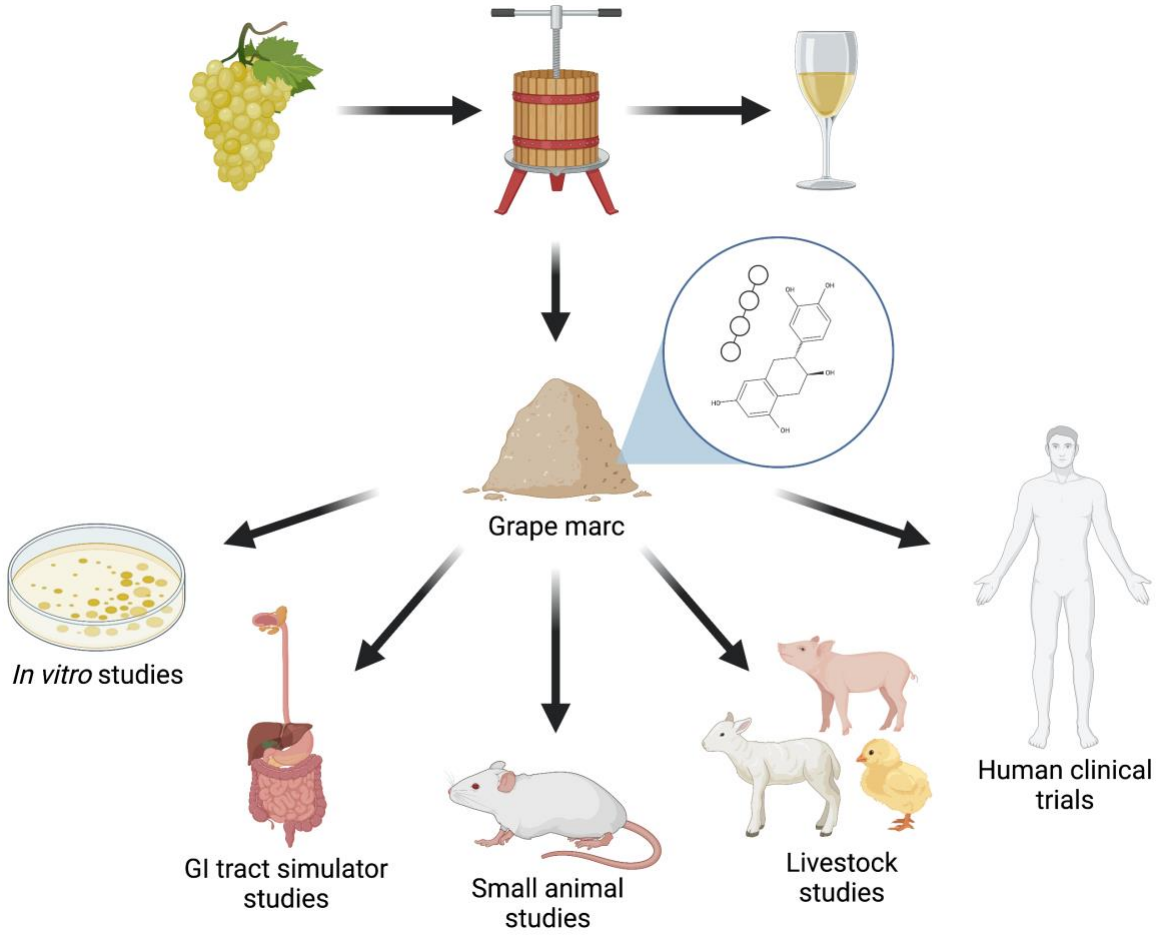

Figure 10. Creation of grape marc containing phenolic compounds and oligosaccharides with grape pressing. The marc has been used in in vitro, GI tract simulator, small animal, livestock, and human studies to evaluate its potential health benefits. Figure created with BioRender.com.

prebiotic-probiotic combination supplement to improve human gut health. Prebiotic and probiotic supplements have become increasingly prevalent as knowledge of and emphasis on the

importance of maintaining the gut microbiome on overall health has increased. A prebiotic is "a substrate that is selectively utilized by the host's microorganisms conferring a health benefit (Gibsom et al., 2017)." Probiotics are beneficial bacteria that provide desirable health benefits. This review surveys the current literature on grape marc phenolics and oligosaccharides and the valorization potential of grape marc as a source of compounds able to modulate gut health and decrease diseases in both animals and humans through *in vitro*, GI tract simulation, small animal, livestock, and human studies (Figure 10).

3. The "problem" with current probiotics

The importance of gut health is becoming increasingly studied and known. One of the key factors of gut health is maintaining a healthy gut microbiome that includes sufficient amounts of commensal bacteria such as *B. longum, L. rhamnosus, L. plantarum, and L. acidophilus* (Ding & Shah, 2007). One popular method for improving the gut microbiome is through probiotic supplements where viable commensal bacteria are consumed to repopulate the intestines. However, to be effective these bacteria must remain viable after passing through the harsh conditions of the upper GI tract and then must be able to remain in the gut and consume the carbon sources available in situ. Eight strains of commonly used probiotic bacteria demonstrated rapid decreases in viability when exposed to *in vitro* acidic conditions that replicated those of the stomach (Ding & Shah, 2007). Ingested probiotic bacteria are also exposed to bile salts including oxgall and taurocholic acid in the upper GI tract. Similarly to stomach acid, oxgal and taurocholic bile acids significantly reduce probiotic viability (Ding & Shah, 2007). The loss of probiotic viability during digestion decreases or eliminates the ability of probiotics to colonize the gut with beneficial bacteria.

To solve this challenge, probiotics can be co-delivered with compounds that not only help protect them through the upper GI tract but also selectively feed the probiotic bacteria to help aid colonization while hindering the growth of less desirable bacteria already present in the gut. Initial *in vitro* studies indicate that grape marc has this functionality. When administered with grape marc, the survival of certain commensal lactic acid bacteria and *bifidobacteri*a (*L. plantarum* 12A*, L. plantarum* PU1*, L. paracasei* 14A, *B. breve* 15A) increased during exposure to GI tract conditions, indicating that grape marc is an effective food matrix to deliver probiotics to the colon (Campanella et al., 2017).

4. Interactions between grape marc phenolics and the gut microbiome

Grape marc and grape seeds contain high concentrations of a diverse array of phenolic compounds (Table 14). Phenolics remain intact through digestion and can reach the small

Table 14. Concentrations of key phenolic compounds in grape marc and grape marc extracts from multiple varietals (Hervert-Hernández, Pintado, Rotger, & Goñi, 2009; Sinrod et al., 2021; Tabasco et al., 2011; Antoniolli, Fontana, Piccoli, & Bottini, 2015; Xu, Burton, Kim, & Sismour, 2016).

Polyphenol	Malbec (red) grape marc extract (µg/g) (Antoniolli, Fontana, Piccoli, & Bottini, 2015)	Chardonnay marc (µg/g dw) (Sinrod et al., 2021)	Vitaflavan® Grape Seed Extract (mg/g) (Tabasco et al., 2011)	Cencibel grape marc (mg/ml) (Hervert-Hernández, Pintado, Rotger, & Goñi, 2009)	Voignier marc extract (mg/g) (Xu, Burton, Kim, & Sismour, 2016)	Vidal Blanc marc extract (mg/g) (Xu, Burton, Kim, & Sismour, 2016)	Cabernet Franc marc extract (mg/g) (Xu, Burton, Kim, & Sismour, 2016)	Chambourcin marc extract (mg/g) (Xu, Burton, Kim, & Sismour, 2016)
Gallic acid	252.8 ± 18.5	116 ± 2	9.11 ± 0.10	99.6				
Vanillic acid		15 ± 2						
Syringic acid	1731.7 ± 156.3							
Caffeic acid	16.0 ± 2.6			100.5				
(+)-Catechin	3387.5 ± 374.7	670 ± 10	74.54 ± 0.09	99.6	910 ± 10.5	631 ± 13.4	560 ± 4537	214 ± 4.80
(-)-Epicatechin	1763.4 ± 221.8	890 ± 30	67.68 ± 0.75	99.9	625 ± 9.20	451 ± 22.2	215 ± 4.67	109 ± 4.17
(-)-Gallocatechin		1490 ± 40						
Epicatechingallate					427 ± 11.7		122 ± 2.995	56.9 ± 5.36
(-)-Epigallocatechin		250 ± 4						
(-)-Epigallocatechin gallate		24.2 ± 0.2			96.1 ± 3.47	62.8 ± 0.78	171 ± 7.26	
(-)-Epicatechin gallate		28 ± 1						
(-)-Epicatechin-3-O-gallate			26.21 ± 0.41					
Gallocatechin gallate					99.1 ± 1.29		232 ± 3.19	146 ± 3.37
Trans-resveratrol		26.3 ± 0.5						
Quercetin-3-glucoside	112.2 ± 12.1							
Quercetin-3-rhamnoside					27.1 ± 2.59	33.5 ± 1.57		
Quercetin	557.3 ± 83.9			100.9	17.3 ± 0.38	20.7 ± 0.01	56.5 ± 1.95	31.2 ± 2.26
Rutin					255 ± 16.7	435 ± 14.0	343 ± 11.0	99.5 ± 0.39
Tyrosol	34.0 ± 2.7							
Trans-resveratrol		26.3 ± 0.5						
Procanidin B3			20.39 ± 0.33					
Procyanidin B1			60.99 ± 1.42					
Procyanidin T2			6.81 ± 0.06					
Procyanidin B4			15.04 ± 0.13					
Procyanidin B2			45.13 ± 0.95					
Procyanidin C1			7.07 ± 0.08					
B1-3-O-gallate			0.32 ± 0.04					
B2-3-O-gallate			1.80 ± 0.06					
B2-3'-O-gallate			1.61 ± 0.00					

intestine where 5-10% of them, primarily monomers and dimers, are absorbed (Faria, Fernandes, Norberto, Mateus, & Calhau, 2014; Gil-Sánchez et al., 2017). The remaining 90-95% of phenolic compounds continue to the colon where they can reach millimolar concentrations (Faria, Fernandes, Norberto, Mateus, & Calhau, 2014). There, (-)-epigallocatechin gallate, a prominent phenolic compound in grape marc, is broken down by either *R. ornithinolytica* or *R. planticola* as demonstrated with an *in vitro* dynamic gastrointestinal digestion model (Gil-Sánchez et al., 2017). Bacterial metabolism transforms grape marc phenolics into more bioavailable metabolites like 3,5-dihydroxybenzoic acid and phenylacetic acid which can be absorbed into the body (Gil-Sánchez et al., 2017). It has been suggested that the many health benefits linked to phenolic compound intake, such as decreased blood pressure and cholesterol, could be a result of the interactions between phenolics and gut bacteria (Queipo-Ortuño et al., 2012).

Grape marc supplementation enhances the growth and survival of some commensal bacteria strains. Studies have shown that certain commensal bacteria metabolize grape phenolics and can therefore grow when exposed to grape marc. *Lactobacillus* and *Bifidobacterium* are the most widely used genera in commercial probiotics, and other researchers indicated that their growth can be stimulated by grape phenolics. For example, *Bifidobacterium breve* 26M2 and *Bifidobacterium bifidum* HDD541 increased when exposed to grape seed extract (Tabasco et al., 2011). Additionally, lactic acid bacteria and bifidobacteria have shown increasing growth with increasing total concentrations of phenolics and procyanadinin, a specific phenolic compound, from grape seed extracts (Tabasco et al., 2011). Furthermore, Tabasco et al. (2011) and Hervert-Hernández et al. (2009) found that grape marc and seed extracts stimulated the growth of *L. acidophilus* CECT 903 (Hervert-Hernández, Pintado, Rotger, & Goñi, 2009; Tabasco et al., 2011).

The sensitivity of gut bacteria to grape marc and grape seed phenolic compounds however was shown to vary greatly depending on the strain. For example, *L. plantarum* IFPL 724 and *L. casei* LC-01 could not grow in media with grape seed extract concentrations above 0.25 mg/ml, whereas the growth of *L. plantarum* IFPL 935 and *L. casei* IFPL 7190 increased with increasing grape seed extract concentration (Tabasco et al., 2011). Some commensal lactic acid bacteria species including *S. thermophilus*, *L. fermentum*, *L. acidophilus*, and *L. vaginalis*

Table 15. Compilation of effects of grape marc, grape seeds, and their extracts on commensal bacteria grown under various in vitro conditions and the carbohydrate and phenolic analysis performed (Karamati Jabehdar, Mirzaei Aghjehgheshlagh, Navidshad, Mahdavi, & Staji , 2018; Hervert-Hernández, Pintado, Rotger, & Goñi, 2009; Costa et al., 2019; Campanella et al., 2017; Gil-Sánchez et al., 2017; Tabasco et al., 2011). In vitro growth assays were performed in MRS broth. Note the studies that demonstrated bacterial growth but did not analyze the carbohydrates did not consider the potential growth caused by the carbohydrates (sugars and oligosaccharides) and thus are not necessarily indicative of the effects of the phenolics on the commensal bacteria.

Bacteria	Growth Effect	Marc Intervention	Study Type	Measured Carbohydrates	Measured Phenolics	Source
B. animalis 13A	Decreased	Red grape marc	*In vitro* growth assay	N/A	N/A	(Campanella et al., 2017)
B. breve 15A	Increased	Red grape marc	*In vitro* growth assay	Total carb before and after incubation	TPC, individual phenolics before and after incubation	(Campanella et al., 2017)
Bacteroides	Increased	Grape marc extract	GI tract simulator	Extract dietary fiber and monosaccharides	Extract TPC and individual phenolics	(Gil-Sánchez et al., 2017)
Bifidobacterium	Increased	Grape marc extract	GI tract simulator	Extract dietary fiber and monosaccharides	Extract TPC and individual phenolics	(Gil-Sánchez et al., 2017)
Bifidobacterium animalis Bo	Increased	Enzymatic grape marc extract	*In vitro* growth assay	Total dietary fiber, monosaccharide, XOS, and polysaccharide quantification before and after incubation	TPC and individual phenolics before and after incubation	(Costa et al., 2019)
Bifidobacterium animalis spp. Lactic Bb12	Increased	Enzymatic grape marc extract	*In vitro* growth assay	Total dietary fiber, monosaccharide, XOS, and polysaccharide quantification before and after incubation	TPC and individual phenolics before and after incubation	(Costa et al., 2019)
Bifidobacterium longum BG3	Increased	Enzymatic grape marc extract	*In vitro* growth assay	Total dietary fiber, monosaccharide, XOS, and polysaccharide quantification before and after incubation	TPC and individual phenolics before and after incubation	(Costa et al., 2019)
Bifidobacterium spp. ATCC 29521	Increased	Grape marc extract	*In vitro* growth assay	N/A	Extract TPC	(Karamati Jabehdar, Mirzaei Aghjehgheshlagh, Navidshad, Mahdavi, & Staji , 2018)
L. acidophilus ATCC 43121	No effect	Grape marc extract	*In vitro* growth assay	N/A	Extract TPC	(Karamati Jabehdar, Mirzaei Aghjehgheshlagh, Navidshad, Mahdavi, & Staji , 2018)
L. acidophilus CECT 903	Increased	Red grape marc extract	*In vitro* growth assay	N/A	Extract TPC, individual phenolics	(Hervert-Hernández, Pintado, Rotger, & Goñi, 2009)
L. casei FC1-13	Decreased	Red grape marc	*In vitro* growth assay	N/A	N/A	(Campanella et al., 2017)
L. casei FPL7190	Increased	Grape seed extract	*In vitro* growth assay	N/A	Extract Individual phenolics	(Tabasco et al., 2011)
L. casei LC-01	Decreased	Grape seed extract	*In vitro* growth assay	N/A	Extract Individual phenolics	(Tabasco et al., 2011)
L. fermentum LC-40	Decreased	Grape seed extract	*In vitro* growth assay	N/A	Extract Individual phenolics	(Tabasco et al., 2011)
L. fermentum PNA1	Decreased	Grape seed extract	*In vitro* growth assay	N/A	Extract Individual phenolics	(Tabasco et al., 2011)
L. paracasei 14A	Increased	Red grape marc	*In vitro* growth assay	Total carb before and after incubation	TPC, individual phenolics before and after incubation	(Campanella et al., 2017)
L. plantarum 12A	Increased	Red grape marc	*In vitro* growth assay	Total carb before and after incubation	TPC, individual phenolics before and after incubation	(Campanella et al., 2017)
L. plantarum CIC17	Decreased	Grape seed extract	*In vitro* growth assay	N/A	Extract Individual phenolics	(Tabasco et al., 2011)
L. plantarum CLB7	Decreased	Grape seed extract	*In vitro* growth assay	N/A	Extract Individual phenolics	(Tabasco et al., 2011)
L. plantarum IFPL711	Decreased	Grape seed extract	*In vitro* growth assay	N/A	Extract Individual phenolics	(Tabasco et al., 2011)
L. plantarum IFPL715	Decreased	Grape seed extract	*In vitro* growth assay	N/A	Extract Individual phenolics	(Tabasco et al., 2011)
L. plantarum IFPL722	Decreased	Grape seed extract	*In vitro* growth assay	N/A	Extract Individual phenolics	(Tabasco et al., 2011)
L. plantarum IFPL724	Decreased	Grape seed extract	*In vitro* growth assay	N/A	Extract Individual phenolics	(Tabasco et al., 2011)
L. plantarum IFPL935	Decreased	Grape seed extract	*In vitro* growth assay	N/A	Extract Individual phenolics	(Tabasco et al., 2011)
L. plantarum PU1	Increased	Red grape marc	*In vitro* growth assay	Total carb before and after incubation	TPC, individual phenolics before and after incubation	(Campanella et al., 2017)
L. reuteri DSM20016	Decreased	Red grape marc	*In vitro* growth assay	N/A	N/A	(Campanella et al., 2017)
L. rhamnosus SP1	Decreased	Red grape marc	*In vitro* growth assay	N/A	N/A	(Campanella et al., 2017)
L. rossiae DSM15814	Decreased	Red grape marc	*In vitro* growth assay	N/A	N/A	(Campanella et al., 2017)
Lactobacillus	Increased	Grape marc extract	GI tract simulator	Extract dietary fiber and monosaccharides	Extract TPC and individual phenolics	(Gil-Sánchez et al., 2017)
Lactobacillus casei 01	Increased	Enzymatic grape marc extract	*In vitro* growth assay	Total dietary fiber, monosaccharide, XOS, and polysaccharide quantification before and after incubation	TPC and individual phenolics before and after incubation	(Costa et al., 2019)
Lactobacillus rhamnosus R11	Increased	Enzymatic grape marc extract	*In vitro* growth assay	Total dietary fiber, monosaccharide, XOS, and polysaccharide quantification before and after incubation	TPC and individual phenolics before and after incubation	(Costa et al., 2019)

have shown high levels of sensitivity to grape seed extract (Tabasco et al., 2011), thus indicating that consuming grape seed extract could potentially decrease the populations of some strains of commensal bacteria while increasing the prominence of others. Table 15 enumerates the responses of specific gut bacteria to grape marc and grape marc extracts measured in various *in vitro* studies.

However, it is vital to point out that neither study purified the phenolics extracted from grape marc and seeds, thus non-phenolic compounds like sugars or oligosaccharides could have been present in the extracts and promoted the bacterial growth instead of the phenolics. Furthermore, neither of these studies analyzed the extracts for the presence of oligosaccharides nor simple sugars. A deeper analysis of the effects of isolated grape marc phenolics on commensal bacteria is therefore required to either validate or refute the claims of commensal bacteria growth on grape phenolics made by Tabasco et al. (2011) and Hervert-Hernández et al. (2009).

As a matter of fact, grape marc and its extracts have been shown to repress the growth of several strains of pathogenic bacteria. Oligomer phenolic compounds are more antimicrobial than monomer phenolic compounds to pathogens including *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus epidermis*, and *Enterococcus faecalis* (Tabasco et al., 2011). Several of the oligomer phenolics in grape marc are specifically known to prevent the growth of bacterial pathogens. For example, catechins hinder *E. coli*, *Bacillus cereus*, and *Serratia marcescens* growth; gallic acid is antimicrobial for *E. coli*, *S. aureus*, and *Pseudomonas aeruginosa*; quercetin suppresses *E. coli*, *Proteus mirabilis*, and *Klebsiella pneumoniae* growth; and caffeic acid is antibacterial for *E. coli*, *P. aeruginosa*, *S. aureus*, and *P. mirabilis* (Hervert-Hernández, Pintado, Rotger, & Goñi, 2009; Antoniolli, Fontana, Piccoli, & Bottini, 2015;

Vaquero, Alberto, & de Nadra, 2007). Karamati Jabehdar, Mirzaei Aghjehgheshlagh, Navidshad, Mahdavi, & Staji (2018) tested the ability of a variety of bacterial strains to grow on grape marc phenolic extract and found that while *E. coli* ATCC 35218 was able to grow at every tested concentration of grape marc, the growth of *Streptococcus* was inhibited when exposed to the extract.

Grape seed and marc extracts have similar phenolic profiles, however the seed extract's phenolics are over three times more concentrated than the marc extract (Sinrod et al., 2021). Grape seed extract increases bacterial growth significantly more than marc extract, whose impact on growth was found to be concentration-dependent (Hervert-Hernández, Pintado, Rotger, & Goñi, 2009). While this could have been due to carbohydrates potentially present in the extracts, the phenolics may have played a key role in the bacteria growth. Of the phenolic compounds tested as standards and present in the extracts, only tannic acid and catechin promoted concentration-dependent growth, while the others had no effect. It is therefore possible that the growth of *L. acidophilus* CECT 903 and other lactic acid bacteria in the marc and seed extracts were linked to their tannic acid and catechin contents (Hervert-Hernández, Pintado, Rotger, & Goñi, 2009). Grape marc extract also increases the growth of certain *Bacteroides*, *Enterobacteriaceae*, and *Clostridial* strains, which are thought to be the primary microbes that metabolize phenolics (Gil-Sánchez et al., 2017). However it is important to note that these do not necessarily include commensal strains of these bacteria and could include strains that have the potential to become opportunistic pathogens as full characterization was not described in Gil-Sánchez et al. (2017).

Some bacteria have the ability to enzymatically break down grape phenolics into smaller metabolites that can have greater bioavailability and bioactivity than the original phenolic

compound. Each phenolic compound is digested in multiple ways, generating a wide array of distinct metabolites. Grape phenolics are catabolized into benzoic acids, phenylacetic acids, phenylpropionic acids, valeric acids, phenyl acetic acids, cinnamic acids, and valerolactones. All of these metabolites can be absorbed by the large intestine and induce further health benefits (Gil-Sánchez et al., 2017).

Scientists have determined how some bacteria in the human gut microbiome interact with flavan-3-ols like (-)-epicatechin gallate and (-)-epigallocatechin gallate which are present in grape marc. Human gut bacteria produce esterases which cleave the flavan-3-ol's salic acid ester (gallic acid) which is decarboxylated into pyrogallol. The carbon ring of flavan-3-ol is then opened to form diphenylpropan-2-ol which is converted to 5-(3', 4'-dihydroxyphenyl)-γ-valerolacetone. The valerolacetone ring is broken to generate 5-(3', 4'-dihydroxyphenyl) valeric acid and 4-hydroxy-5-(3', 4'-dihydroxyphenyl) valeric acid. These compounds are dehydroxylated into mono-hydroxylated phenolic acids which are absorbed through the intestine and metabolized by the liver (Meselhy, Nakmura, & Hattori, 1997; Kohri, Suzuki, & Nanjo, 2003; Roowi et al., 2010).

Current literature indicates that grape marc phenolics may be able, directly or more likely indirectly, to increase the growth of multiple commensal bacteria strains whilst inhibiting the growth of infection causing pathogens in the gut. Altering the composition of the gut microbiome to be more favorable and ingesting increased levels of phenolics which the commensal bacteria can transform into bioactive metabolites suggests great promise for grape marc phenolics as a functional food product. More studies are needed, however, to determine the exact mechanisms by which phenolics supposedly exert prebiotic effects (including determining

the mechanisms of incorporation and utilization as carbon source as well as gene expression) of highly purified grape marc phenolics to verify their health benefits.

5. Potential health impacts of grape marc oligosaccharides

Oligosaccharides are a class of non-digestible carbohydrates with between 3 and 20 degrees of polymerization. The majority of oligosaccharide research has been conducted on milk; however, oligosaccharides are also present in plants.

Recent studies found multiple types of oligosaccharides in grapes, wine, and wine coproducts. Blanch et al. (2011) found 5 fructo-oligosaccharides (1-kestose, neokestose, nystose, nystose b, and kestopentaose) in red table grapes while Dos Santos Lima et al (2019) identified 1-ketose, nystose, and raffinose oligosaccharides in grape juice and wine. Oligosaccharides are present in both red and white wines as shown in the study conducted by Bordiga et al. (2012) which discovered 45 distinct oligosaccharides in wine with degrees of polymerization of 3-14 monosaccharide building blocks of glucose, arabinose, xylose, mannose, rhamnose, fucose, galacturonic acid, and glucuronic acid. Oligosaccharides containing arabinose, mannose, galactose, glucose, and rhamnose have been found in seeds isolated from red wine marc (Table 16) (Bordiga, Montella, Travaglia, Arlorio, & Coïsson, 2019). Furthermore, our recent study of chardonnay marc, its seed and skin components, and a seed extract collectively identified 36 distinct oligosaccharides among the samples that were composed of 11 individual hexose, pentose, N-acetylhexosamine, and hexuronic acid monosaccharides (Table 16). While significant overlap of the oligosaccharides existed between the chardonnay marc fractions, each fraction had a set of unique oligosaccharides not found in the other fractions. The chardonnay skins had the largest number of naturally occurring oligosaccharides and the most unique oligosaccharides compared to the other fractions. Interestingly, applying subcritical water extraction to

chardonnay seeds dramatically increased the number of oligosaccharides present in the seeds (Sinrod et al., 2021).

Table 16. Complex oligosaccharides in grape marc and grape marc components. Oligosaccharides are described by their hexose (Hex), hexuronic acid (HexA), pentose (Pent), and N-acetyl hexosamine (HexNAc) monosaccharides (Sinrod et al., 2021; Bordiga, Montella, Travaglia, Arlorio, & Coïsson, 2019; Bordiga et al., 2019). Monosaccharides are abbreviated as Ara for arabinose, GalA for galuronic acid, and Rha for rhamnose.

Material	Oligosaccharide composition	Source
Nebbiolo red grape seeds	Hex_3, Hex_4, Hex_5, Hex_6, Hex_7, Ara_8, Ara_9, Ara_10, Ara_11, GalA-Rha-Ara_7, GalA- Rha-Ara_8, GalA-Rha-Ara_9, GalA-Rha-Ara_10, GalA-Rha-Ara_11, GalA-Rha-Ara_12, GalA-Rha-Ara_13, GalA-Rha- Ara_14, GalA-Rha-GalA-Ara_5, GalA-Rha-GalA-Ara_6, GalA-Rha-GalA-Ara_7, GalA-Rha-GalA-Ara_8, GalA-Rha-GalA-Ara_9, GalA-Rha-GalA-Ara_10, GalA-Rha-GalA-Ara_11, GalA-Rha-GalA-Ara_12, GalA-Rha-GalA-Ara_13, GalA-Rha-GalA-Ara_14, GalA-Rha-GalA-Ara_15	(Bordiga et al., 2019)
Chardonnay marc	Hex_3, Hex_4, Hex_5, Hex_6, Hex_2 HexA_1, Hex_3 HexA_1 Pent_4 HexA_1, Pent_5 HexA_1, Hex_2 Pent_1, Hex_2 Pent_2, Hex_2 Pent_3, Hex_3 Pent_2, Hex_3 Pent_1 HexA_1, Hex_3 Pent_2 HexA_1, Hex_3 Pent_3 HexA_1	(Sinrod et al., 2021)
Chardonnay skins	Hex_3, Hex_4, Hex_6, Hex_7, Hex_2 Pent_1, Hex_2 Pent_2, Hex_2 Pent_3, Hex_3 Pent_1, Hex_4 Pent_1, Hex_4 Pent_2, Hex_5 Pent_1, Hex_2 HexA_1, Pent_4 HexA_1, Pent_5 HexA_1, Hex_2 HexNAc_1, Hex_3 HexNAc_1, Hex_4 HexNAc_1, Hex_5 HexNAc_1, Hex_2 Pent_3 HexA_1	(Sinrod et al., 2021)
Chardonnay seeds	Hex_3, Hex_4, Hex_5, Hex_6, Hex_7, Hex_8, Hex_2 HexA_1, Pent_5 HexA_1, Hex_2 Pent_1, Hex_2 Pent_2, Hex_2 Pent_3, Hex_3 HexNAc_1, Hex_4 HexNAc_1, Hex_1 HexNAc_1 HexA_1, Hex_3 Pent_2 HexA_1	(Sinrod et al., 2021)
Chardonnay seed extract	Hex_3, Hex_4, Hex_5, Hex_6, Hex_7, Hex_8, Hex_9, Hex_2 Pent_1, Hex_2 Pent_2, Hex_2 Pent_3, Hex_3 HexNAc_1, Hex_4 HexNAc_1, Hex_3 HexA_1, Hex_3 HexA_2, Hex_4 HexA_3, HexNAc_3, Hex_1 HexNAc_2 HexA_1, Hex_2 HexNAc_1 Pent_1, Hex_3 HexNAc_1 Pent_1, HexNAc_4 Pent_1 HexA_2	(Sinrod et al., 2021)
Nebbiolo seeds	Not specified	(Bordiga, Montella, Travaglia, Arlorio, & Coïsson, 2019)

Like phenolics, oligosaccharides boast many significant health benefits. Oligosaccharides can act as prebiotics as they selectively promote the growth of beneficial gut bacteria and inhibit pathogen growth and binding to host-cells. Grape marc oligosaccharides hold great potential to serve as prebiotics. Previous studies indicate grape marc's ability to increase the growth of commensal gut bacteria. Beneficial gut bacteria found in human feces like *L. acidophilus* NRRL B-1910, *L. plantarum* NRRL B-4495, and *B. bifidum* ATCC 15696 are able to ferment naturally occurring plant fructo-oligosaccharides and arabinogalactans similar to those in grape marc (Pedreschi, Campos, Noratto, Chirinos, & Cisneros-Zevallos, 2003; Englyst, Hay, & Macfarlane,

1987). Oligosaccharides discovered in red grape seeds also improve the growth of probiotic *L. acidophilus* P18806 when used in small concentrations (Bordiga, Montella, Travaglia, Arlorio, & Coïsson, 2019). Additionally, an enzymatically produced grape marc extract stimulated the growth of commensal *Bifidobacterium animalis* sp. *lactis* Bb12, *Bifidobacterium animalis* Bo, *Bifidobacerium longum* BG3, *Lactobacillus casei* 01, and *Lactobacillus rhamnosus* R11 potentially because of the xylo-oligosaccharides present in the extract (Costa et al., 2019). Furthermore, the enzymatic extract exhibits antimicrobial activity towards multiple pathogens including *E. coli* ATCC 25922, *P. aeruginosa* ATCC 10145, *S. aureus* ATCC 25923, and *S. aureus* CCUG 60578, likely caused by a combination of the xylo-oligosaccharides, phenolics, and other compounds present in the extract (Costa et al., 2019). Utilizing oligosaccharides as prebiotics to beneficially alter the gut microbiome has great potential systemic effects beyond the gastrointestinal tract. Supplementing high fat diets with short-chain fructo-oligosaccharides increases Bifidobacteria and *Clostridium coccoides* abundance and decreases *Clostridium leptum* in the mouse gut microbiome. These alterations to the gut microflora induced great metabolic changes and eliminated total weight and fat mass gain in the mice, indicating that the fructo-oligosaccharides have anti-obesity effects (Respondek et al., 2013). Other studies have also shown that oligosaccharides have anti-diabetic, anti-cancer, and cardio-protective benefits (Kumar et al., 2009; Kapoor & Dharmesh, 2017; Zhang, Cai, & Ma, 2015).

6. Preliminary studies of the prebiotic potential of isolated chardonnay marc phenolics and oligosaccharides

The published studies described throughout this chapter that investigate the effects of grape phenolics and oligosaccharides are inherently limited in their abilities to determine the individual effects of grape marc phenolics and oligosaccharides on human health. Until the work

detailed in Chapter 2, which has yet to be published and distributed, no one had determined how to separate and collect oligosaccharides and phenolics when they are simultaneously present in a matrix. Thus, all these previous studies either tested commercial standard versions of grape marc compounds, compounds that are approximations of those in grape marc, or grape marc extracts that contain both oligosaccharides and phenolics.

The separation and collection of grape marc oligosaccharides and phenolics using PVPP and C18 SPE, as detailed in Chapter 2, enabled the first microbial tests on purified grape marc phenolics and oligosaccharides which were performed by our collaborator, Dr. Ishita Shah in the Mills Lab at UC Davis. In preliminary direct kill assays, the purified chardonnay marc phenolics destroyed the viability of *S. aureus* and *L. monocytogenes*, two gram-positive pathogens. Meanwhile the chardonnay marc phenolics did not affect *E. coli or K. pneumoniae,* the two gram-negative pathogens analyzed thus far. These results are in agreement with the findings of Karamati Jabehdar et al. (2018) where *S. aureus* viability was eliminated by grape marc while *E. coli* was unaffected by the presence of grape marc phenolics (Karamati Jabehdar, Mirzaei Aghjehgheshlagh, Navidshad, Mahdavi, & Staji, 2018), as discussed in Section 3. Additional research, which is currently in progress, is needed to conclusively verify the effects of grape marc phenolics on both commensal and pathogenic bacteria *in vitro* and *in vivo*.

Furthermore, we analyzed the effects of purified chardonnay marc oligosaccharides on commensal and pathogenic bacteria growth. All four common commensal bacteria tested underwent robust growth when incubated with the isolated grape marc oligosaccharides. *L. plantarum* experienced similar growth on the grape marc oligosaccharides compared to the positive glucose control (Figure 11). *B. animalis*, *B. infantis lactis*, and *L. rhamnosus* LGG all fermented the oligosaccharides but had less growth than when fed the glucose positive control

(Figure 11). Meanwhile, three pathogenic bacteria (*E. coli, S. aureus,* and *K.* pneumoniae) were tested in the presence of grape marc oligosaccharides and the assay resulted in minimal growth compared to the positive control. These results indicate that the grape marc oligosaccharides likely have prebiotic activity by stimulating vigorous growth of commensal bacteria over that of pathogenic bacteria. Further research is currently being done to better understand the commensal bacterial fermentation of the chardonnay marc oligosaccharides.

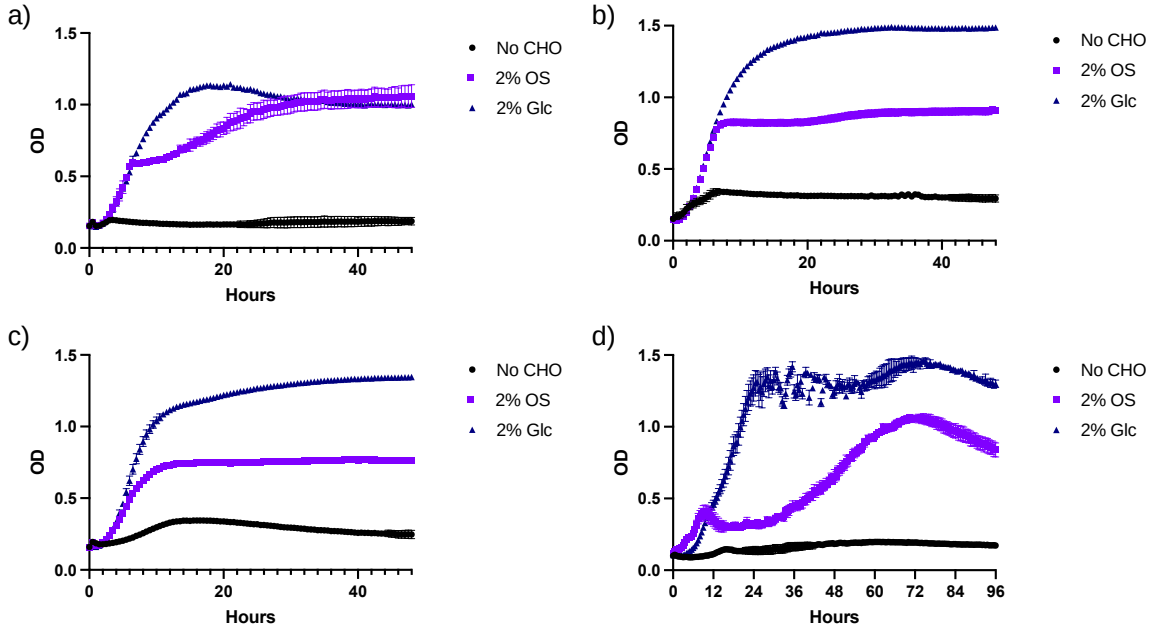

Figure 11. Example growth curves of commensal (a) *L. plantarum* b) *L.* rhamnosus LGG c) *B. animalis* and d) *B. infantis lactis* on isolated chardonnay marc oligosaccharides (2% OS), glucose positive control (2% Glc), and without a carbon source negative control (No CHO). Experiments were performed by Dr. Ishita Shah.

7. Culturing commensal bacteria, pathogenic bacteria, and colonic epithelial cells (Caco-2) on grape marc

In addition to testing the ability of commensal bacteria to grow when exposed to grape phenolics and oligosaccharides, studies have begun analyzing the effects of the marc as well as marc combined with resistant starch on the growth of commensal bacteria. *L. acidophilus* ATCC

43121 was shown to grow on medium containing low concentrations of grape marc extracts, however its growth improved significantly when resistant starch was added. Meanwhile the growth of *Bifidobacteria* ATCC 29521 increased at every concentration of grape marc extract both with and without added resistant starch (Karamati Jabehdar, Mirzaei Aghjehgheshlagh, Navidshad, Mahdavi, & Staji, 2018). Commensal *L. plantarum* 12A, *L. plantarum* PU1, *L. paracasei* 14A, and *B. breve* 15A were able to grow on media made with whole red grape marc both with and without 1% glucose supplementation (Campanella et al., 2017). All of these bacteria fermented the grape marc to produced lactic acid, titratable acids, and volatile acids. These results were particularly dramatic for *L. plantarum* PU1 and *B. breve* 15A. Each of the four tested strains also decreased the concentrations of free amino acids, gallic acid, (-)-epicatechin, and syringic acid but the bacterial fermentation of grape marc did not significantly alter its antioxidant activity (Campanella et al., 2017). The ability of grape marc to inhibit linoleic oxidation increased with fermentation, particularly with *L. plantarum* PU1. Additionally, all four fermenting bacteria strains analyzed consumed all of the citric acid and glycerol present in the medium in addition to the added 1% glucose supplementation, suggesting that these bacteria may be able to use the marc, including phenolics such as gallic acid, as an alternative carbon source for metabolism instead of sugars (Campanella et al., 2017). From these studies, it can be seen that grape marc meets the first requirement for prebiotics as it can enhance the growth of commensal gut bacteria by acting as a food source for these bacteria. *In vitro* studies identifying the ability of grape marc to inhibit pathogen growth are still needed to better assess grape marc's prebiotic functionality. However, the studies demonstrating pathogen reduction from phenolics that are abundant in grape marc discussed in section 3 indicate that grape marc likely hinders pathogen growth.

Beyond being prebiotic, the benefit of consuming grape marc on human health could be further increased by administering marc in conjunction with probiotics. When combined with probiotics, low marc concentrations decreased the oxidative damage to Caco-2 cells (human epithelial colorectal adenocarcinoma cells), thus improving the antioxidant protection of the cells (Campanella et al., 2017). Another study combined grape marc extract with *L. rhamnosus* IBNA02, *L. paracasei* 13239, and *L. acidophilus* 11692 and found the marc extract protected the *lactobacilli* strains. This prebiotic/probiotic combination also decreased the lipopolysaccharide (LPS) O-antigen instigated inflammation of Caco-2 intestinal cells, down-regulating most inflammation cytokine genes, proteins, and signaling molecules that were activated by LPS. This indicates the grape marc and lactobacilli pre/probiotic combination has potential to act as an intestinal inflammation treatment (Pistol, Marin, Dragomir, & Taranu, 2018). More studies should be done combining grape marc with probiotics to determine their potential health benefits as this could be a highly effective method to valorize grape marc.

8. Evaluation of grape marc through in vitro gastrointestinal digestion

Determining the bioaccessiblity of the bioactive compounds like phenolics and oligosaccharides present in grape marc is crucial to evaluating its potential health benefits. Bioaccessibility is the amount of a compound released from its food matrix in the lumen of the gastrointestinal tract that then becomes available for absorption into the body to produce its health effects. Simulated gastrointestinal digestion enables scientists to closely monitor the effects of each stage of digestion as well as the stabilities of individual compounds.

Multiple studies found grape marc phenolics partially degraded before they reached the large intestine. Many phenolics are pH sensitive, making them vulnerable to gastric acid. Both flavanol and anthocyanin contents decreased after passing through the stomach section of the

simulator. The gastric acid and pancreatic conditions of this segment likely induced the degradation (Costa et al., 2019; Jara-Palacios, Gonçalves, Hernanz, Heredia, & Romano, 2018). Additionally, the gastric acid conditions in the stomach also degraded grape marc oligosaccharides (Costa et al., 2019). Gil-Sánchez et al (2017), however, found that large phenolics reached the large intestine intact. Encapsulating grape antioxidants and oligosaccharides is a potential method to increase the amount of the potentially bioactive compounds that reach the large intestine for microbial fermentation. Alginate encapsulation of grape marc extract increases the concentration of intact phenolics in the large intestine where they were released with fermentation (Li, Loo, Cheng, Howell, & Zhang, 2019). The food matrix delivering bioactive compounds could also be optimized to improve phenolic and oligosaccharide stability, thus decreasing their degradation during the early stages of digestion without encapsulation.

After surviving the simulated upper GI tract, the phenolics content of the grape marc digestate increased during the intestinal phases. The gut microbes from the fecal inoculum induced this increase through two mechanisms. First, enzymes produced by gut microbes released phenolics bound to the cell walls and dietary fiber in the grape matrix. This made the phenols bioavailable and detectable (Gil-Sánchez et al., 2017; Li, Loo, Cheng, Howell, & Zhang, 2019). Second, gut microbes metabolize phenolics into metabolites that are more bioactive than the original larger phenolic compounds. This was determined as the phenolics originally present in the grape marc, including (+)-catechin and (-)-epicatechin, decrease significantly with digestion, while smaller phenolics that were not in the original marc are detected in the digestate (Li, Loo, Cheng, Howell, & Zhang, 2019; Corrêa et al., 2017). Furthermore, 21 grape marc phenolic metabolites were identified including benzoic acids, phenols, phenylpropionic acids,

phenlyactic acids, cinnamic acids, valeric acids, and valerolactones. The abundance of these metabolites decreased when grape marc supplementation stopped, indicating that they were generated during the microbial metabolism of the marc (Gil-Sánchez et al., 2017). The smaller grape marc phenolic metabolites were absorbed by the large intestine and provided at least some of the health benefits associated with grape marc consumption (Gil-Sánchez et al., 2017).

In addition to increasing the bioavailability of grape marc phenolics, supplementing the "diet" of a GI simulator with grape marc improved the composition and functionality of its "artificial" gut microbiome, generated with fecal inoculum from two healthy human donors, in every section of the colon. The abundance of *Lactobacillus*, *Bacteroides*, *Bifidobacterium*, *Enterococaceae*, *Clostridia XIVa*, *Enterobacteriaceae*, and *Faecalobacterium prausnitzii* derived from healthy human feces increased with chronic grape marc feeding. Of these, *Lactobacillus*, *Bacteroides*, *Clostridia XIVa*, *Enterobacteriaceae*, and *Faecalibacterium pausnitzii* growth increased most (Gil-Sánchez et al., 2017). Unfortunately, the specific components of the grape marc that lead to this increased growth are unknown as the marc contained simple sugars, oligosaccharides, and phenolics.

Gastrointestinal tract simulation studies illustrate that the phenolics present in grape marc have enough stability to reach the large intestine intact in high enough concentrations to affect the gut microbiome. While interacting with phenolics, commensal bacteria convert large phenolics into metabolites which are more readily absorbed by the intestines, increasing the biological functionality of grape marc while improving the composition of the gut microbiome. These studies further support the valorization of grape marc as a beneficial prebiotic and functional ingredient to supplement the diet.

9. Effects of grape marc supplementation on livestock

Grape marc is widely used as animal feed in wine regions to bring minimal value to this abundant waste material. However, in addition to acting as an economical feed source, recent studies indicate that whole grape marc diet supplementation increases livestock health and meat quality. Incorporating red grape marc as 9% of feed solids greatly improved both lamb and piglet gut microbiomes. In both cases, an increase in the abundance of commensal *Bifidobacterium* and a decrease in the abundance of *E. coli* and *Enterobacteriacae,* a family containing many pathogens, was observed (Kafantaris et al., 2017; Kafantaris et al., 2018). Grape marc had no effect on lactic acid bacteria, *Campylobacter*, or *Clostridia* populations in lambs but increased lactic acid bacteria growth and decreased *Campylobacter jejuni* growth in piglets (Kafantaris et al., 2017; Kafantaris et al., 2018). Meanwhile supplementing the diet of broiler chicks with 2% grape seeds reduced the abundance of *E. coli* and *Streptococcuss* while increasing beneficial bacteria growth including *Lactobacilli* in the ileum of chicks (Abu Hafsa & Ibrahim, 2018). These results indicate that grape marc can improve the gut microbiomes of animals, thus increasing the abundance of commensal bacteria and reducing the growth of pathogenic bacteria. Results from these studies also demonstrate improvement in gut barrier function, which has been tied to the fructan, polysaccharides, and phenolic compounds present in the grape marc (Kafantaris et al., 2017; Kafantaris et al., 2018).

In addition to curating a healthier gut microbiome, the marc demonstrated further positive effects on the animals. The lambs, piglets, and chicks fed grape marc or seeds experienced improved antioxidant mechanisms which was the likely cause of the decreased lipid peroxidation and protein oxidation throughout their bodies (Kafantaris et al., 2017; Kafantaris et al., 2018; Abu Hafsa & Ibrahim, 2018), thus producing not only healthier animals but also higher meat quality. The piglets also had an increased daily weight gain of 23.65% over the control group,

which is potentially the result of improved gut functionality stemming from the increased antioxidants reducing reactive oxygen species in the gut and therefore preventing damage to the intestinal membrane (Kafantaris et al., 2017). Similarly, the grape seeds significantly increased the chicks' weight gain without affecting their feed intake (Abu Hafsa & Ibrahim, 2018).

Grape marc proves to be a valuable potential feed additive for drastically different livestock as illustrated by ruminants (lambs), nonruminants (pigs), and poultry (chicks). These feeding studies indicate that grape marc increased animal welfare by improving their health particularly regarding their gut microbiomes. The grape marc also improved meat quality by decreasing lipid and protein oxidation and increasing yield in piglets and chicks. With further research with positive results, grape marc should be increasingly utilized and valued for its ability to improve livestock health and quality. Utilizing grape marc as a livestock health supplement would likely be a relatively rapid avenue for grape marc valorization as livestock already safely consume grape marc. However, the market value of grape marc as animal feed should increase from its current low value as a waste. As grape marc becomes better known and used as a valuable functional feed ingredient for improving livestock products it should increase in value, making livestock feed supplementation a potentially viable valorization strategy for grape marc.

10. Potential for human health improvement from grape marc through small animal studies and a human clinical trial

Grape marc's benefits have the potential to extend beyond improving livestock to positively impact human health as a functional food ingredient. Initial studies of the effects of grape marc and grape seed phenolics on commensal and pathogenic bacteria suggests that grape marc and seed diet supplementation could positively impact human health. Small animal studies

of rats and mice ingesting significant quantities of grape marc in its various forms have been performed as the precursor to human trials to determine the impact of marc consumption.

In one study, rats received red grape marc extract which positively altered their gut microbiomes by promoting beneficial bacteria with a 21-27% increase of *Bifidobacterium* bacteria with marc extract consumption while inhibiting the growth of pathogenic bacteria. The abundance of *Bifidobacterium* plateaued with high marc extract doses likely because catechin reached its saturation limits within the plasma of the rats. Meanwhile, commensal *Enterococcus* bacteria were unaffected by the marc. *Lactobacillus* growth decreased with grape marc supplementation, however data from other studies suggest a higher marc dose than was delivered in this study is likely to remedy this. Additionally, pathogenic *Clostridium* growth was inhibited by the marc treatment (Chacar et al., 2018), an observation that can have translational relevance in modern medical foods with the ability to reduce Clostridia.

Seo, Kim, Jeong, Yokoyama, & Kim (2017) used an obese mice model and after supplementing a high fat diet with chardonnay seeds, observed dramatic overall health improvements correlated with improved gut microbiome health from the chardonnay seed intervention. The mice fed grape seeds had decreased body, liver, and adipose tissue weights and lower LDL levels despite having increased food intake. The abundance *Lactobacillus* and *Bifidobacterium*, two well classes well known for containing commensal and prebiotic bacteria, were found to be dependent on the flavonoid presence in mice feces. *Akkermansia* abundance increased with grape marc and seed extract supplementation in mice and, in another study was linked to improved gut barrier function and mucus thickness as well as decreased fat mass, insulin resistance, endotoxemia, and adipose tissue inflammation, thus helping to decrease obesity and diabetes in obese mice (Lu, Liu, Zhou, Hu, & Zhang, 2019; Everard et al., 2013).

Meanwhile, the abundance of Firmicutes bacteria including Firmicutes include *Clostridium*, *Rosebuira*, *Lactobacillus*, *Enterococcus,* and *Oscillibacter* decreased in the mice feces (Seo, Kim, Jeong, Yokoyama, & Kim, 2017). *Lactobacillus* and *Oscillibacter* abundance are correlated with weight gain as they can ferment polysaccharides, releasing additional energy that can be absorbed, thus their decreased abundance likely contributed to the weight loss observed in the mice. *Roseburia*, *Adlercreutzia*, and *Enteroccoccus* are also correlated with body and adipose tissue weight gain. For example, *Aldercreutzia* was able to biotransform epigallocatechin, a phenolic compound present in grape marc (Antoniolli, Fontana, Piccoli, & Bottini, 2015; Seo, Kim, Jeong, Yokoyama, & Kim, 2017). Therefore, the decreased concentrations of these bacteria as well as the increased abundance of *Akkermansia* within the mice feces indicates that the influence of chardonnay seeds on the gut microbiome could have potentially contributed to the weight loss and health benefits observed in mice.

Chardonnay seeds, grape marc extract, and grape seed extracts improved gut microbiomes in mice which resulted in numerous health benefits.

In addition to having the potential to improve the gut microbiome of healthy individuals and decrease obesity related health problems, grape marc has the potential to help regenerate healthy gut microbiomes that have been altered by antibiotics use. Antibiotic treatment of mice decreased the diversity and abundance of microbes in their feces, particularly regarding commensal bacteria. The gut bacteria repopulate following antibiotic treatment and can develop into unhealthy gut microbiomes if sufficient commensal bacteria are not present, resulting in dysbiosis. Consuming grape marc and seed extracts following antibiotic treatment increased both the diversity and abundance of commensal microbiota within mice intestines (Lu, Liu, Zhou, Hu, & Zhang, 2019). Compared to control mice whose gut microbiomes repopulated without

intervention, mice given grape marc and seed extracts showed increased commensal *Verrucomicrobia*, *Akkermansia,* and *Alloprevotella* within their feces. The seed extract supplementation also produced increased levels of *Prevotella*. Additionally, the grape marc and seed extract supplementation decreased the abundance of pathogenic *Streptococcus* and *Actinobacteria* (Lu, Liu, Zhou, Hu, & Zhang, 2019). Based on these results, grape marc and grape seeds have the potential to act as therapeutic prebiotics following antibiotic intervention to repopulate the gut with a healthy microbiome favoring commensal bacteria and reducing pathogenic bacteria.

These small animal studies were followed by a 2020 human clinical trial to examine the cardiovascular health effects of chardonnay seeds and particularly chardonnay seed phenolics. Initial trials and the primary study found chardonnay seed supplements to be safe in low doses of 4.8 g/day but to cause non-severe negative gastrointestinal effects in higher doses of 24 g/day (Corban et al., 2020). Interestingly, consuming phenolic rich chardonnay seed supplements or phenolic free supplements that attempted to mimic the composition of chardonnay seeds minus the phenolics had similar effects on the health of the participants. Both groups experienced similar levels of endothelial function improvement and peripheral endothelial function including decreased systolic and diastolic blood pressure (Corban et al., 2020). Due to the similarities in results induced by the phenol rich and phenol free chardonnay seed supplements, Corban et al. (2020) hypothesized that phenolic compounds, which have largely been assumed to cause the health benefits in earlier *in vitro* and small animal studies, are not the impactful bioactive compound in chardonnay seeds for cardiovascular health. Instead, Corban et al. (2020) speculated that the dietary fiber fraction which was consistent in both supplements caused the

observed cardiovascular health benefits as dietary fibers have previously demonstrated cardioprotective effects.

11. Conclusion

Grape marc is an exceedingly abundant, underutilized, and undervalued coproduct of the wine industry. Grape marc contains phenolic compounds including gallic acid, (-)-epicatechin, and (+)-catechin. Microbial analysis found that grape marc selectively promotes the growth of many commensal bacteria strains while other types of bacteria, including various pathogens, are highly sensitive to the marc and its components. Overall, the compounds within the marc's matrix stimulate commensal bacteria growth while largely preventing pathogen proliferation in cell cultures, small animal studies, and larger animal studies (Tables 2, 4, 5, 6), thus leading to diverse gut microbiota composition and resulting in improved intestinal health and function. Grape marc also protects probiotic strains from the harsh conditions of the upper GI tract, enabling them to reach the colon, thus indicating the tremendous potential of grape marc in a prebiotic/probiotic combination supplement to improve human gut health. Additionally, cell and animal studies show that grape marc may have the ability to greatly affect animal health and likely human health by decreasing intestinal cell inflammation, obesity and its related illnesses, and preventing lipid and protein oxidation. Chardonnay seed consumption also demonstrated cardioprotective effects in humans, likely caused by the dietary fiber and the oligosaccharides present within the seeds. With the impressive results of the small animal and small livestock studies, more research should be performed to target additional grape marc applications such as with further human trials and large livestock studies with animals like beef cattle to determine the effects of grape marc on the organisms that would likely be the commercial targets for grape marc supplementation. Future studies utilizing well-characterized and purified fractions of grape

marc will enable to differentiate the contribution of phenolics and oligosaccharides to human and animal health. Grape marc has immense potential as a functional ingredient to improve health, yield, and quality of livestock as well as human health while helping the environment by utilizing a waste product.

Table 17. Compilation of effects of grape marc, grape seeds, and their extracts on non-commensal bacteria including opportunistic pathogens grown under various in vitro conditions and the carbohydrate and phenolic analysis performed (Karamati Jabehdar, Mirzaei Aghjehgheshlagh, Navidshad, Mahdavi, & Staji, 2018; Costa et al., 2019; Gil-Sánchez et al., 2017; Tabasco et al., 2011). In vitro growth assays were performed with BHI broth.

Bacteria	Growth Effect	Marc Intervention	Study Type	Measured Carbohydrates	Measured Phenolics	Source
Clostridia XIVa	Increased	Grape marc extract	GI tract simulator	Extract dietary fiber and monosaccharides	Extract TPC and individual phenolics	(Gil-Sánchez et al., 2017)
E. coli ATCC 25922	Decreased	Enzymatic grape marc extract	*In vitro* growth assay	Total dietary fiber, monosaccharide, XOS, and polysaccharide quantification before and after incubation	TPC and individual phenolics before and after incubation	(Costa et al., 2019)
E. coli ATCC 25922	No effect	Grape seed extract	*In vitro* growth assay	N/A	Extract Individual phenolics	(Tabasco et al., 2011)
E. coli ATCC 35218	Increased	Grape marc extract	*In vitro* growth assay	N/A	Extract TPC	(Karamati Jabehdar, Mirzaei Aghjehgheshlagh, Navidshad, Mahdavi, & Staji, 2018)
E. coli BW13711	Decreased	Grape seed extract	*In vitro* growth assay	N/A	Extract Individual phenolics	(Tabasco et al., 2011)
E. coli CECT 5947	Increased	Grape seed extract	*In vitro* growth assay	N/A	Extract Individual phenolics	(Tabasco et al., 2011)
E. coli WTT1	Decreased	Grape seed extract	*In vitro* growth assay	N/A	Extract Individual phenolics	(Tabasco et al., 2011)
Enterococaceae	Increased	Grape marc extract	GI tract simulator	Extract dietary fiber and monosaccharides	Extract TPC and individual phenolics	(Gil-Sánchez et al., 2017)
Eterobacteriaceae	Increased	Grape marc extract	GI tract simulator	Extract dietary fiber and monosaccharides	Extract TPC and individual phenolics	(Gil-Sánchez et al., 2017)
Faecalibacterium prausnitzii	Increased	Grape marc extract	GI tract simulator	Extract dietary fiber and monosaccharides	Extract TPC and individual phenolics	(Gil-Sánchez et al., 2017)
Pseudomonas aeruginosa ATCC 10145	Decreased	Enzymatic grape marc extract	*In vitro* growth assay	Total dietary fiber, monosaccharide, XOS, and polysaccharide quantification before and after incubation	TPC and individual phenolics before and after incubation	(Costa et al., 2019)
S. salivarius ZL50-7	Decreased	Grape seed extract	*In vitro* growth assay	N/A	Extract Individual phenolics	(Tabasco et al., 2011)
S. salivarius ZL93-3	Decreased	Grape seed extract	*In vitro* growth assay	N/A	Extract Individual phenolics	(Tabasco et al., 2011)
S. sobrinus ATCC 33478	No effect	Grape marc extract	*In vitro* growth assay	N/A	Extract TPC	(Karamati Jabehdar, Mirzaei Aghjehgheshlagh, Navidshad, Mahdavi, & Staji, 2018)
S. thermophilus STY-31	Decreased	Grape seed extract	*In vitro* growth assay	N/A	Extract Individual phenolics	(Tabasco et al., 2011)

Table 18. Compilation of effects of grape marc, grape seeds, and their extracts on commensal bacteria grown under various in vivo conditions as well as the carbohydrate and phenolics analysis performed in each study (Chacar et al., 2018; Kafantaris et al., 2017; Kafantaris et al., 2018; Abu Hafsa & Ibrahim, 2018; Seo, Kim, Jeong, Yokoyama, & Kim, 2017).

Bacteria	Growth Effect	Marc Intervention	Study Type	Measured Carbohydrates	Measured Phenolics	Source
Bifidobacterium	Increased	Red grape marc extract, red grape marc	Rat gut microbiome, lamb and piglet gut microbiomes	N/A, crude fiber of diet	Extract TPC and individual phenolics, TPC of diet	(Chacar et al., 2018; Kafantaris et al., 2017; Kafantaris et al.)
Bifidobacterium	Decreased	White grape seed	Mice gut microbiome	N/A	N/A	(Seo, Kim, Jeong, Yokoyama, & Kim, 2017)
Lactobacillus	Decreased	Red grape marc extract, white grape seed	Rat gut microbiome, mice gut microbiome	N/A	Extract TPC and individual phenolics, N/A	(Chacar et al., 2018; Seo, Kim, Jeong, Yokoyama, & Kim, 2017)
Lactobacillus	Increased	Red grape seed	Broiler chick ileum	N/A	TPC of diet	(Abu Hafsa & Ibrahim, 2018)

Table 19. Compilation of effects of grape marc, grape seeds, and their extracts on non-commensal bacteria including opportunistic pathogens grown under various in vivo conditions as well as the carbohydrate and phenolics analysis performed in each study (Chacar et al., 2018, Lu, Liu, Zhou, Hu, & Zhang, 2019; Kafantaris et al., 2017; Kafantaris et al., 2018; Abu & Ibrahim, 2018; Seo, Kim, Jeong, Yokoyama, & Kim, 2017).

Bacteria	Growth Effect	Marc Intervention	Study Type	Measured Carbohydrates	Measured Phenolics	Source
Adlercreutzia	Increased	White grape seed	Mice gut microbiome	N/A	N/A	(Seo, Kim, Jeong, Yokoyama, & Kim, 2017)
Akkermansia	Increased	Red grape marc extract, red grape seed extract	Mice gut microbiome	N/A	Extract individual phenolics	(Lu, Liu, Zhou, Hu, & Zhang, 2019)
Allobaculum	Decreased	White grape seed	Mice gut microbiome	N/A	N/A	(Seo, Kim, Jeong, Yokoyama, & Kim, 2017)
Alloprevotella	Increased	Red grape marc extract, red grape seed extract	Mice gut microbiome	N/A	Extract individual phenolics	(Lu, Liu, Zhou, Hu, & Zhang, 2019)
Anaerosporobacter	Increased	White grape seed	Mice gut microbiome	N/A	N/A	(Seo, Kim, Jeong, Yokoyama, & Kim, 2017)
Anaerotruncus	Decreased	White grape seed	Mice gut microbiome	N/A	N/A	(Seo, Kim, Jeong, Yokoyama, & Kim, 2017)
Blautia	Increased	White grape seed	Mice gut microbiome	N/A	N/A	(Seo, Kim, Jeong, Yokoyama, & Kim, 2017)
Campylobacter jejuni	No effect	Red grape marc	Lamb gut microbiome	N/A	TPC of diet	(Kafantaris et al., 2017)
Campylobacter jejuni	Decreased	Red grape marc	Piglet gut microbiome	Crude fiber of diet	TPC of diet	(Kafantaris et al., 2018)
Clostridia	No effect	Red grape marc	Lamb and piglet gut microbiomes	N/A, crude fiber of diet	TPC of diet	(Kafantaris et al., 2017; Kafantaris et al., 2018)
Clostridium	Increased	Red grape marc extract	Rat gut microbiome	N/A	Extract TPC and individual phenolics	(Chacar et al., 2018)
Clostridium	Decreased	White grape seed	Mice gut microbiome	N/A	N/A	(Seo, Kim, Jeong, Yokoyama, & Kim, 2017)
E. coli	Decreased	Red grape marc	Lamb and piglet gut microbiomes	N/A, crude fiber of diet	TPC of diet	(Kafantaris et al., 2017; Kafantaris et al., 2018)
E. coli	Decreased	Red grape seeds	Broiler chick ileum	N/A	TPC of diet	(Abu & Ibrahim, 2018)
Enterobacteriaceae	Decreased	Red grape marc	Lamb and piglet gut microbiomes	N/A, crude fiber of diet	TPC of diet	(Kafantaris et al., 2017; Kafantaris et al., 2018)
Enterococcus	No effect	Red grape marc extract	Rat gut microbiome	N/A	Extract TPC and individual phenolics	(Chacar et al., 2018)
Enterococcus	Increased	White grape seed	Mice gut microbiome	N/A	N/A	(Seo, Kim, Jeong, Yokoyama, & Kim, 2017)
Enterorhabdus	Decreased	White grape seed	Mice gut microbiome	N/A	N/A	(Seo, Kim, Jeong, Yokoyama, & Kim, 2017)
Flavonifractor	Increased	White grape seed	Mice gut microbiome	N/A	N/A	(Seo, Kim, Jeong, Yokoyama, & Kim, 2017)
Odoribacter	Decreased	White grape seed	Mice gut microbiome	N/A	N/A	(Seo, Kim, Jeong, Yokoyama, & Kim, 2017)
Oscillibacter	Decreased	White grape seed	Mice gut microbiome	N/A	N/A	(Seo, Kim, Jeong, Yokoyama, & Kim, 2017)
Parabacteroides	Decreased	White grape seed	Mice gut microbiome	N/A	N/A	(Seo, Kim, Jeong, Yokoyama, & Kim, 2017)
Peptococcus	Decreased	White grape seed	Mice gut microbiome	N/A	N/A	(Seo, Kim, Jeong, Yokoyama, & Kim, 2017)
Prevotella	Decreased	Red grape marc extract	Mice gut microbiome	N/A	Extract individual phenolics	(Lu, Liu, Zhou, Hu, & Zhang, 2019)
Prevotella	Increased	Red grape seed extract	Mice gut microbiome	N/A	Extract individual phenolics	(Lu, Liu, Zhou, Hu, & Zhang, 2019)
Roseburia	Increased	White grape seed	Mice gut microbiome	N/A	N/A	(Seo, Kim, Jeong, Yokoyama, & Kim, 2017)
Streptococcus	Decreased	Red grape marc extract, red grape seed extract	Mice gut microbiome	N/A	Extract individual phenolics	(Lu, Liu, Zhou, Hu, & Zhang, 2019)
Streptococcus	Decreased	Red grape seed	Broiler chick ileum	N/A	TPC of diet	(Abu & Ibrahim, 2018)

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