ABSTRACT

VONG, CHI IN. Small Phenolic Acids in Rum Fermentation Products. (Under the direction of Dr. Slavko Komarnytsky).

Polyphenols are a versatile group of phenolic compounds that can be found in many dietary sources, including fruits, vegetables, legumes, nuts and seeds, grains, spices, and herbs used in medicinal and beverage preparations. The polyphenolic structures are very diverse and can be generally classified into several major groups, including phenolic acid and their esters, flavonoids, chalcones, stilbenes, tannins, and lignans. Flavonoids are further differentiated based on the structural modification of the C-ring into anthocyanins, flavones, flavonols, flavanones, flavanols, and isoflavones. The C-ring fission reactions during degradation and microbial fermentation of polyphenols produce a diversity of small phenolic acid metabolites that differ in structure, polarity, stability, absorption and metabolism, tissue distribution, and associated biological activity upon ingestion. Precise understanding of phenolic metabolite pools that originate from different groups of polyphenols is critical for the development of future nutritional interventions and dietary guidelines. In this work, we also highlighted the recent findings on the distribution and metabolism of dietary polyphenols with a particular focus on the phase II metabolism that results in a wide variety of glucuronidated, sulfated, methylated, and glycine/glutamine conjugated products both in circulation (whole blood or plasma) and in the waste liquids destined for excretion (urine and fecal water). In doing so, we specifically focused on the radiolabeled tracer studies that provided a unique opportunity to eliminate food matrix effects and provide *in vivo* human data on the precise origin and metabolism of small phenolic compounds otherwise obscured by the diversity of polyphenols found in each food source. The hopes to apply these findings successfully and safely to tackle future dietary reference intakes, nutritional interventions, and public health outcomes rely in part on our ability to address the

identification of all small phenolic metabolites and understand the subtle differences in personalized metabolism and microbiome profiles that can have a disproportionate role on human health and wellbeing outcomes.

Sugarcane (*Saccharum officinarum* L.) is the world's largest crop by production quantity, yet it is one of the lesser-known sources of polyphenols. It is an important agricultural commodity in tropical and subtropical countries because its stalks (culms) accumulate sucrose that is used in the production of table sugar. Sugarcane raw materials are also used in fermentation industries to produce biofuels (ethanol) and distilled spirits (cachaça and rum). After sugarcane juice or the concentrated product (molasses) is fermented, a significant quantity of sugarcane polyphenols ends up in the final, partially dehydrated, byproduct (vinasse). Large amounts of vinasse effluents pose a significant recycling and environmental threat. We, therefore, attempted to determine the small phenolic acid metabolite profiles of molasses (pre-fermentation) and vinasse (post-fermentation) materials generated by fermentation and distillation of rum. Stability in the fermentation medium decreased, and the vinasse end products were enriched with diosmetin, vanillic, and syringic acids. Phenolic acid metabolites found in vinasse reduced pro-inflammatory gene expression profiles in the LPS-stimulated macrophages, with benzoic and cinnamic acids showing maximum anti-inflammatory effects. This study lays the foundation for future investigations evaluating the effects of phenolics present in sugarcane waste streams and their putative applications to managing immune health outcomes.

Small Phenolic Acids in Rum Fermentation Products

by
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DEDICATION

To my beloved parents, for their endless love, support, and encouragement, and for teaching me to make plans, set goals and enjoy everything that I choose to do.

BIOGRAPHY

Chi In “Angela” Vong was born in Macau and spent thirteen years of her youth in Macau before moving to Dublin, Ireland, to continue her middle school, high school, and college educations. After she graduated with her first bachelor’s degree in Accounting from the National College of Ireland in 2014, she decided to pursue her passion in the science field in the U.S. She then moved to Ocala, Florida, and subsequently to Atlanta, Georgia, where she completed her second bachelor’s degree in Nutrition Science from Georgia State University in 2020. During her time at Georgia State University, Angela was fortunate to meet her food science professor who allowed her to work in his nutrition research lab, which opened a door to her interests in food science and research. Her passion and hands-on work in the lab motivated her to further her education with a master’s degree in Food Science at North Carolina State University. She began her Master's studies in Raleigh, North Carolina, in August 2020 and her Master's research in the Plants for Human Health Institute in Kannapolis, North Carolina, under the direction of Dr. Slavko Komarnytsky. During her time at North Carolina State University, Angela served as Treasurer in the Food Science Club during the 2021-2022 academic year. Following graduation, Angela intends to expand and utilize her scientific knowledge and gain valuable experience in the industry.

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CHAPTER 1

ALL POLYPHENOLS ARE NOT CREATED EQUAL: EXPLORING THE DIVERSITY OF PHENOLIC METABOLITES

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Abstract

Dietary intake of plant polyphenols is significant, yet many of these compounds are not bioavailable and enter the human body as a highly diverse pool of ring-fission phenolic metabolites arising from digestion and microbial catabolism of the parental polyphenols in the gastrointestinal tract. Difficulty in designing the uniform intervention studies, the variety of nomenclatures, and limited tools calibrated to detect and quantify the inherent complexity of phase I and phase II phenolic metabolites hindered efforts to establish and validate protective health effects of these molecules. Here we highlight the recent findings that describe complex downstream metabolite profiles with a particular focus on dihydrophenolic (phenylpropanoic) acids of microbial origin, ingested and phase II-transformed methylated phenolic metabolites (methylated sinks), and small phenolic metabolite signatures derived from the breakdown of different classes of flavonoids, stilbenoids, and tannins. There is a critical need for precise identification of phenolic metabolites in diverse biological fluids that accounts for polyphenol sources, interindividual variability, microbial strains and a consortium of a model microbiome, and host physiological outcomes to enable the future translation of these findings into break-through nutritional interventions and dietary guidelines.

Keywords: polyphenols, phenolic acids, microbiome, microbial catabolism, methylation

Abbreviations:

ADME	absorption, distribution, metabolism, and excretion
BA	benzoic acid
2HBA	2-hydroxybenzoic
3HBA	3-hydroxybenzoic
4HBA	4-hydroxybenzoic
CA	cinnamic acid
2HCA	*o*-coumaric acid
3HCA	*m*-coumaric acid
4HCA	*p*-coumaric acid
3,4DHCA	caffeic acid
4H3MCA	ferulic acid
COMT	catechol-*O*-methyltransferase
HSA	human serum albumin
SAM	S-adenosyl-l-methionine

1.1 Introduction

For this work, we propose to loosely define polyphenols as a group of plant, algae, and fungal secondary metabolites that contain at least one hydroxylated aromatic ring (phenyl) moiety as found in phenolic acids, stilbenes, flavonoids, and lignans (**Figure 1.1**). This definition builds upon other historical definitions of polyphenols [1], including the White-Bate-Smith-Swain-Haslam WBSSH limit of 5-7 aromatic rings per 1000 Da and the Quideau limit of the shikimate/phenylpropanoid and/or the polyketide origin [2]. Oligomeric or polymeric forms of polyphenols capable of binding and precipitating proteins are further classified as hydrolysable tannins (derived from gallic acid or ellagic acid and a carbohydrate core, typically D-glucose or quinic acid), condensed tannins (derived from flavans with no sugar residues), and phlorotannins (derived from phloroglucinol) [3]. Soluble polyphenols are sequestrated in small vesicles and vacuoles, while insoluble forms are deposited as structural components of cell walls and represent the next significant portion of terrestrial biomass after cellulose and hemicellulose [4]. Decomposing plant matter, and live plants to a smaller degree, leach on average 20-200 kg/ha/yr of soluble polyphenols into their immediate surroundings where they contribute to tannins and humic substances found in soils and black water streams [5]. Advanced imaging studies of subcellular localization of phenolic compounds also point to phenyloplasts, a specialized subset plastids which are used for accumulation and storage of excessively high levels of phenolics as shown for 4-O-(3-methoxybenzaldehyde) β-d-glucoside in vanilla beans [6]. Here phenolic constituents may serve as primary targets for chloroplast-localized polyphenol oxidases producing phenolic-derived quinones that subsequently polymerase into post-harvest browning oxidation products and cross-linked proteins [7]. Overall concentration of polyphenols is highly variable among different plant taxa and within individuals based on geographical area, climate,

and biotic interactions as they show remarkable plasticity in response to a variety of environmental factors [8].

Polyphenols originate from two biosynthetic pathways: polyacetate (polyketide) and phenylpropanoid, while flavonoids constitute a combination of these two routes. The acetate pathway provides malonyl-CoA that is catalyzed by a two-step reaction shared between the production of flavonoids and the elongation of long-chain fatty acids [9]. The phenylpropanoid metabolic pathway uses phenylalanine (all plants) or tyrosine (Poaceae) to produce the phenylpropanoid core of all polyphenols including cinnamic acids (C6-C3), benzoic acids (C6-C1), flavonoids (C6-C3-C6), proanthocyanidins [(C6-C3-C6)n], stilbenes (C6-C2-C6), lignans (C6-C3-C3-C6) and lignins [(C6-C3)n] [10]. The derivatives of cinnamic acid form the basis for a wide variety of these phenolic metabolites, however, the exact mechanisms, transporters, and metabolic fluxes in the phenylpropanoid pathway have not been elucidated as they require tightly synchronized compartmentalization into the chloroplast (phenylalanine synthesis), cytoplasm (phenolic acids), and vacuole (phenolic acid esters) [11].

Throughout history humans, both actively and passively, ingested, absorbed, and inhaled a wide range of phenolic substances as a part of a regular diet (**Table 1.1**), food additives and preservatives (i.e., sodium benzoate), pharmaceutical drugs (i.e., acetylsalicylic acid or aspirin), and environmental pollutants (coal tar, creosote, industrial and agricultural wastes such as chlorophenols). Upon ingestion, phenolic compounds are metabolized by digestive enzymes and intrinsic microbiota populations that show high interindividual variability [12], produce complex metabolite profiles not indicative of bioavailability of the original parental structures [13], and therefore make predictions of the microbiome and host responses to a dietary intervention difficult. Microbial biotransformation of phenolic metabolites in foods such as the conversion of

ferulic acid to 4-vinyl guaiacol [14] or vanillic acid [15] is not uncommon, as well as mobilization of simple phenolics after fermentation [16,17]. Phenol (hydroxybenzene) is also produced naturally during the decomposition of organic matter and released as a tyrosine metabolic waste product by the gastrointestinal microbiome together with p-cresol [18].

Stomach absorption of intact polyphenols is very limited, however passive absorption of parental aglycones in the upper gastrointestinal tract can be facilitated by enzymatic hydrolysis of the respective glycosides by the transmembrane lactase-phloridzin hydrolase and cytosolic β-glucosidases found within the small intestine epithelial cells in the brush border [19]. Absorbed phenolics further undergo phase I (oxidation, reduction) and phase II (conjugation) xenobiotic metabolism, and this transformation is required for the conversion of less-polar phenolic constituents to their water-soluble metabolites that allows excretion by kidneys. In humans, however, phase I metabolism of polyphenols via cytochrome P450 monooxygenases is rather limited in favor of phase II conjugation [20]. Phenolic molecules that enter phase II metabolism diffuse into the endoplasmic reticulum lumen where they are glucuronidated by UDP-glucuronosyltransferases or remain in the cytoplasm where they are sulfated by cytoplasmic sulfotransferases. Methylation occurs both in the cytosol or in the endoplasmic reticulum depending on the presence of soluble or membrane-bound catechol-O-methyltransferase [21]. Alternatively, low molecular weight phenolics can enter mitochondria where they are conjugated with glycine (human, benzoic acid), glutamine (human, phenylacetic acid), ornithine (chicken, benzoic acid), taurine (pigeon, phenylacetic acid), or arginine (spiders, benzoic acid), to name a few, by the respective N-acyltransferases, and this process differs both qualitatively and quantitatively between animals that may be reflective of their evolutionary physiology and dietary habits [22]. In some extreme instances, certain phase II metabolism pathways show a

remarkably reduced capacity as seen with glucuronidation in cats and sulfation in pigs [23]. Additionally, aromatization plays a secondary role in adding to the pool of circulating phenolic metabolites, as evident from the transformation of dietary alicyclic quinic and shikimic acids into benzoic and hippuric acids prior to excretion [24]. The degree of conjugation of a phenolic metabolite with a particular phase II pathway must therefore relate to its physiochemical properties, and different fragmentation patterns of phenolic catabolites originating from various parental polyphenolic structures are expected to produce different metabolic signatures and physiological outcomes; thus current understanding and extrapolation of dietary phenolic intakes and their relationship to human health and wellbeing outcomes need critical rethinking.

1.2 Low molecular weight phenolic acids

Low molecular weight phenolic acids available from various dietary sources of primarily plant origin generally belong to two major groups, benzoic (C6-C1) and cinnamic (C6-C3) acids [25]. There are no significant food sources of benzoic (BA), 2-hydroxybenzoic (2HBA), 3-hydroxybenzoic (3HBA), and 4-hydroxybenzoic (4HBA) acids or their esters and glycosides with a possible exception of raw olives (**Table 1.1**). Moderate amounts of free dihydroxybenzoic and trihydroxybenzoic acids can be found in certain spices, berries, and black tea, with several niche foods containing large amounts as shown for cloves [26] and chestnuts [27] at 250-900 mg/100 g. As such, most benzoic acid metabolites found in healthy human circulation will likely originate from microbiota catabolism or degradation of larger phenolic structures including flavonoids and hydrolysable tannins.

Likewise, there are no good dietary sources of free or glycosylated *trans*-cinnamic acid (CA), *o*-coumaric acid (2HCA), *m*-coumaric acid (3HCA), and *p*-coumaric acid (4HCA) (**Table**

1.1). Among dihydroxycinnamic acids, only caffeic acid (3,4DHCA) and ferulic acid (4H3MCA) are present in the human diet in significant quantities, while other natural isomers such as umbellic acid (2,4DHCA), 2,3DHCA, 2,5DHCA, and 3,5DHCA only appear in blood circulation as trace metabolites [28]. Large amounts of free caffeic acid were reported for black chokeberry [29] at 140 mg/100 g, while ferulic acids were common in whole grain durum wheat [30]. These two phenolic acids, however, appear in much larger quantities in our diets as soluble esters of quinic acid including caffeoylquinic acids and their respective methyl and ethyl esters [31], tartaric acid (chicoric acid), and 3,4-dihydroxyphenylacetic acid (rosmarinic acid), as well as cell wall polysaccharide-linked esters such as diferulic acids [32]. In rare instances, these molecules are also conjugated to anthranilic amino acids to form phenolic alkaloids avenanthramides found in oats [33]. Typically, all these structures are reduced and/or cleaved by microbiota following ingestion to release caffeic, ferulic, dihydrocaffeic, and dihydroferulic acid metabolites into circulation [34]. Another often overlooked class of small phenolic metabolites lacking quantitative data is cinnamoyl glucoses (glycosides and glucose esters of hydroxycinnamic acids) that exist in multiple isomeric arrangements totaling to 2-8 mg/100 g in tomatoes [35] and 1-5 mg/100 g in berries [36]. This class of phenolic compounds retains biological activity similar to its unconjugated counterparts [37].

5-Caffeoylquinic acid (chlorogenic acid, 5CQA) is one of the most abundant plant soluble phenolic constituents and is found in significant amounts in sunflower seeds (450 mg/100 g), chicory and artichoke (160-200 mg/100g), coffee (40-120 mg/100 ml) and certain fruits such as prune, blueberry, and apple (40-80 mg/100g). Coffee and prunes are also a rich source of their natural isomers, 3-caffeoylquinic acid (neochlorogenic acid, 3CQA) at 50-120 mg/100 g and 4-caffeoylquinic acid (cryptochlorogenic acid, 4CQA) at 20-60 mg/100 g (**Table**

1.1). These compounds, like other classes of polyphenols, are found in plant tissues in large different sets of isomeric structures as exemplified by green coffee beans where up to 40-90 isomeric metabolites were detected, and this complexity is dramatically increased to 200-250 distinct molecular formulas after food processing such as roasting [38].

Dicaffeoylated CQAs such as 3,5CQA, 3,4CQA, and 4,5CQA are typically present in foods in much lower quantities in the range of 2-6 mg/100 g [39], however, they represented 40-60% of chlorogenic acids found inmate (*Ilex paraguariensis* A.St.-Hill.) and were quantified at 18-20 mg/100 ml beverage [40]. As such, people who regularly consume coffee or mate obtain their bulk of dietary phenolic constituents from chlorogenic acids [41]. Most bioavailability and metabolism studies of the caffeoylquinic acids are based on ingestion of coffee or coffee bean extracts, and therefore it is difficult to correctly determine the origin of all phenolic metabolites observed in these reports. The size and lipophilicity of phenolic acid esters generally limit their passive diffusion across the cell membranes and result in a biphasic appearance in blood circulation, peaking at 1h (absorption in the small intestine) and 5h (colonic metabolism) after ingestion [42]. The most recent study looking at healthy volunteers consuming coffee that contained 310 mg chlorogenic acid reported the presence of 56 phenolic metabolites in plasma [43]. It is interesting to note that caffeic acid metabolites were predominantly sulfated with 3´-O-sulfate moiety being the most abundant, ferulic acids were equally glucuronidated and sulfated, while coumaric acids were present in very small amounts. However, dihydro metabolites of all phenolic acids presumably formed in the colon by microbiota-mediated hydrogenation of the double bonds are most abundant in plasma in their free, nonconjugated forms (**Table 1.3**). When compared with the earlier study reporting urinary caffeoylquinic acid metabolites under similar conditions showed only trace amounts of parental structures but a similar distribution of

conjugated phenolics [44]. Finally, although caffeic acid derivative dominates the coffee phenolic profile, a prominent shift towards ferulic and isoferulic metabolites indicates extensive m-hydroxyl and lesser p-hydroxyl methylation, presumably via catechol-*O*-methyltransferase (COMT) to significantly increase plasma pool of methylated phenolic structures, with an overall preference for 3′-methylation [41]. This was also observed earlier both in humans and rats giving chlorogenic acid and caffeic acid, although rats produced 3-hydroxyphenylpropanoic acid as a major metabolite [45]. Among the downstream benzoic acid metabolites, vanillic and 3-hydroxybenzoic acids derived presumably from partial endogenous β-oxidation [45], as well as gallic acid derived presumably from quinic acid metabolism [46], were found in lesser quantities. Stable isotopes can be efficiently used to determine the metabolic fate of a specific metabolite, as it was described for possible aromatization of quinic acid to protocatechuic acid and dehydroxylation to cyclohexanoic acid [47].

A recent study on the metabolic fate of chlorogenic acids derived from artichoke identified 76 molecular structures in human fluids using UHPLC-MS/MS and authentic standards [48]. These findings further confirmed 5-10-fold abundance of sulphated phase II metabolites of phenolic acids over glucuronidated and unconjugated counterparts in human fluids reaching individual Cmax values as high as 1-3 μM, as well as overall dominance of methylated molecular structures such as ferulic acid (4H3MCA), 3-methoxycinnamic acid sulfate (3MCA), dihydroferulic acid (4H3MPPA), and 4-methoxyphenylpropanoic acid glucuronide (4MPPA) in plasma, while benzoic acid metabolites were detected only in small quantities. High levels of artichoke chlorogenic acids reached the colon and were metabolized by microbiota as evident from the detection of large amounts of dihydroferulic acid (4H3MPPA), 3-

(4′-methoxyphenyl)propanoic acid (4MPPA), and 3-(3′-hydroxyphenyl) propanoic acid (3HPPA) [48].

Despite their presence in the human metabolome (**Table 1.2**), there are no significant dietary sources of phenylacetic and phenylpropanoic acids. Colonic metabolism of dietary polyphenols by microbiota is a major source of these metabolites [13]. In the case of microbial transformation of caffeoylquinic acids, hydrogenation of cinnamic acids to their dihydrocinnamic (phenylpropanoic) counterparts was observed in large quantities [49], with m-dihydrocoumaric acid (3HPPA) being one of the major metabolites [50]. There was a strong interindividual variability in the ability to perform and the exact order of the individual transformation steps, which likely arose from differences in the gastrointestinal microbial composition among subjects [51]. Several recent analyses of fecal water or matrix have pointed to proportionally large amounts of dihydro-p-coumaric (4HPPA) [52], dihydrocaffeic (3,4DHPPA) [53,54], dihydroferulic (4H3MPPA) [53,55], and/or 3-hydroxyphenylpropanoic acid (3HPPA) microbial metabolites found in the gastrointestinal (colonic) lumen. These results provided a clear rationale to further explore the potential of fecal microbial phenylpropanoic metabolites as possible biomarkers of health status, as well as colon function and integrity [56].

1.3 Catabolism of flavanols (epicatechin) and condensed tannins

Flavanols are a group of flavonoids that possess a flavan-3-ol core that are present in foods predominantly as monomeric structures (diastereoisomers (+)-catechin and (-)-epicatechin), their hydroxylated derivatives (gallocatechin, epigallocatechin), gallic acid esters (epigallocatechin gallate), polymeric condensed tannins such as pronathocyanidins, and oxidized polymers created during food processing (theaflavins or thearubigins) [57]. Fermentation

technology vastly expands the range of phenolic structures in foods as shown for conversion of 6 major green tea catechins into 30,000 novel molecular structures present in the brewed black tea [58]. Modern diets provide as high as 540 mg/p/d flavanols in populations that drink large amounts of tea [59], and 150-400 mg/p/d otherwise [60]. Proanthocyanidins (mostly from pome fruits, cocoa, wine, and cranberries) add another 100-175 mg/p/d flavanols to the diet [61]. The flavanol metabolic signature was established with 60 mg of [2-^{14}C](-)-epicatechin in 8 healthy subjects [62]. Epicatechin phase II metabolites peaked in plasma at 1220 nM 0-1h post bolus, with most of the parent structures undergoing methylation and sulfation and only 25% metabolites found as glucuronides. The dominant phenolic metabolites were identified at γ-valerolactones that peaked in plasma at 590 nM 5-6h post bolus. 5-(4′-Hydroxyphenyl)-γ-valerolactone-3′-sulfate was a major breakdown metabolite in plasma, although near equal amounts of glucuronidated derivatives were observed as well. Metabolite profile in urine followed a similar pattern with the addition of 3-hydroxyphenylhydracrylic and hippuric acids, suggesting that the breakdown pathway from γ-valerolactones to hippuric acid may be mediated in part by phenylhydracrylic metabolites. It is interesting to note that 3-hydroxyphenyl-hydracrylic acid was also noted in the metabolism of the flavanone hesperetin [63]. No significant amounts of other benzoic and cinnamic acids were detected. While clearly, the radiolabeled tracer studies are the highest quality attempts to determine flavanol metabolism, they have an inherent limitation in the availability of authentic reference standards at the time of the study. However, most of these findings were further confirmed in multiple dietary intervention studies with tea, cocoa, and other flavanol-rich foods as reported previously [64].

1.4 Catabolism of anthocyanins (cyanidin)

Anthocyanins are the flavonoid pigments that accumulate in slightly acidic plant vacuoles (pH=5) in contrast to the rest of the cytosol (pH=7.2) and thus are inherently unstable at neutral and alkaline conditions [65]. The current estimates suggest that people consume on average 10-25 mg anthocyanins/person/day (mg/p/d) depending on the geographical region [66,67], although higher estimates up to 80-150 mg/p/d were reported for the Mediterranean diets [68]. Once ingested, anthocyanins undergo rapid degradation which can include hydration and ring-opening reactions to a chalcone pseudo-base, or a proton transfer reaction to a quinonoidal base, resulting in the low (1%) bioavailability of the original structures. The resulting metabolic signature undergoes extensive phase II metabolism and microbial biotransformation as revealed by the 500 mg oral bolus dose of ^{13}C-labelled cyanidin-3-glucoside [69]. The study included 8 healthy male volunteers that provided blood, urine, breath, and fecal samples between 0 and 48 hours. Free, methylated, and glucuronidated conjugates of the parent anthocyanin represented 2% of metabolites found in circulation and reached a cumulative concentration of 760 nM 1-2h post bolus. Most small phenolic metabolites followed biphasic plasma kinetics with two peaks at 0-5h and 6-48h post bolus. The methylated benzoic series metabolite vanillic acid (4H3MBA) dominated both plasma and urine profiles over protocatechuic acid (2,3DHBA), and both compounds showed a preference for sulfation over glucuronidation. Hydroxycinnamic metabolites such as caffeic acid (2,3DHCA) and its methylated metabolite ferulic acid (4H3MCA) were found in lesser amounts, and ferulic acid dominated over caffeic acid in all samples, once again indicating extensive methylation of the ingested phenolic substrates [69]. Larger amounts of benzoic series metabolites over cinnamic and dihydrocinnamic series

counterparts may suggest that ring fission reactions in the anthocyanin phenylpropanoid core are skewed towards the generation of benzoic acid metabolites, but this remains to be investigated.

1.5 Catabolism of other flavonoids (quercetin, hesperitin, apigenin, daidzein)

Flavonols are a group of flavonoids with a characteristic 3-hydroxyflavone backbone that are found in plants as mostly glycosides, with quercetin, myricetin, and kaempferol being most common. These compounds are present in diets in lesser quantities averaging to 20-50 mg/p/d depending on the population, with some countries like Spain reaching 80 mg/p/d [60]. Unfortunately, there no radiolabeled tracer ^{14}C-qurcetin glucoside studies in humans and data from the rats studies [70] must be viewed with caution as we know that polyphenol metabolism in rodents doesn't extrapolate well to humans due to differences in phase II conjugation [62]. Nevertheless, the rats study reported different methylated, glucuronidated, and/or sulfated quercetin conjugates, and several small phenolic acids, principally 3-hydroxyphenylacetic, 3,4-dihydroxyphenylacetic acid, but also lesser quantities of benzoic and hippuric acids [70]. While informative, human dietary intervention studies do not allow us to distinguish between different parental structures that contribute to the observed small phenolic metabolites [71].

Flavanones are flavonoid ketones that enter the human diet primarily through consumption of citrus fruits and beverages, with average daily intakes estimated at 20-60 mg/p/d similar to flavonols [60]. No ^{13}C-labeled tracer studies have been reported for these compounds. Some hints at the possible metabolism of hesperitin and naringenin can be extrapolated from orange juice feeding studies. Glucuronide conjugation was a preferred route for phase II metabolism of the parent aglycones, although some sulfates were also noted. Small phenolic catabolites found in circulation and excreted in urine included 3′-hydroxyphenyl-hydracrylic acid

and its methylated derivative, dihydroferulic and dihydroisoferulic acids, methylated 4′-hydroxyphenylacetic acid, and hippuric acids [63].

Flavones (apigenin, luteolin) is a class of flavonoids typically underrepresented in dietary and food questionaries, as it enters human diets mostly through culinary seasonings (spices) and herbal preparations (teas), both of which are often underreported. Several less common flavones (tricin, diosmetin) are also found in sugarcane and can be consumed as a part of fresh and fermented beverages in tropical areas. As such, flavone intake is estimated at 1-5 mg/p/d but is projected to increase to 5-20 mg/p/d if all dietary sources are accounted for [60]. There are no ^{13}C-labeled tracer studies with flavones, but their metabolism can be extrapolated from dietary feeding studies with apigenin. The parent aglycone is typically glucuronidated, sulfated, or converted to luteolin [72]. However, very little current knowledge exists on microbial transformation of apigenin to small phenolic acid metabolites outside of *in vitro* fermentation studies summarized elsewhere [73].

Isoflavones are a class of flavonoids mostly restricted to legumes, and they appear in the human diet in significant quantities mainly through soy and its derived products, averaging at 0.2-2 mg/p/d and reaching up to 15-60 mg/p/d in some Asian cultures [60]. Their inherent phytoestrogenic activity mediated in part by selective activation of the estrogen α/β receptor is the major reason why we have advanced ADME data on their metabolism in humans using ^{13}C-labeled tracers [74]. Unlike other flavonoids, isoflavones have higher bioavailability of the parent aglycones, with both daidzein and genistein peaking in plasma at 5-7h post bolus of 25-50 mg dose in 16 healthy premenopausal women, and up to 30% of the compounds being later recovered from urine. Equol (4',7-isoflavandiol) was observed as a major daidzein metabolite found in some subjects that reached peak urine concentrations at 24-48h post bolus, however, no

other small phenolic metabolite were reported [74]. O-desmethylangolensin (ODMA) was a phenolic ring fission microbial metabolite of daidzein, and subjects ability to produce ODMA showed a negative correlation with their obesity status [75].

1.6 General notes on catabolism of chalcones, stilbenes, and hydrolysable tannins

Chalcones and dihydrochalcones are a minor class of flavonoids that are present in foods in small quantities equal to 0.01-3 mg/p/d [60], although they can be found in certain herbal preparations in larger amounts. The parent aglycones and their phase II metabolites could be found in circulation and urine in trace quantities due to limited solubility, and their contribution to small phenolic acid metabolism is largely unexplored [76].

Stilbenoids share their biosynthetic pathway with chalcones, however, they are classified as non-flavonoid polyphenols and are best known for resveratrol (grapes), piceatannol (berries, grapes, rhubarb, and passion fruit), and rhapontigenin (rhubarb) at 0.1-2 mg/p/d dietary intake [77]. Resveratrol is also metabolized into piceatannol in humans [78], while its methylated derivative pterostilbene is more metabolically stable due to enhanced tissue distribution and reduced affinity for glucuronidation or sulphation [79], although physiologic levels of resveratrol achieved with the diet alone had negligible effects on health outcomes [80]. ^{13}C-labeled tracer study using 25 mg bolus in 6 healthy adults established mostly glucuronidated, but also sulfated, conjugates of the parent aglycone and microbiota-derived dihydroresveratrol [81]. A subsequent study using 1 g resveratrol plus 5 mg ^{13}C-resveratrol bolus in 12 patients with prostate biopsies confirmed these findings, but also provided no information about the possible downstream small phenolic metabolites if any [82].

Hydrolysable tannins in the form of gallotannins (based on the gallic acid backbone as found in larger quantities in mango, strawberry, raspberry, barley, and black tea) or ellagitannins (based on the ellagic acid backbone as found in pomegranate, strawberries, raspberries, and walnuts) contribute significantly to the downstream pool of small phenolic metabolites after digestion and microbial catabolism [83]. The ability to breakdown highly abundant ellagitannins seems to critically depend on the microbiome, resulting in the production of three distinctive urolithin metabolite phenotypes: urolithin A conjugates, isourolithin A and urolithin B conjugates, or no conjugates at all [84]. Likewise, degradation of gallotannins releases mostly methylated and sulfated metabolites of gallic acid, as well as methylated and sulfated conjugates of pyrogallol, decarboxylated derivatives of gallic acid formed via microbial metabolism that were observed over 8-24h [85]. Additional chlorogenic acid, vanillic acid, p-coumaric acid, ferulic acid, and sinapic acid observed after a mango juice feeding study [86] probably originated from the food matrix itself and cannot be associated with gallotannin metabolism in the absence of the radiolabeled tracer studies.

1.7 Structure-activity relationships in the phenolic core

The number and positional modification of functional groups and conjugates of small phenolic metabolites define their hydrophilicity and play a critical role in their ability to be absorbed, metabolized, distributed within the circulation system and target tissues, as well as excreted. Molecular mass is one of the important factors that determine the excretion route of the hydrophilic metabolites, with higher mass metabolites predominantly eliminated via bile [87]. As such, metabolites conjugated with glucuronic acid are more likely to enter enterohepatic circulation than the respective sulfate or methylated derivatives, and this observation alone may

partially explain lesser amounts of glucuronidated phenolic metabolites found in circulation. Complex glycosylation [88] and acylation patterns [37] generally reduce the biological activity of these structures [37], but increase their stability in the gut and excretion in feces [89]. Urinary excretion of phenolic metabolites is regulated by a family of organic anion transporters in the proximal kidney tubules, with different transporters OAT1 (SLC22A6), OAT2 (SLC22A7), OAT3 (SLC22A8), and OAT4 (SLC22A11) also showing a variable affinity for free, glucuronidated, and sulfated conjugates [90].

Functional groups also directly affect the antioxidant properties of small phenolic metabolites, however, this property has likely little relationship with respect to their biological activity [91]. The functional groups substituted in the ortho (2-hydroxy), meta (3-hydroxy), or para (4-hydroxy) positions lead to significant differences in antiradical activities, with a pyrogallol moiety being a better antioxidant than a catechol moiety, and methylation generally reducing the antioxidant effects [92]. This also directly affects the ability of small phenolic metabolites to reduce the rate of hydroperoxides formation during the initial stages of lipid oxidation, with vanillic and syringic acids [92] as well as dihydroxylated metabolites [93], developing pro-oxidant activities to some extent. Hydroxyphenylacetic acids present in feces in large quantities (**Table 1.2**) also showed the highest radical scavenging activity in both water and ethanol systems [94]. Considering the enhanced tissue uptake and distribution of the methylated phenolic metabolites, such an effect may suggest that antioxidant properties of phenolic acids are more pronounced in the gastrointestinal lumen where the multiple hydroxylated forms have high potential to reduce peroxidation, scavenge oxygen radicals, and contribute to low oxygen environment to ensure proper anaerobic fermentation in the gut [88].

Another peculiar difference in the structural and spatial distribution of phenolic metabolites in the human body is the high accumulation of presumably microbial-derived phenylacetic acid in the gastrointestinal lumen in excess of 450 μM [52], while this metabolite remains at least 10-fold lower in both blood and urine (**Table 1.2**). High levels of phenylacetic acid in the gastrointestinal tract could be directly related to its microbiota content. The colonic microbiome consists of predominantly obligate and facultative anaerobes (mostly *Bacteroidetes* and *Firmicutes*, comprising near 90% of the total bacterial diversity in the gut), and a relatively smaller contribution from the *Proteobacteria*, *Fusobacteria*, *Verrucomicrobia*, and *Actinobacteria* phyla [95]. Colonic microorganisms widely utilize not only the saccharolytic catabolic energy pathway that relies on partially undigested polysaccharides and yields short-chain fatty acids, lactic acid, carbon dioxide, methane, and ethanol among other metabolites [96], but also the second catabolic pathway for fermentation of partially digested proteins leading to the production of ammonia, amines, thiols, phenols, and indoles [97]. Humans also have the capacity to form glutamine conjugates of phenylacetic acid to form phenylacetylglutamine that is excreted in urine [98]. Traditionally, phase II metabolism of phenolic structures is viewed as a part of the xenobiotic detoxification response to eliminate foreign substances from the human body [99]. However, the opposite function of using dietary phenolic metabolites from benzoic and phenylacetic series, mostly benzoic and phenylacetic acids, to form the respective glycine and glutamine conjugates may be considered as a critical function to regulate physiological levels of two abundant biosynthetic precursors (protein synthesis) and neurotransmitters (GlyR and Glu/ GABA-ergic receptors the central nervous system) by serving as amino acid deportation system that can freely cross the inner mitochondrial membrane [100], modulate mitochondrial energy metabolism [101], and contribute to detoxification of waste nitrogen.

The structure-activity relationships in regard to their specific biological activities among the discussed polyphenols are extensive and cannot be reviewed here in full detail, see elsewhere [102]. We can speculate that if the metabolism of anthocyanins is confirmed to produce larger amounts of benzoic acid metabolites, their biological activity may be greater in the areas of inflammation via inhibition of cyclooxygenase-mediated prostaglandin formation and NADPH oxidase activities as evident from studies on salicylic acid (2HBA) and apocynin (acetovanillone) [103]. Along the same line of thought, higher amounts of cinnamic acid metabolites associated with caffeoylquinic metabolism may promote greater vasodilation and blood pressure as shown for ferulic acid [104], similar to flavan-3-ol metabolites (γ-valerolactones) and their effects on endothelial function and flow-mediated dilation [105]. There also seems to be a trend to produce more phenylacetic acids from ring fission reactions of flavonols (see quercetin), hydracrylic acids from flavanones (see hesperetin), and flavandiols from isoflavones (see daidzenin). While these initial observations are not certain, they critically highlight the current shortcomings and gaps in our understanding of the metabolism of small phenolic metabolites originating from a diverse pool of parental structures.

1.8 Structure-activity relationships: focus on methylation

The apparent discrepancy between the original phenolic metabolite profiles from different dietary sources to those found in circulation is strongly affected by catechol-*O*-methyltransferase (COMT) activity, both at the level of microbiome [106], as well as the majority of mammalian tissues that express high levels of COMT [107]. While the primary and best characterized biological function of COMT is the metabolism of catecholamines, this widely conserved enzyme effectively transfers a methyl group from S-adenosyl-l-methionine (SAM) to

a hydroxyl group of many catechol-like structures, thus directly affecting their metabolism and tissue distribution. For example, homovanillic acid (4H3MPAA) is a methylated phenylacetic metabolite shared between tyrosine/dopamine metabolism and the breakdown of dietary polyphenols (**Figure 1.2**). Both COMT-3 and COMT-4 methylation activities are observed simultaneously at different ratios [48]. The human COMT exhibits functional SNPs such as Val158Met, and in addition to the association with disorders of catecholamine metabolism, likely participates in metabolic disorders such as obesity and diabetes. Subjects with a 3-4-fold lower-activity allele (Met/Met) displayed higher blood pressure and abdominal obesity [108], while the high activity rs4680 G-allele (Val/Val) was found to associate with lower HbA1c [109]. Excessive caloric intake significantly downregulates COMT protein levels in the liver, as well as increases lipid deposition in the liver and macrophage accumulation in the epididymal fat [110]. These findings may suggest that COMT-mediated transformation of phenolic metabolites is also diminished in obese states, and this topic warrants further exploration.

Absorption of phenolic metabolites from the gastrointestinal tract increases with hydrophobicity, and more polar structures are often converted to their respective methylated derivatives to ensure efficient uptake via MCT1 (SLC16A1) and transfer to the blood via MCT4 (SLC16A3) transporters [104], resulting in a disproportionately increased pool of methylated phenolic metabolites in circulation [111]. These effects are facilitated by endogenous esterases found in the pancreatic secretions, brush border layers of the small intestine, as well as bacterial and fungal esterases associated with colonic microbiota that efficiently release phenolic metabolites from their parental structures [112]. While the majority of phase II metabolism including glucuronidation, sulfation, and methylation reactions occur both in the enterocyte and liver, demethylation and dehydrogenation seem to be restricted to liver tissues [113], thus pointing

at the liver as a major site that regulates the intrinsic levels of methylated phenolic metabolites in the body. Finally, the extent of endogenous β-oxidation of small phenolic acid metabolites is unclear and probably limited; this partially explains why benzoic acid metabolites are detected in smaller quantities in clinical studies that use flavonoid-free polyphenol supplementation strategies [111].

An additional source of variation observed among different phenolic metabolites in circulation is their affinity for binding human serum albumin (HSA), as the strength of the binding increases with the number of free aromatic hydroxyls [114]. HSA is the most abundant protein in human plasma, it contains two independent binding sites for small organic molecules, including the vast majority of phenolic acids and polyphenols [115]. Binding to HSA controls the free, active concentration of a metabolite in the blood, and methylation of free aromatic hydroxyls generally decreases the HSA affinity of the phenolic metabolite [116], thereby potentially increasing the free fraction of methylated phenolic metabolites, enhancing their potency, and tissue distribution. This is illustrated by the detection of ferulic acid, but not caffeic acid, in blood cells although both metabolites were present in serum after ingesting 200 mg of polyphenol-rich maritime pine bark extract [117].

Methylated phenolic metabolites are also more resistant to hepatic metabolism and show a higher intestinal absorption [118]. Indeed, *O*-methylated anthocyanin metabolites were characterized with increased hydrophobicity at the B-ring of the molecule that reduced the plasma residence time and increased tissue affinity [119]. A direct comparison of whole berry supplementation on metabolic risk factors among six berries with different anthocyanin profiles supported the findings that methylated polyphenols are more effective at ameliorating obesity and improving glucose metabolism [88]. The potency of reducing lipid accumulation in adipocytes

was also reported in the order of $–OCH_3$ > –OH > –H, and thus the contribution of less polar B ring substitutes to the efficacy of phenolic metabolites was further confirmed [120]. The increase in the basal oxygen consumption rate observed in this study in response to the physiological concentration of 1 μM malvidin glucoside suggested that phenolic metabolites may directly modulate oxidative phosphorylation and mitochondrial coupling efficiency, and the increased hydrophobicity associated with methylation allowed for enhanced tissue distribution and crossing of multiple membranes *en route* to the mitochondrial matrix [121]. Methylated derivatives of phenolic metabolites could be also obtained synthetically under mild and practical conditions using dimethyl carbonate both as solvent and reagent [122].

Taken together, while cytosolic sulfation, endoplasmatic glucuronidation, and mitochondrial glycine/glutamine conjugation are clearly positioned in place to increase hydrophilicity and reduce the reactivity of the target phenolic metabolites to facilitate their excretion, the methylation that occurs both in the cytosol and the endoplasmic reticulum serves a less obvious function. Given the totality of the evidence presented here, we are inclined to propose the existence of "methylated sinks", the endpoint pools of small methylated phenolic metabolites (**Figure 1.2**) better capable of crossing the cell membranes, achieving greater tissue penetration and/or distribution, and allowing for easier access to the inner mitochondria matrix where they may directly affect central energy metabolism.

1.9 Conclusions

In this review, we highlighted the recent findings on the distribution and metabolism of dietary polyphenols and phenolic acids, with a particular focus on the ring-fission reactions that generate small phenolic metabolites from the diverse parental aglycones, and the subsequent

phase II metabolism that produces a wide variety of glucuronidated, sulfated, methylated, and glycine/glutamine conjugated products both in circulation (blood/plasma) as well as in the waste liquids (urine and fecal water) destined for excretion. In doing so, we specifically focused on the radiolabeled tracer studies that provided a unique opportunity to eliminate food matrix effects and provide *in vivo* human data on the precise origin and metabolism of small phenolic compounds otherwise obscured by the diversity of polyphenols found in each food source. The preliminary overview of the data accumulated so far identifies several trends that need to be further confirmed in clinical studies and isolated cultures/mixed fecal cultures fermentation experiments, and specifically the notion that different classes of polyphenols may be associated with distinct biochemical signatures of small phenolic metabolites that affect their downstream distribution and function. Novel combinations of LC-MS based chemometrics visualization strategies may improve untargeted discovery and analysis of additional phenolic metabolites in human tissues and fluids [123].

The hopes to apply these findings successfully and safely to tackle future dietary reference intakes, nutritional interventions, and public health outcomes rely in part on our ability to address the following critical gaps in the field: (1) develop new robust technological solutions and metabolomic pipelines to accelerate discovery and identification of all small phenolic metabolites as pure authentic and conjugated standards become available for calibration; (2) fund additional clinical studies that use radiolabeled tracers or stable isotopes to precisely define metabolic fate of specific compounds and the ring-fission reactions of the respective phenolic backbones; (3) attain high throughput microbial isolation and cultivation systems individually or as a consortium of a model microbiome for validation and manipulation of the candidate metabolites and genes; and (4) understand the relatively subtle differences in metabolism and

microbiome profiles that can have a disproportionate role on human health and wellbeing outcomes. In all likelihood, there will be no one healthy class of polyphenols nor there will be a healthy level of a total polyphenol intake, but instead, there could be multiple healthy configurations of microbial populations and phenolic metabolites signatures that define the desired and personalized health outcomes.

Table 1.1 Major dietary sources of phenolic acids and polyphenols. Data was retrieved and averaged across multiple studies summarized in the Phenol-Explorer database [39]. Dry herbs (culinary spices and herbal preparations such as teas and tinctures) are expected to contain on average 5-8-fold higher amounts of polyphenols than fresh plant tissues.

Phenolic constituents	Exemplary compounds and abbreviation	Normalized dietary content (mg/100 g FW)
Phenolic acids		
Benzoic		
Monohydroxy	Benzoic (BA), 2-hydroxybenzoic (2BA), 3-hydroxybenzoic (3BA), or 4-hydroxybenzoic (4BA)	Raw olives in the range of 2-5 mg
Dihydroxy	Protocatechuic acid (3,4DHBA), resorcyclic acid (2,4DHBA) – *see also flavonoids*	Raw chicory greens and star anise at 16-32 mg
	Vanillic acid (4H3MBA)	Chestnuts at 480-4200 mg; date, sage, oregano and basil at 4-14 mg
Trihydroxy	Gallic acid (3,4,5THBA) – *see also hydrolysable tannins*	Celery seeds at 1330 mg; chokeberry at 770-3000 mg; cloves, chestnuts at 250-900 mg; grape wines, blackberry, raspberry, mulberry, black tea, chicory, and black currant leaves at 3-26 mg
	Syringic acid (4H3,5DMBA)	Dried sage, thyme, oregano, raw olive, and walnuts at 3-34 mg
Cinnamic		
Monohydroxy	Cinnamic acid (CA), o-coumaric acid (2HCA), m-coumaric acid (3HCA), and p-coumaric acid (4HCA)	Celery seeds at 50 mg; raw olives at 2-14 mg; lingonberry, cranberry, cloudberry, cloves, rosemary, oregano, thyme, sage, date, and peanuts at 2-6 mg
Dihydroxy	Caffeic acid (3,4DHCA) – *see also phenolic acid esters*	Celery seeds at 160 mg; chokeberry at 140 mg; lingonberry, caraway, dried spearmint, sage, thyme, oregano, rosemary, sunflower seeds, cumin, dried ginger, nutmeg, star anise, and cinnamon at 5-26 mg
	Ferulic acid (4H3MCA), isoferulic acid (3H4MCA) – *see also phenolic acid esters*	Durum wheat at 70 mg; celery seeds at 30 mg; dates, and dark chocolate at 10-24 mg
Trihydroxy	Sinapic acid (4H3,5DMCA)	Raw olives at 10-44 mg; raw cauliflower at 4 mg
Phenolic acid conjugates		
Caffeoylquinic	Chlorogenic acid (5-caffeoylquinic acid, 5CQA)	Sunflower seeds at 450 mg; raw chicory and artichoke heads at 160-202 mg; coffee beverages at 40-120 mg/100 ml, prune, blueberry, and apple at 40-87 mg; potato at 4-90 mg
	3-Caffeoylquinic acid (3CQA)	Coffee beverages, fresh plum, and plum prunes at 50-120 mg
	4-Caffeoylquinic acid (4CQA)	Coffee beverages, and plum prunes at 20-60 mg
	3,5-Dicaffeoylquinic acid (3,5DCQA)	Mate at 18-20 mg/100 ml; coffee beverages at 2-6 mg/100 ml
Cinnamoyl glucoses	6-*O*-caffeoyl glucose	Tomato at 2-8 mg; berries at 1-5 mg
Phenylethanoids	Verbascoside, echinacoside	Verbena at 4-1370 mg; olives at 17-68 mg; *Echinacea* at 5-10 mg
Various	Chicoric acid	Chicory at 30-40 mg
	Rosmarinic acid	Sage, thyme, rosemary, peppermint, and sweet basil at 90-150 mg
	Diferulic acids	Corn, rye, wheat at 1-11 mg
	Avenanthramides	Whole grain oats at 0.9-1 mg
Tannins		
Dilactone	Ellagic acid	Chestnut at 735 mg, walnuts, black raspberry, blackberry at 30-40 mg
Hydrolysable	Ellagitannins	Chestnut, pecan, walnut, jaboticaba at 150-860 mg; blackberry, raspberry, strawberry at 80-300 mg
	Gallotannins	Mango at 30-80 mg
Condensed	Proanthocyanidins	Chokeberry, rosehips at 700-1800 mg; cranberry, blueberry, strawberry at 140-500 mg
Flavonoid glycosides		
Anthocyanidins	Cyanidin, delphinidin, malvidin, pelargonidin, petunidin, peonidin	Elderberry at 800 mg; berries, grapes, plums, cherries at 100-550 mg
Flavones	Apigenin, luteolin, diosmetin, tricetin	Mint, sage, oregano, thyme, artichoke heads, buckwheat at 10-120 mg
Flavonols	Quercetin, kaempferol, myricetin	Capers, cloves, cumin, caraway, oregano, elderberry, buckwheat, onion, dark chocolate at 20-104 mg
Flavanones	Hesperetin, naringenin	Oregano at 370 mg; mint, orange at 10-50 mg
Flavanols	Catechin, epicatechin, epigallocatechin, epigallocatechin-3-gallate	Chestnuits at 120-220 mg; dark chocolate, cocoa powder, chocolate milk beverages, broad bean, apple, tea at 10-150 mg
Isoflavones	Genistein, daidzein, glycitein	Soybeans, soymilk at 30-100 mg, tofu at 20-30 mg
Chalcones	Resveratrol, rhapontigenin	Tomato, apple, licorice at 1-15 mg
Stilbenoids	Resveratrol, rhapontigenin	Grape wine at 0.1-3 mg; currants, cranberry, lingonberries at 1-3 mg
Lignans	Matairesinol, pinoresinol	Flaxseeds at 250-300 mg; sesame seeds at 30-50 mg

Table 1.2 Major phenolic acid metabolites identified in human circulation, urine, and feces. Data was retrieved and averaged across multiple studies summarized in the Human Metabolome database [124]. Detected, but not quantified, metabolites are listed as nq.

Phenolic acid metabolites	Chemical and traditional names	Molecular weight	Range of normal concentrations		
			Blood, μM	Urine, μmol/ mmol creatinine	Feces, nmol/g
Benzoic acids					
BA	Benzoic acid	122.1230	10.49-77.75	0.1-145.04	6.55-355.38
2HBA	2-hydroxybenzoic acid Salicylic acid	138.1220	0.02	1.1-57.7	0.753-6.66
3HBA	3-hydroxybenzoic acid m-hydroxybenzoic acid	138.1220	0.08-1.27	0.003-2.51	0.34-7.53
4HBA	4-hydroxybenzoic acid p-hydroxybenzoic acid	138.1220	2.32-20.88	0.23-5.9	1.44-29.68
3,4DHBA	3,4-dihydroxybenzoic acid Protocatechuic acid	154.1210	0.20-1.23	0.03-1.11	0.97-8.11
4H3MBA	4-hydroxy-3-methoxybenzoic acid Vanillic acid	168.1480	0.09-8.71	0.07-19.2	2.87-6.66
3,4,5THBA	3,4,5-trihydroxybenzoic acid Gallic acid	170.1200	0-2.09	0.003-0.38	11.87-13.87
Phenylacetic acids					
PAA	Phenylacetic acid	136.1479	47.24-80.36	0.2-8.99	498.72-1344.12
3HPAA	3-hydroxyphenylacetic acid m-hydroxyphenylacetic acid	152.1473	0.01-2.31	0.6-17.6	3.74-122.25
4HPAA	4-hydroxyphenylacetic acid p-hydroxyphenylacetic acid	152.1473	0.23-42.41	1.68-917	3.71-63.22
3,4DHPAA	3,4-dihydroxyphenylacetic acid Dopacetic acid, DOPAC	168.1467	0.003-0.02	0.034-4.34	13.56-40.85
4H3MPAA	4-hydroxy-3-methoxyphenylacetic acid Homovanillic acid	182.1733	0.02-0.16	0-20.6	2.19-34.52
Phenylpropanoic acids					
3HPPA	3-hydroxyphenylpropanoic acid m-dihydrocoumaric acid	166.1739	0.03-0.77	0.04-1.5	9.05-290.65
4HPPA	4-Hydroxyphenylpropanoic acid p-dihydrocoumaric acid, desaminotyrosine	166.1739	0.57-3.18	0.08-0.2	16.12-184.75
3,4DHPPA	3,4-dihydroxyphenylpropanoic acid Dihydrocaffeic acid	182.1733	nq	nq	nq
4H3MPPA	4-Hydroxy-3-methoxyphenylpropanoic acid Dihydroferulic acid	196.1999	0.0006	nq	nq
3H4MPPA	3-Hydroxy-4-methoxyphenylpropanoic acid Dihydroisoferulic acid	196.1999	nq	nq	nq
Cinnamic acids					
3HCA	3-Hydroxyphenylcinnamic acid m-Coumaric acid	164.1580	0-3.4	0.01-11.19	0.2-2.07
4HCA	4-Hydroxyphenylcinnamic acid p-Coumaric acid	164.1580	0.04-0.94	0.01-2.94	0.32-9.38
3,4DHCA	3,4-Dihydroxyphenylcinnamic acid Caffeic acid	180.1574	0-18.88	0.006-3.5	11.26-14.98
4H3MCA	4-Hydroxy-3-methoxyphenylcinnamic acid Ferulic acid	194.1840	0-1.58	0.24-36.3	0.34-13.85
3H4MCA	3-Hydroxy-4-methoxyphenylcinnamic acid Isoferulic acid	194.1840	0.08-1.54	0.04-0.39	18.59-21.98
Amino acid conjugates					
HA	Hippuric acid	179.1727	1.0-27.93	1.51-969.8	0.23
4HHA	4-Hydroxyhippuric acid	195.1721	nq	0-27.48	0.36-4.09
PAG	Phenylacetylglutamine	264.2771	3.34-17.8	4.5-71.0	nq

Table 1.3 Plasma phenolic metabolites after consumption of coffee with 310 mg chlorogenic acid. Data was retrieved and averaged across different metabolite groups [43].

Phenolic acids	**Maximum free and conjugated forms, nM**			
	Free	**Gluc**	**Sulf**	**Meth**
Plasma				
Caffeoylquinic	2-19	--	1-2	--
Caffeic	3-5	1-2	1-68	Ferulic
Dihydrocaffeic	2-64	1-27	1-167	Dihydroferulic
Feruloylquinic	1-17	12-16	1-3	--
Ferulic	1-9	7-25	3-43	14-96
Dihydroferulic	32-247	11-60	4-31	22-27
Isoferulic	1-14	8-53	1-9	--
Dihydroisoferulic	52-189	14-30	8-20	--
Coumaric	1-2	1-3	1-2	--
Dihydrocoumaric	4-335	1-36	2-12	--
Cinnamic	3-4	--	--	6-23
Dihydrocinnamic	349-394	--	--	8-13

Table 1.4 Major plasma phenolic metabolites originating from dietary flavonoids. Data was retrieved and averaged across different metabolite groups after a bolus dose of 500 mg cyanidin-3-glucoside [69].

Phenolic metabolites	Maximum metabolite forms, nM			
	Free	Gluc	Sulf	Meth
Plasma				
Cyanidin-3-glucoside	141	--	--	
Phloroglucinaldehyde	582	--	--	
4-Hydroxybenzaldehyde	667	--	--	
Benzoic	--	74	--	
Protocatechuic	146	79	157	Vanillic
Vanillic	1845	24	430	
Isovanillic	195	35	--	
Ferulic (B ring)	827	--	--	
Ferulic (A ring)	87	--	--	
Hippuric	1962	--	--	
Urine				
Cyanidin-3-glucoside	334	107	--	
Phloroglucinaldehyde	8	--	--	
4-Hydroxybenzaldehyde	97	--	--	
Benzoic	--	129	--	
3 or 4-Hydroxybenzoic	49	--	--	
Protocatechuic	337	534	2356	Vanillic
Vanillic	3412	762	1682	
Isovanillic	212	699	822	
4-Hydroxyphenylacetic	391	--	--	
3,4-Dihydroxyphenylacetic	82	--	--	
Ferulic (B ring)	1839	--	--	
Ferulic (A ring)	474	--	--	
Hippuric	5417	--	--	
Feces (µg recovered)				
Cyanidin-3-glucoside	89	--	--	
Phloroglucinaldehyde	113	--	--	
4-Hydroxybenzaldehyde	1	--	--	
Benzoic	--	--	--	
3 or 4-Hydroxybenzoic	22	--	--	
Protocatechuic	581	108	--	Vanillic
Vanillic	104	76	69	
Isovanillic	19	15	176	
4-Hydroxyphenylacetic	62	--	--	
3,4-Dihydroxyphenylacetic	22	--	--	
Caffeic	379	--	--	Ferulic
Ferulic (B ring)	3837	--	--	
Ferulic (A ring)	709	--	--	
Hippuric	39	--	--	

Figures and legends

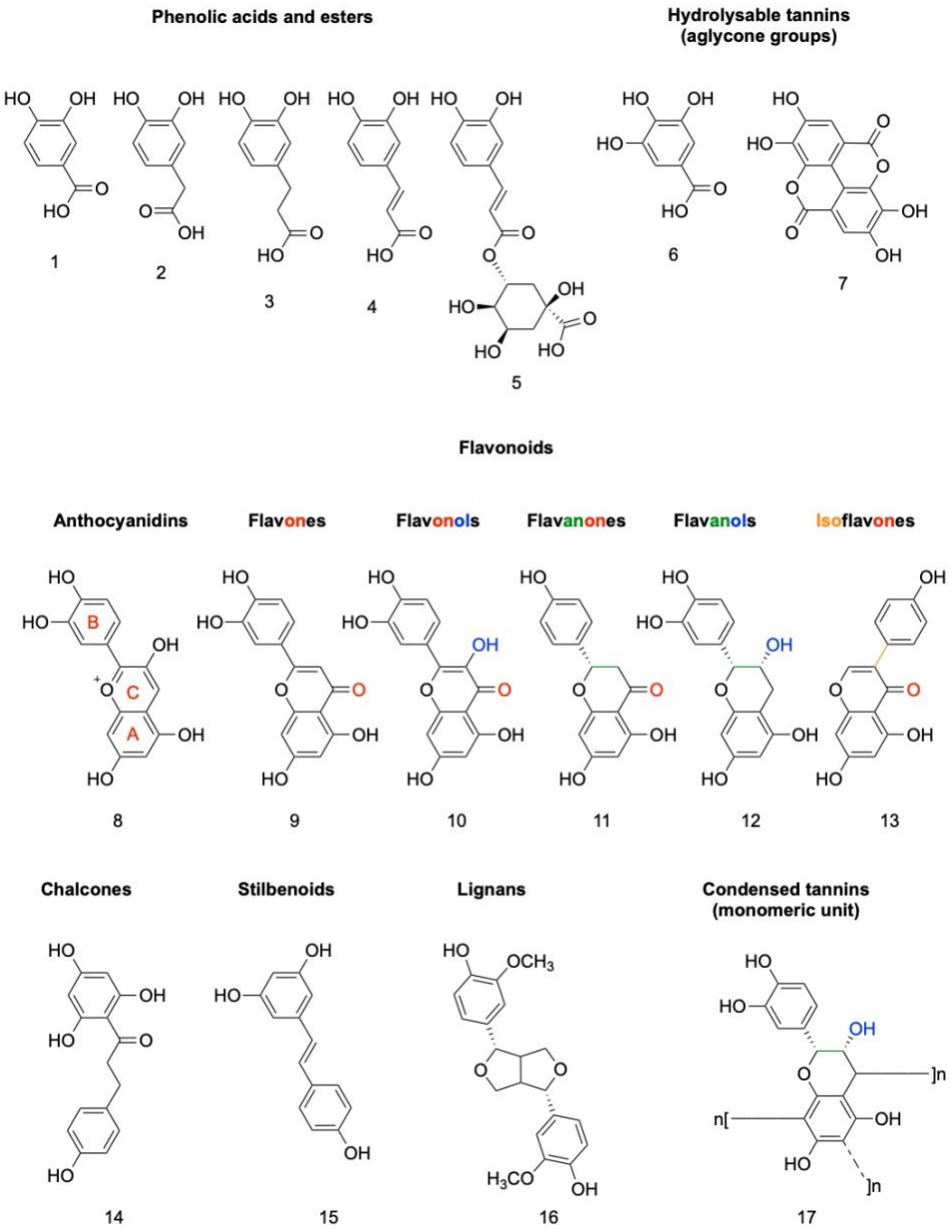

Figure 1.1 Major classes of phenolic acids and polyphenols with the representative chemical structures. Numbers denote (1) protocatechuic acid, (2) 3,4-dihydroxyphenylacetic acid, (3) 3,4-dihydroxyphenylpropanoic acid, (4) caffeic acid, (5) 5-caffeoylquinic acid, (6) gallic acid, (7) ellagic acid, (8) cyanidin, (9) luteolin, (10) quercetin, (11) naringenin, (12) epicatechin, (13) genistein, (14) phloretin, (15) resveratrol, (16) pinoresinol, (17) proanthocyanidin.

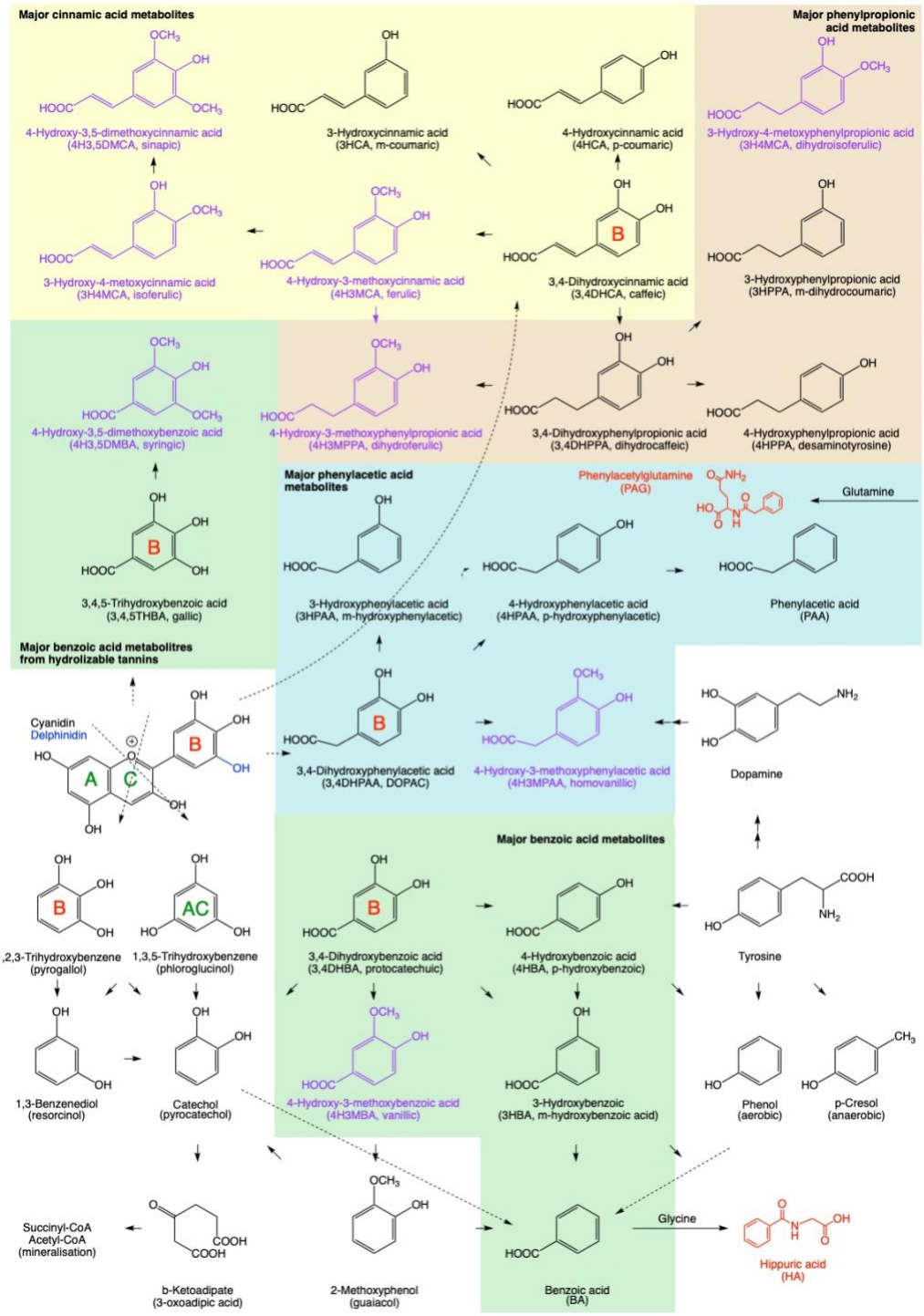

Figure 1.2 Different series of small phenolic acid metabolites and their interactions with amino acid and catecholamine metabolism. The breakdown of a parental polyphenol structure is shown using cyanidin or delphinidin as an example. Letters denote A, B, and C rings of the phenylpropanoid core. Hippuric acid and phenylacetylglutamine are highlighted in red as a part of the putative amino acid deportation systems. Methylated derivatives of phenolic acids are highlighted in purple as a part of the proposed methylated metabolite pools (sinks).

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CHAPTER 2

DIFFERENCES IN PHENOLIC ACID COMPOSITION AND BIOLOGICAL ACTIVITY AMONG RUM FERMENTATION PRODUCTS

Abstract

Sugarcane (*Saccharum officinarum* L.) is the world's largest crop by production quantity, yet it is one of the lesser-known sources of polyphenols. It is an important agricultural commodity in tropical and subtropical countries because its stalks (culms) accumulate sucrose that is used in the production of table sugar. Sugarcane raw materials are also used in fermentation industries to produce biofuels (ethanol) and distilled spirits (cachaça and rum). After sugarcane juice or the concentrated product (molasses) is fermented, a significant quantity of sugarcane polyphenols ends up in the final, partially dehydrated, byproduct (vinasse). Large amounts of vinasse effluents pose a significant recycling and environmental threat. We, therefore, attempted to determine the small phenolic acid metabolite profiles of molasses (pre-fermentation) and vinasse (post-fermentation) materials generated by fermentation and distillation of rum. Stability in the fermentation medium decreased, and the vinasse end products were enriched with diosmetin, vanillic, and syringic acids. Phenolic acid metabolites found in vinasse reduced pro-inflammatory gene expression profiles in the LPS-stimulated macrophages, with benzoic and cinnamic acids showing maximum anti-inflammatory effects. This study lays the foundation for future investigations evaluating the effects of phenolics present in sugarcane waste streams and their putative applications to managing immune health outcomes.

2.1 Introduction

Rum is an alcoholic beverage made from fermented and distilled sugarcane juice. Distilled rum is well known to have originated in Caribbean countries, with the manufacturing processes varying according to the traditional style of the production countries [1]. The different grades of rum production also define the types of rum, such as white, dark, amber, over-proof, and spiced. Rum production begins with obtaining the raw materials of sugarcane juice or its boiled concentrate after the crystalized sugar is removed (molasses). The initial step in sugarcane juice processing occurs at the sugar mills, where the sugarcane crop is crushed and expressed to release the juice that then boils into syrups containing 30% (w/w) sugar and goes into a clarification process. The clarification process includes the addition of crystallized sugar to the syrup. The syrup is then boiled again under vacuum and the table sugar is separated by centrifugation once the syrup cools down. This process is repeated twice to create a dark brown thick, and sticky liquid consistency called molasses. A desirable pH level of molasses is usually insured in commercial rum distilleries to prepare for the next production steps [2].

Sugarcane crop used in rum production is an important agricultural commodity in the tropical economies, where Brazil is the top country that produces the largest share of sugarcane mass. Other subtropical or tropical countries such as China and Central America also produce large amounts of sugarcane. Aside from sugar metabolite production, the phytochemical profile of sugarcane remains inadequately explored. Previous analytical studies reported the presence of phenolic acids, flavonoids, and other phenolic compounds found in sugarcane, and attributed a series of health beneficial effects to the physicochemical, antioxidant, and antimicrobial components found in these compounds [3,4]. Such effects are found in sugarcane raw juice or molasses, culms, and leaves. Mainly flavones including tricin, apigenin, or luteolin are the

primary flavonoid, and hydroxycinnamic acids, including chlorogenic, caffeic, coumaric, or ferulic acids, are identified in sugarcane [3,5]. Additionally, sugarcane extracts also have health-promoting properties of anticarcinogenic, hepatoprotective, gastroprotective, regulating and stimulating the immune system, protecting against DNA damage, protecting against hypertension and diabetes disorders, and playing a significant role in scavenging free radicals [3].

Sugarcane juice or molasses is a significant industrial raw material from sugar mills where sugarcane crops are extracted and boiled into a dark brown thick and sticky syrup. In the past years, sugarcane juice or molasses has been reported to contain various health benefits such as the protection against metabolic disorders, DNA oxidative damage, and scavenging free radicals due to its rich source of antioxidant activity from polyphenols [6]. The polyphenol composition of sugarcane molasses is very complex. A recent study shows the composition of polyphenols present in sugarcane molasses with the highest concentration of diosmin, followed by syringic acid and chlorogenic acid [6]. Furthermore, apigenini-*C*-glycosides, methoxyluteolin-*C*-glycosides, tricin-*O*-glycosides, isomers isoschaftoside, and schaftoside have also been identified [6].

Sugarcane vinasse is an acidic brown-colored byproduct of liquid waste (effluent) generated from sugarcane molasses fermentation and distillation in ethanol processing [7]. Generally, 10 to 13 liters of sugarcane vinasse are generated with each one liter of ethanol produced [7]. Vinasse is often used as fertilizers on sugarcane crops due to its high contents of organic compounds and plant nutrients, but it is believed that this colored waste poses significant environmental problems including soil and groundwater contaminations [7]. The brown color of this effluent comes from the Millard reaction, alkaline degradation reactions, and sugar degradation during sugarcane processing [8,9]. These reactions can form enzymatically and non-

enzymatically-derived colorants including melanins, melanoidins, alkaline degradation products of hexose (HADPs), and caramels from sucrose hydrolysis in the production process [9]. Recent literature on polyphenol classes and phenolic acids in sugarcane vinasse is limited. In a previous study, the retention times of several flavonoids and phenolic acids including p-coumaric, ferulic, caffeic, vanillic, and gallic acids (in the order of hydrophobicity) were reported. The presence of vanillic and caffeic acids in sugarcane vinasse was confirmed through HPLC with pulse amperometry [7].

Fermentation is one of the most used and one of the oldest food bioprocessing techniques to increase food stabilization, shelf-life, and nutritional qualities based on the microorganisms' growth and metabolic actions such as bacteria, yeasts, and fungi [10,11]. Chemoheterotrophic microorganisms are often used in the food industry by utilizing energy derived from the oxidation of organic molecules [12]. A chemoheterotrophic microorganism, lactic acid bacteria (LAB) are the most widely used bacteria group in food production, notably in the process of dairy products [12]. LAB are gram-positive, facultative anaerobes, which undergoes homofermentative or heterofermentative metabolism, depending upon the genus of the bacteria. In general, LAB in dairy processing include the genus of *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, and *Streptococcus* [12]. In the metabolism of homofermentation, aldolase acts as the primary enzyme, and lactic acid acts as the end product. In contrast, in heterofermentative metabolism, phosphoketolase is the primary enzyme that catalyzes a cascade of reactions leading to lactic acid, ethanol, and carbon dioxide formation as the end products [12,13]. Other than LAB, Saccharomyces yeasts are also a significant microorganism used in food production, including beer, bread, and wine processing. In the production of beer, *Saccharomyces* yeasts are essential, especially *Saccharomyces cerevisiae* and

Saccharomyces pastorianus. *Saccharomyces cerevisiae* in carbohydrate metabolism results in two adenosine triphosphate molecules from one hexose unit, and pyruvate from glucose is converted to acetaldehyde by decarboxylation and given ethanol and carbon dioxide as the end products [12].

Many other microbial organisms are present in the gastrointestinal microbiome and capable of fermenting polyphenolic compounds. Among these are *Aspergillus* (also known for external fermentation aid in the form of koji mold, *Candida* (that also can become a pathogenic fungus in the gut when its growth is not controlled), and two model species of bacteria, gram-positive *Staphylococcus*, and gram-negative *Escherichia*. When there are suitable substrates, microorganisms, and gastrointestinal environments such as relevant factors of pH, temperature, and moisture content, the fermentation occurs but great differences are observed due to variations in microbial and environmental factors [11]. During fermentation, biochemical changes happen to modify the contents of bioactive phenolic compounds and antioxidant properties in plant-based foods, including fruits and vegetables [10,14]. Similar processes occur during external fermentation of foods through the bioconversion of the bound forms of phenolic compounds into free forms, which therefore increase the health functions of foods [14]. For instance, lactic acid bacteria act as bacteria that modulate hypertension since lactic acid bacteria are used in foods to produce angiotensin-converting enzyme (ACE) inhibitory peptides and γ-aminobutyric acid [10,14,15]. The application of fermentation is a valuable tool for food production to improve the antioxidant activity of food due to the possible high antioxidant content found in foods through the hydrolyzation of phenolic glycosides and the release of free aglycones by ligninolytic and carbohydrate metabolizing enzymes. The antioxidant content in foods acts as auto-oxidation prevention, which offsets the undesired free radicals in the human body [10]. Most antioxidants,

particularly polyphenols, are excellent antioxidants due to a 3'-4' dihydroxy group in the B ring and the galloyl ester in the C ring of flavonoids, which contain the ability to reduce the risk of developing chronic diseases [10].

Upon fermentation, the antioxidative activity in plant-based foods increases and causes the release of natural antioxidants of flavonoids from the foods [16]. These effects of flavonoids can scavenge hydroxyl and peroxyl radicals, thereby inhibiting lipid oxidation initiated by metals [10]. Flavonoids have the strongest antioxidant activity among the phenolic compounds due to their chemical structures of an o-diphenolic group, a 2-3 double bond linked to a 4-oxo functional group, and hydroxyl groups at the 3 and 5 positions [10,17]. The antioxidant capacity found in phenolic acids is primarily based on the number and orientation of the hydroxyl groups relative to the withdrawal of electrons of the functional groups of carboxylic acid (CO_2H), oxaloacetic acid (CH_2CO_2H), or $(CH)_2CO_2CH$ [18]. Fermentation causes structural destruction of plant cell walls and produces microbial enzymes (glycosidase, amylase, cellulase, chitinase, inulinase, phytase, xylanase, tannase, esterase, invertase, or lipase), which leads to bioactive compound synthesis and hydrolyzation of glucosides [19,20]. In addition, fermentation of plant-based foods increases antioxidant activity caused by the structural changes in phytochemicals, where fermentation by lactic acid bacteria promotes simple phenolic conversion and depolymerization of high molecular weight phenolic compounds [10,21].

The changes to polyphenols and phenolic acid profiles that occur in sugarcane juice during partial dehydration (molasses), and subsequent rum fermentation (vinasse) are virtually not explored. In this study, we examined the ability of major phenolic acids from sugarcane to remain stable in different stages of the rum fermentation process with a focus on molasses and

vinasse byproducts and to modulate biomarkers of inflammation *in vitro*, in order to assess their potential to support immune health outcomes.

2.2 Materials and methods

2.2.1 Chemicals and reagents

Chemicals and general laboratory reagents were purchased from Sigma (Saint Louis, MO) unless otherwise specified. All solvents were of HPLC grade and purchased from VWR (Radnor, PA). Water was purified with a Milli-Q water purification system (Millipore, Burlington, MA). Phenolic acids including benzoic acids (benzoic acid, 2-hydroxybenzoic acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, protocatechuic acid (Aldrich, St. Louis, MO, USA), vanillic acid (Sigma-Aldrich, St. Louis, MO, USA), gallic acid, syringic acid), cinnamic acids (caffeic acid, ferulic acid, isoferulic acid (Aldrich), 3-hydroxycinnamic acid, and 4-hydroxycinnamic acid), phenylacetic acids (3-hydroxyphenylacetic acid, 4-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid, and 4-hydroxy-3-methoxyphenylacetic acid), and phenylpropanoic acids (3-hydroxyphenylpropanoic acid, 4-hydroxyphenylpropanoic acid, 3,4-dihydroxyphenylpropanoic acid (Aldrich), and 4-hydroxy-3-methoxyphenylpropanoic acid) used were purchased from Sigma, Aldrich, or Sigma-Aldrich (St. Louis, MO, USA). DMEM, TRIzol, and cDNA kit were purchased from Life Technologies (Grand Island, NY, USA). Penicillin and streptomycin were obtained from Fisher Scientific (Atlanta, GA, USA). All other solvents and chemicals used in this investigation were obtained from VWR International (Suwanee, GA, USA).

2.2.2 In vitro stability of phenolic acids in a model system

Phenolic acids including benzoic acids (benzoic acid BA, 2-hydroxybenzoic acid 2HBA, 3-hydroxybenzoic acid 3HBA, 4-hydroxybenzoic acid 4HBA, protocatechuic acid 3,4DHBA, vanillic acid 4H3MBA, gallic acid 3,4,5THBA, and syringic acid 4H3,5DMBA), phenylacetic acids (3-hydroxyphenylacetic acid 3HPAA, 4-hydroxyphenylacetic acid 4HPAA, 3,4-dihydroxyphenylacetic acid 3,4DHPAA, and 4-hydroxy-3-methoxyphenylacetic acid 4H3MPAA), phenylpropanoic acids (3-hydroxyphenylpropanoic acid 3HPPA, 4-hydroxyphenylpropanoic acid 4HPPA, 3,4-dihydroxyphenylpropanoic acid 3,4DHPPA, and 4-hydroxy-3-methoxyphenylpropanoic acid 4H3MPPA), and cinnamic acids (3-hydroxycinnamic/m-coumaric acid 3HCA, 4-hydroxycinnamic/p-coumaric acid 4HCA, 3,4-dihydroxycinnamic/caffeic acid 3,4DHCA, 4-hydroxy-3-methoxycinnamic/ferulic acid 4H3MCA, and 3-hydroxy-4-methoxycinnamic/isoferulic acid 3H4MCA) were used in this study.

Initial experiments to establish *in vitro* fermentation parameters for stability studies of unmethylated and methylated phenolic acids were performed in benzoic acids (using unmethylated protocatechuic acid and the corresponding methylated vanillic acid pair) and cinnamic acids (using unmethylated caffeic acid and the corresponding methylated ferulic acid pair) in the presence of a model microbial organism, a gram-negative bacteria *Escherichia coli*. The time course of incubation treatments was established under different fermentation time points (0, 3, 24, and 48 hours). All treatments were initiated by adding 100 μg/ml of the respective phenolic acid to 1:100 freshly diluted overnight culture of *Escherichia coli* (approximately 10^9 CFU/ml) grown in LB media at 37C and 200 rpm. Media samples were collected at the indicated timepoints and extracted following the established protocol. Starting (0

h) samples were collected immediately following the introduction of phenolic acids into the fermentation conditions.

Fermented bacteria media were transferred to 15 ml centrifuge tubes and acidified by adding 20 µl of 2M HCl solution to each tube. Tubes were then vortexed twice for 15 seconds, extracted with 2 ml of ethyl acetate, and vortexed twice for 15 seconds before centrifugation at 3500 rpm for 5 minutes to remove bacterial membrane fragments. The supernatant organic layer was separated into glass vials for the removal of ethyl acetate with the rotary evaporator to obtain a dried residue. The residue was then dissolved with 1 ml methanol for HPLC analysis.

2.2.3 In vitro stability of phenolic acids in presence of different microorganisms

Additional studies to establish *in vitro* fermentation parameters to evaluate the stability of unmethylated and methylated phenolic acids were performed in benzoic acids (using unmethylated protocatechuic acid and the corresponding methylated vanillic acid pair) in the presence of other model microbial organisms including a gram-positive bacteria *Staphylococcus aureus*, single-cell yeasts *Saccharomyces cerevisiae* and *Candida albicans*, and a filamentous fungus *Aspergillus niger*. The stability of individual phenolic acids in these mixtures was estimated at the 16 h time point established from the *Escherichia* model culture. Media samples were collected and processed in the same way as described for *E. coli* cultures.

2.2.4 Extraction of rum fermentation products

Samples of rum fermentation products were from a local distillery (Charlotte, NC). Diluted sugarcane molasses and vinasse were suspended in acetone to achieve 70% concentration. The tubes were vortexed strongly twice for 15 seconds each before shaking in the

shaker at 200 rpm for 15 minutes and centrifuged for 15 minutes at 3500 rpm. The resulting supernatant was pipetted to a new tube, while a black precipitated residue was discarded. Following acetone evaporation, the remaining aqueous solution was sequentially extracted with dichloromethane (DCM) and ethyl acetate (EA). The organic layers were concentrated to dryness and designated as phenolic-rich DCM and EA fractions. The fractions were also pooled together and then dried completely with nitrogen flow at room temperature. The remained residue was dissolved in ethanol to obtain 50 mg/ml stocks and stored in a −20ºC freezer until use.

2.2.5 Assay to determine total phenol content

The total phenolic content assays were performed following modified quantification procedure protocols in existing literature [22]. The assays were accomplished using a modified Folin-Ciocalteu colorimetric method and results of total phenolic content were expressed as milligram of gallic acid per 100 g fresh weight or dried weight (mg GAE/100 g dry weight) [22–24]. Acidified methanol (80%) was prepared by adding 830 µL of concentrated HCl to 80 ml methanol, and 20 ml of distilled water. Serial dilution of gallic acid with the highest concentration of 4 mg/ml was prepared as a reference standard. Sodium carbonate (20%) was obtained by adding 5 g of sodium carbonate to 25 ml of distilled water. Gallic acid standards were prepared by allocating 300 µl of the gallic acid stock solution and 300 µl of acidified methanol with the exception of the blank. A 96 well plate was set up and 140 µl of distilled water was added to each well. A 10 µl standard Folin-Ciocalteu reagent (Sigma-Aldrich, St. Louis, MO, USA) was also added to each well and 20 µl of blacks, standard solutions, and samples were added and allowed to stand for 5 min at room temperature before 30 µl of sodium carbonate solution was added to each well. The solution was mixed by shaking the plate briefly and kept at

room temperature for 20 min to allow the development of color, and absorbance at 765 nm against an appropriate blank was determined using the Synergy H1/Take 3 spectrophotometer (BioTek, Winooski, VT, USA). After subtraction of blank values, data were reported as means, and phenolic concentrations were calculated as gallic acid equivalents from a standard curve X (concentration) vs. Y (absorbance 765).

2.2.6 HPLC analysis of phenolics

HPLC-UV analysis was performed using a Shimadzu HPLC system equipped with a pump (LC-20AT), an autosampler (SIL-20A), a diode array detector (SPD-M20A), and an automatic column temperature control oven (CTO-20A). Separation was performed on the Restek Ultra C18 column (250 x 4.6 mm, 5 μ) at a column temperature of 30^{o}C. The binary mobile phase consisted of 0.1% formic acid in water (Eluent A) and acetonitrile (Eluent B) in a gradient as follows: 0-5 min, 10% acetonitrile; 5-35 min, 55% acetonitrile;35-37 min, 95% acetonitrile; 37-39 min, 95% acetonitrile; 39-40 min, 10% acetonitrile, 40-45 min, 10% acetonitrile. Each run was followed by an equilibration time of 10 min. Ultraviolet (UV) spectra were monitored at 340 nm, and the flow rate was 1.0 ml/min. The data were collected and analyzed with LC solution (Shimadzu, Nakagyo-ku, Kyoto, Japan) software. Peaks were identified based on a comparison of retention times and UV spectra with those of authentic standards.

2.2.7 Macrophage cell culture

The mouse macrophage cell line RAW 267.4 (ATCC TIB-71, obtained from American Type Culture Collection, Livingstone, MT, USA) was maintained in Dulbecco's modified

Eagle's medium (DMEM, Life Technologies, NY, USA), supplemented with 100 IU/ml penicillin/100 μg/ml streptomycin (Fisher) and 10% fetal bovine serum (Life Technologies) at a density not exceeding 5 x 10^5 cells/ml and maintained at 37 ^oC in a humidified incubator with 5% CO_2. Unless otherwise specified, extracts and pure compounds for cell culture use were prepared in DMSO as 1000X stocks and stored at -20 ^oC until use.

2.2.8 Total RNA extraction, cDNA synthesis, and quantitative PCR analysis

The total RNA extraction from the mouse macrophage cell line RAW 267.4 was performed using TRIzol reagent (Life Technologies) following the manufacturer's instructions. The quantification of RNA was used by the Synergy H1/Take 3 spectrophotometer (BioTek, Winooski, VT, USA). Followed by conducting cDNAs synthesis using 2 μg of RNA for each sample with a commercially available high-capacity cDNA Reverse Transcription kit (Life Technologies), following the manufacturer's protocol on an ABI GeneAMP 9700 (Life Technologies). The resulting cDNA was amplified in triplicate by real-time PCR using SYBR green PCR Master Mix (Life Technologies). To avoid interference due to genomic DNA contamination, only intron-overlapping primers were selected using the Primer Express version 2.0 software (Applied Biosystems, Foster City, CA) as follows: : β-actin, forward primer: 5'-AAC CGT GAA AAG ATG ACC CAG AT-3′, reverse primer: 5′-CAC AGC CTG GAT GGC TAC GT-3′; COX2, forward primer: 5′-TGG TGC CTG GTC TGA TGA TG-3′, reverse primer: 5′-GTG GTA ACC GCT CAG GTG TTG-3′; iNOS, forward primer: 5′-CCC TCC TGA TCT TGT GTT GGA-3′, reverse primer: 5′-TCA ACC CGA GCT CCT GGA A-3′; IL-6, forward primer: 5′-TAG TCC TTC CTA CCC CAA TTT CC-3′, reverse primer: 5′-TTG GTC CTT AGC CAC TCC TTC-3′; and IL-1β**,** forward primer: 5′-CAA CCA ACA AGT GAT ATT CTC CAT

G-3′, reverse primer: 5′-GAT CCA CAC TCT CCA GCT GCA-3′. Quantitative PCR (qPCR) amplifications were performed on an ABI 7500 Fast real time PCR (Life Technologies) using 1 cycle at 50 °C for 2 min and 1 cycle of 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The dissociation curve was completed with 1 cycle of 1 min at 95 °C, 30 s at 55 °C, and 30 s at 95 °C. mRNA expression was analyzed using the ΔΔCT method and normalized with respect to the expression of the β-actin housekeeping gene using 7500 Fast System SDS Software v1.3.0 (Life Technologies). Amplification of specific transcripts was confirmed by obtaining melting curve profiles.

2.2.9 Statistical analysis

Statistical analyses in all the experiments were performed using Prism 9.0.2 (GraphPad Software, San Diego, CA) and values are expressed as mean ± SEM. Data were analyzed by one-way ANOVA with treatment as a factor and significance was set at $p < 0.05$.

2.3 Results and discussion

2.3.1 In vitro stability of phenolic acids in microbial cultures

In this experiment, we tested the initial hypothesis that the stability of different phenolic acids depends on the methylation status of the hydroxyl groups found on the phenolic ring. A pair of model benzoic acids (protocatechuic acid 3,4DHBA or PCA; and methylated vanillic acid 3H4MBA or VA) were followed for 16 h (overnight) in presence of model microbial organisms including a gram-positive bacteria *Staphylococcus aureus*, single-cell yeasts *Saccharomyces cerevisiae* and *Candida albicans*, and a filamentous fungus *Aspergillus niger* (**Figure 2.1**). The

stability of individual phenolic acids in these mixtures were estimated at a 16 h time point. Post-incubation media samples were collected and processed for HPLC analysis. *Aspergillus* culture showed moderate metabolism of both phenolic acids, while both *Saccharomyces* and *Candida* consumed significant amounts of supplemented phenolic acids. In both gram-negative *Escherichia coli* and gram-positive *Staphylococcus aureus* bacteria models we observed preferential metabolism of a hydroxylated phenolic acid (benzoic acid) versus its methylated counterpart (vanillic acid).

We next introduced a second pair of hydroxylated (caffeic acid) and its methylated variant (ferulic acid) and tested both pars in fermentation mixtures with *Escherichia coli* over a series of time points in the range of 0-48 hours (**Figure 2.2**). The HPLC results suggested that the concentrations of all phenolic acids (protocatechuic acid, vanillic acid, caffeic acid, and ferulic acid) decreased significantly over time, with hydroxylated phenolic acids disappearing from the treatment media at a higher rate. For example, at 24 h point, an average of 50% of benzoic acid was lost while more than 75% of vanillic acid remained in the medium. A similar dataset was also obtained for the second pair, where hydroxylated caffeic acid remained at 40% at 24 h post-treatment, while its methylated derivative ferulic acid persisted at 70% at this point. Both datasets supported the idea that methylated phenolic acids survived in the microbial fermentation environment for a longer period.

2.3.2 In vitro stability of phenolic acids groups

To analyze the behavior of all major phenolic acid metabolites typically present in human biological fluids (such as plasma, urine, and fecal water) after ingestion of polyphenols, we extended the studies in the *Escherichia coli* model system to four series of phenolic acid

metabolites including benzoic acids (benzoic acid, 2-hydroxybenzoic acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, protocatechuic acid, vanillic acid, gallic acid, and syringic acid), cinnamic acids (3,4,dihydroxycinnamic/caffeic acid, 3-hydroxy-4-methoxycinnamic/isoferulic acid, 4-hydroxy-3-methoxycinnamic/ferulic acid, 3-hydroxycinnamic/m-coumaric acid, and 4-hydroxycinnamic/p-coumaric acid), phenylacetic acids (3,4-dihydroxyphenylacetic acid, 4-hydroxy-3-methoxyphenylacetic acid, 4-hydroxyphenylacetic acid and 3-hydroxyphenylacetic acid), and phenylpropanoic acids (4-dihydroxyphenylpropanoic acid, 4-hydroxy-3-methoxyphenylpropanoic acid, 4-hydroxyphenylpropanoic acid and 3-hydroxyphenylpropanoic acid). When supplemented at 100 μg/ml and kept in the presence of *E. coli* for 24 hours, the concentration of vanillic acid showed the highest survival rate at 67.21%, followed by 3-hydroxybenzoic acid at 55.88%, 4-hydroxybenzoic acid (46.43%), 2-hydroxybenzoic acid (43.22%), benzoic acid (38.56%), and gallic acid was found to have the lowest concentration rate (30.76%) (**Figure 2.3A**). Regarding phenylacetic acids, their concentrations showed the highest survival rate in 4-hydroxy-3-methylphenylacetic acid (41.67%), whereas 3,4-dihydroxyacetic acid had the lowest rate (31.42%) (**Figure 2.3B**). On the other hand, among the phenylpropanoic acids examined, 3,4-dihyroxyphenylpropanoic acid had the highest survival of 43.27%, and the lowest rate was found in 4-hydroxypropanoic acid (19.55%) (**Figure 2.3C**). In cinnamic acid series, 4-hydroxy-3-methoxycinnamic acid was found to have the higher 59.01% survival rate, while 3-hydroxycinnamic acid nearly degraded with the lowest concentration (16.67%) (**Figure 2.3D**).

2.3.3 Phenolic profiles of rum fermentation products

To analyze the behavior of all major phenolic acid metabolites typically found in human biological fluids in different steps and byproducts of rum fermentation, we analyzed the total phenolics, total phenolic HPLC profiles, and quantified major phenolic acid metabolites in molasses (a concentrated substrate used for rum fermentation) and vinasse (a concentrated waste product after fermentation and distillation are completed).

Total HPLC phytochemical profiles of molasses (**Figure 2.4**) and vinasse (**Figure 2.5**) showed the measurable differences in the chromatograms. The proportional intensity of the early polar peaks (0-5 min, mostly sugars) to midrange polarity metabolites that contain most phenolic structures present in these products (5-25 min) showed the natural concentration of phenolic metabolites during the fermentation process from molasses to vinasse. This suggests that a significant amount of sugarcane phenolics survived rum fermentation and accumulated in the vinasse byproduct. To quantify individual phenolic metabolites, both molasses and vinasse were further fractionated into dichloromethane (DCM, less polar) and ethyl acetate (EA, more polar) fractions to simplify the overall HPLC profiles and allow for successful resolution of individual peaks.

DCM fraction of molasses (**Figure 2.6**) contained on average 1.9 μg quercetin, 0.5 μg kaempferol, 219.9 μg diosmetin, 1.2 μg tricetin, 1.4 μg luteolin, 2.0 μg apigenin, 0.7 μg protocatechuic acid, 12.7 μg vanillic acid, 58.0 μg syringic acid, 4.3 μg homovanillic acid, 4.3 μg p-coumaric acid, 9.9 μg sinapic acid.

EA fraction of molasses (**Figure 2.7**) contained on average 0.2 μg quercetin, 0 μg kaempferol, 140.8 μg diosmetin, 0.8 μg tricetin, 1.0 μg luteolin, 0.8 μg apigenin, 29.4 μg

protocatechuic acid, 72.3 μg vanillic acid, 37.2 μg syringic acid, 5.9 μg homovanillic acid, 110.9 μg p-coumaric acid, 2.5 μg chlorogenic acid, 17.2 μg sinapic acid.

DCM fraction of vinasse (**Figure 2.8**) contained on average 5.4 μg quercetin, 1.2 μg kaempferol, 1030.0 μg diosmetin, 1.6 μg tricetin, 1.4 μg luteolin, 9.3 μg apigenin, 5.3 μg protocatechuic acid, 163.5 μg vanillic acid, 271.3 μg syringic acid, 12.2 μg homovanillic acid, 7.3 μg p-coumaric acid, 4.0 μg sinapic acid.

EA fraction of molasses (**Figure 2.9**) contained on average 0.3 μg quercetin, 0 μg kaempferol, 567.4 μg diosmetin, 3.6 μg tricetin, 0.8 μg luteolin, 0.9 μg apigenin, 94.8 μg protocatechuic acid, 72.3 μg vanillic acid, 64.0 μg syringic acid, 5.3 μg homovanillic acid, 2.4 μg p-coumaric acid, 7.1 μg chlorogenic acid, 3.4 μg sinapic acid, 0.6 μg ferulic acid.

When summarized, these combined results indicated enrichment of vinasse byproduct with flavonoids quercetin (a change from 2.1 μg in molasses to 5.7 μg in vinasse), diosmetin (360.7 to 1597.4 μg), tricetin (2.0 to 5.2 μg), apigenin (2.8 to 10.2 μg) and phenolic acids protocatechuic acid (30.1 to 100.2 μg), vanillic acid (85.0 to 235.8 μg), syringic acid (95.2 to 335.3 μg). Methylated metabolites such as diosmetin (4.4 fold), vanillic (2.7 fold), and syringic acids (3.5 fold) showed the most prominent increases. One phenolic acid, specifically p-coumaric acid, showed a dramatic decrease (115.2 to 9.6 μg) indicating that it was metabolized and lost during the rum fermentation process.

2.3.4 Reduction of inflammatory response in macrophages

All groups of phenolic acid metabolites showed varying levels of modulation of gene expression profiles associated with acute and chronic biomarkers of inflammation (Cox-2, iNOS, IL-1β, and IL-6). Cox-2 expression was most strongly affected by 3-caffeoylquinic acid -1.72x (-

72%) and 2-hydroxybenzoic acid -1.76x (-76%), followed by metabolites from hydroxybenzoic and hydroxycinnamic acids in the range of 22-56% and 38-61%, respectively (**Figure 2.10**). A wider array of metabolites affected the expression of iNOS, albeit the overall magnitude of the effect was smaller than that of Cox-2. Many of the metabolites affected the expression levels of the early inflammatory IL-1β gene with no clear specificity towards any subclass used in this study. The strongest inhibition of IL-1β expression was achieved by treatment with 3-caffeoylquinic acid and its major metabolite 3,4-dihydroxycinnamic acid (-1.73x and -1.81x, respectively), as well as 2-hydroxybenzoic acid (-1.65x). The mRNA levels of IL-6 were least affected by phenolic metabolites, as significant IL-6 mRNA decreases were observed only when cells were exposed to cinnamic acid metabolites in the range of 24-55% as observed for 3,4-dihydroxycinnamic, 4-hydroxy-3-methoxycinnamic, and 3-hydroxy-4-methoxycinnamic acids.

2.3.5 Comparative summary of results

Low bioavailability of the high molecular weight polyphenols, at least partially resistant to digestion, has been a matter of research and debate for several decades. We now know that chemical degradation and bacterial catabolism of these molecules into the smaller and more bioavailable catabolites such as phenolic acids form a critical connection between xenobiotic metabolism, bioactivation, and host-gut biochemical interactions with these structures. Microbiome-derived fermentation products from foods naturally rich in polyphenols may also play an important role in regulating the immune outcomes [25].

The integrity of intestinal mucosa is not only the major critical factor for nutrient digestion and absorption but also plays a key role in the recognition and activation of the immune system in response to physiological stimuli and pathogen infection. Lipopolysaccharide

induces an increase in intestinal permeability and subsequently activates residential tissue macrophages that promote differentiation and development of the pro-inflammatory response [26]. These effects are mediated by changes in the expression levels of cytokine biomarkers that drive activation of the classical pro-inflammatory M1 pathway, including the inducible nitric oxide synthase (iNOS), interleukin-1β (IL-1β), and interleukin-6 (IL-6). Additionally, cyclooxygenase-2 (Cox-2)/PGE_2 production in activated macrophages further drives distinct intracellular pathways that modulate inflammation and host immune responses [27].

In this study, phenolic acids reduced gene expression levels of iNOS in macrophages, and this effect was largely absent when cells were exposed to hydroxyphenylacetic and hydroxyphenylpropionic acids (**Figure 2.10**). Among those, 3,4-dihydroxycinnamic (caffeic acid) and chlorogenic acid (that carries the caffeic acid moiety as a part of its structure) showed the strongest inhibition of pro-inflammatory gene expression in macrophages at the concentration tested. This was an interesting parallel to a previous study that showed the importance of the caffeic acid pharmacophore to metabolic health [28]. Methylated derivatives of caffeic acid showed stronger effects on COX-2 and IL-6 expression as compared to iNOS and IL-1β genes, suggesting different efficacies at various pathways of the inflammatory regulatory networks. The yet unexplored structure-activity relationships between different subclasses of phenolic metabolites with respect to their methylation and hydrophobicity, which facilitates passive membrane permeation and interaction with molecular and intercellular targets, may partially explain these findings and warrant further investigation.

Figures and legends

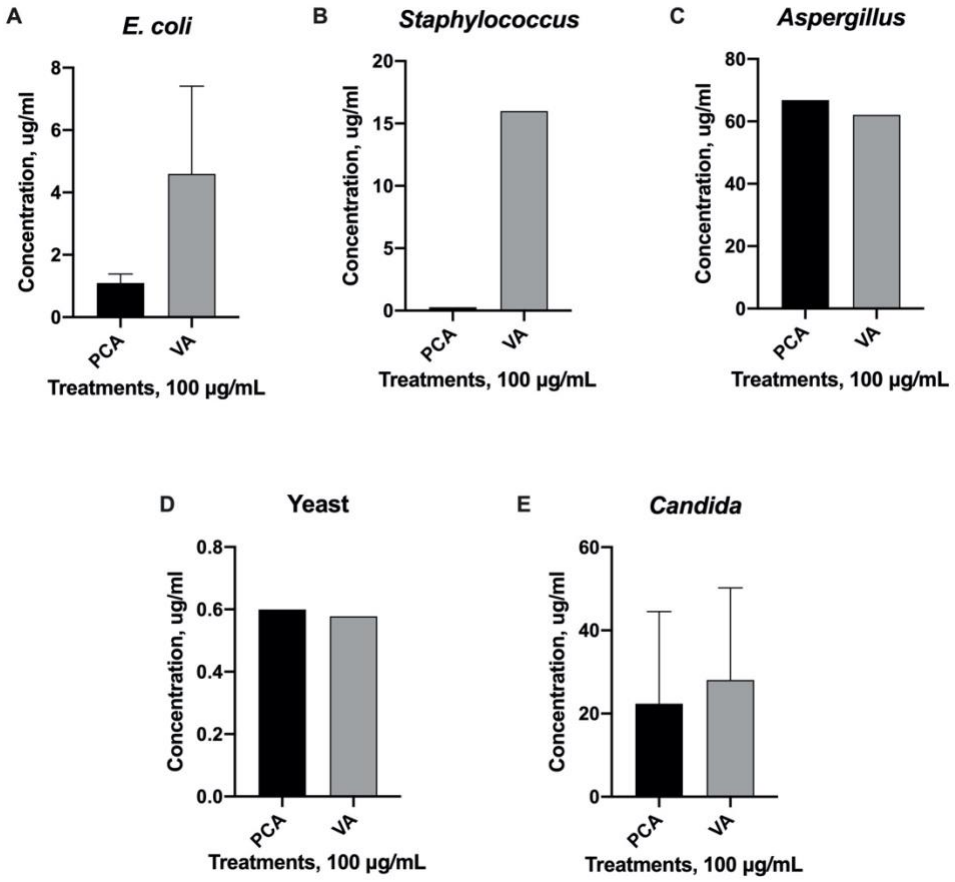

Figure 2.1. Stability of unmethylated and methylated pairs of phenolic acids in a model fermentation conditions in the presence of different microorganisms including (A) gram-negative *Escherichia coli*, (B) gram-positive *Staphylococcus aureus*, (C) filamentous fungus *Aspergillus niger*, (D) budding yeast *Saccharomyces cerevisiae*, (E) single cell yeast *Candida albicans*. Concentrations of benzoic acids pair (protocatechuic acid 3,4DHBA or PCA; vanillic acid 3H4MBA or VA) were followed for 16 h (overnight). Data is reported from two independent experiments as mean ± SEM.

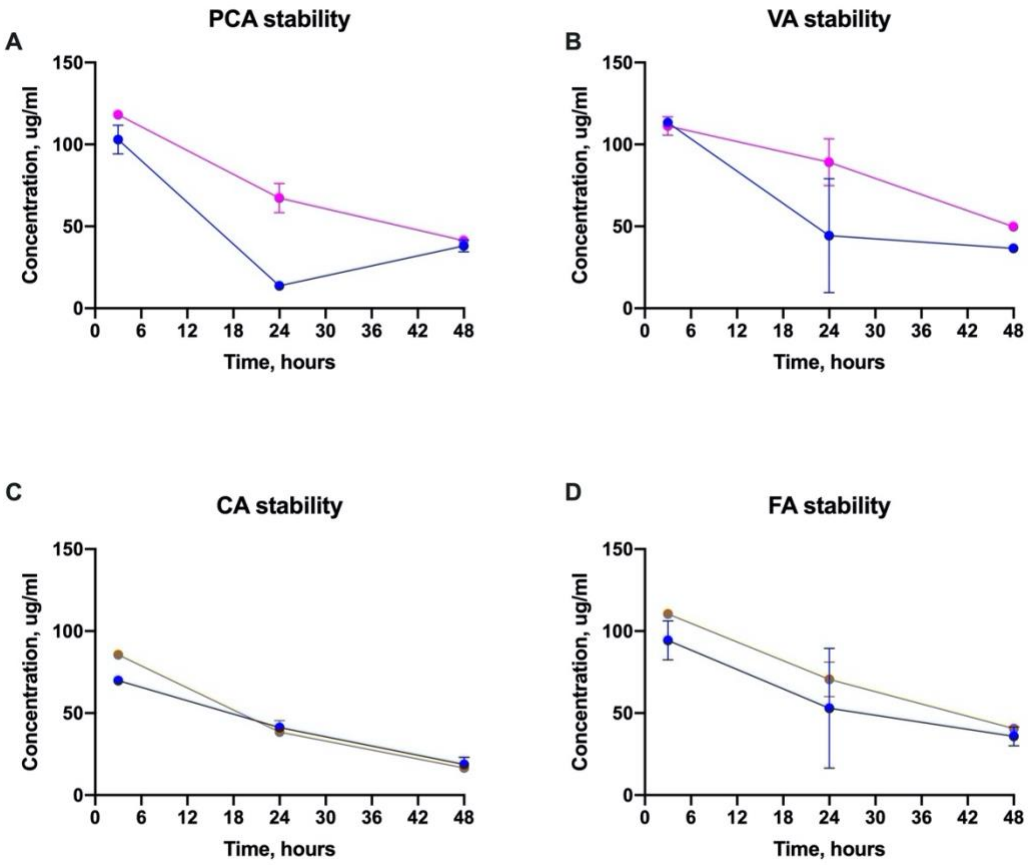

Figure 2.2. Stability of unmethylated and methylated pairs of phenolic acids in a model fermentation conditions in the presence of *Escherichia coli*. Concentrations of benzoic acids pair (protocatechuic acid 3,4DHBA or PCA; vanillic acid 3H4MBA or VA) and cinnamic acids pair (caffeic acid 3,4DHCA or CA; ferulic acid 3H4MCA or FA) were followed for 48 h. Data is reported from two independent experiments as mean ± SEM.

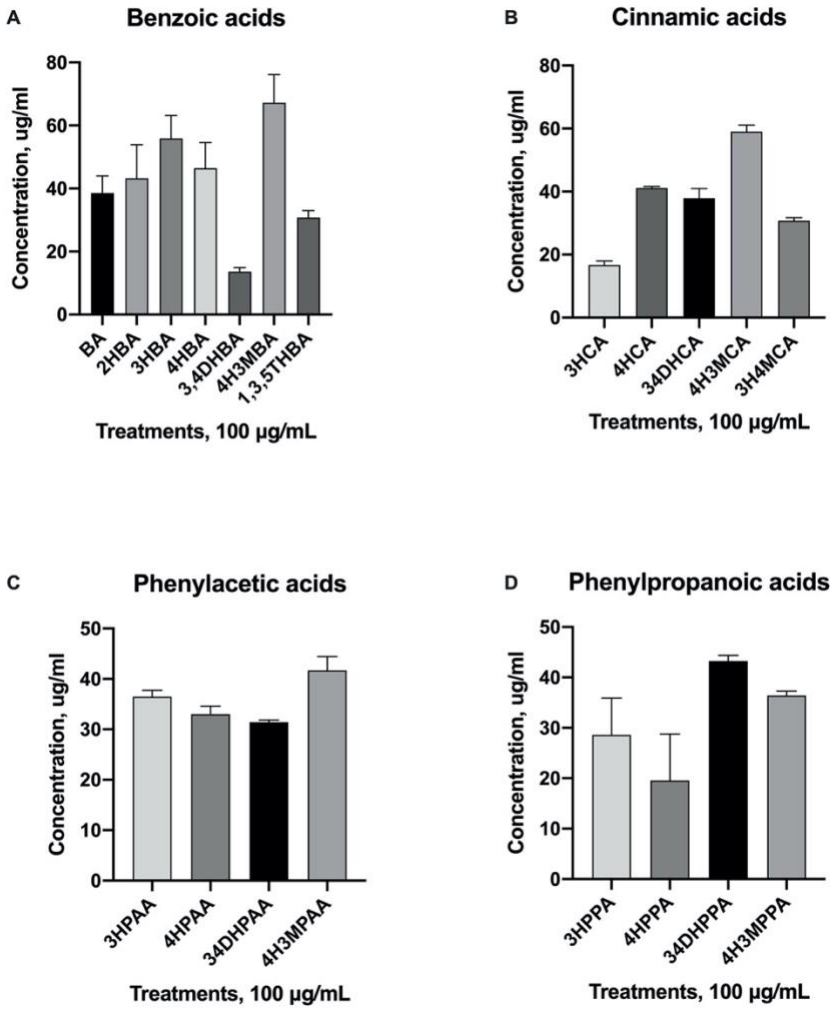

Figure 2.3. Stability of four series of phenolic acid metabolites typically found in biological fluids (such as plasma, urine, and fecal water) in a model fermentation conditions in the presence of *Escherichia coli*. Data is reported as mean ± SEM.

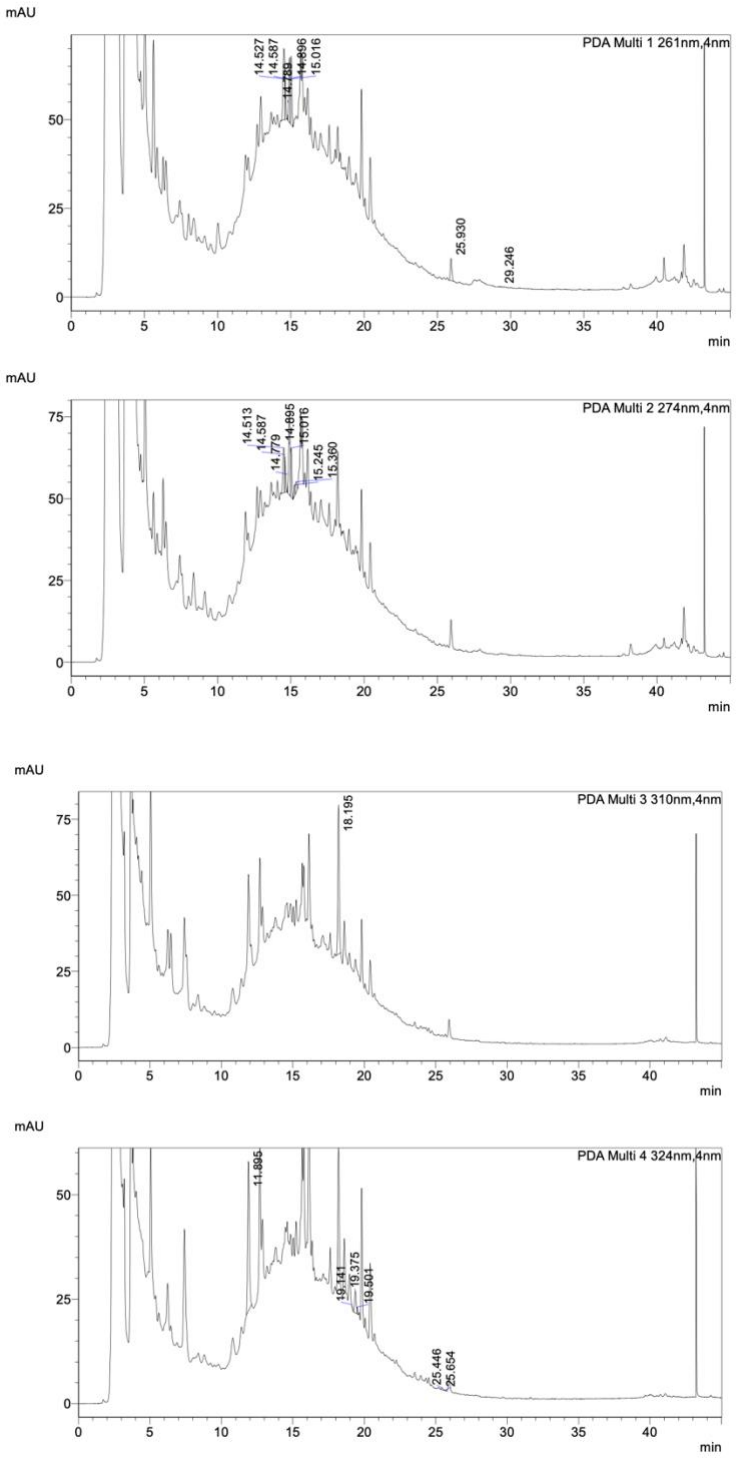

Figure 2.4. Total phytochemical profile of rum **molasses** at different wavelengths of 261, 274, 310, and 324 nm used for detection of flavonoid and phenolic acid metabolites.

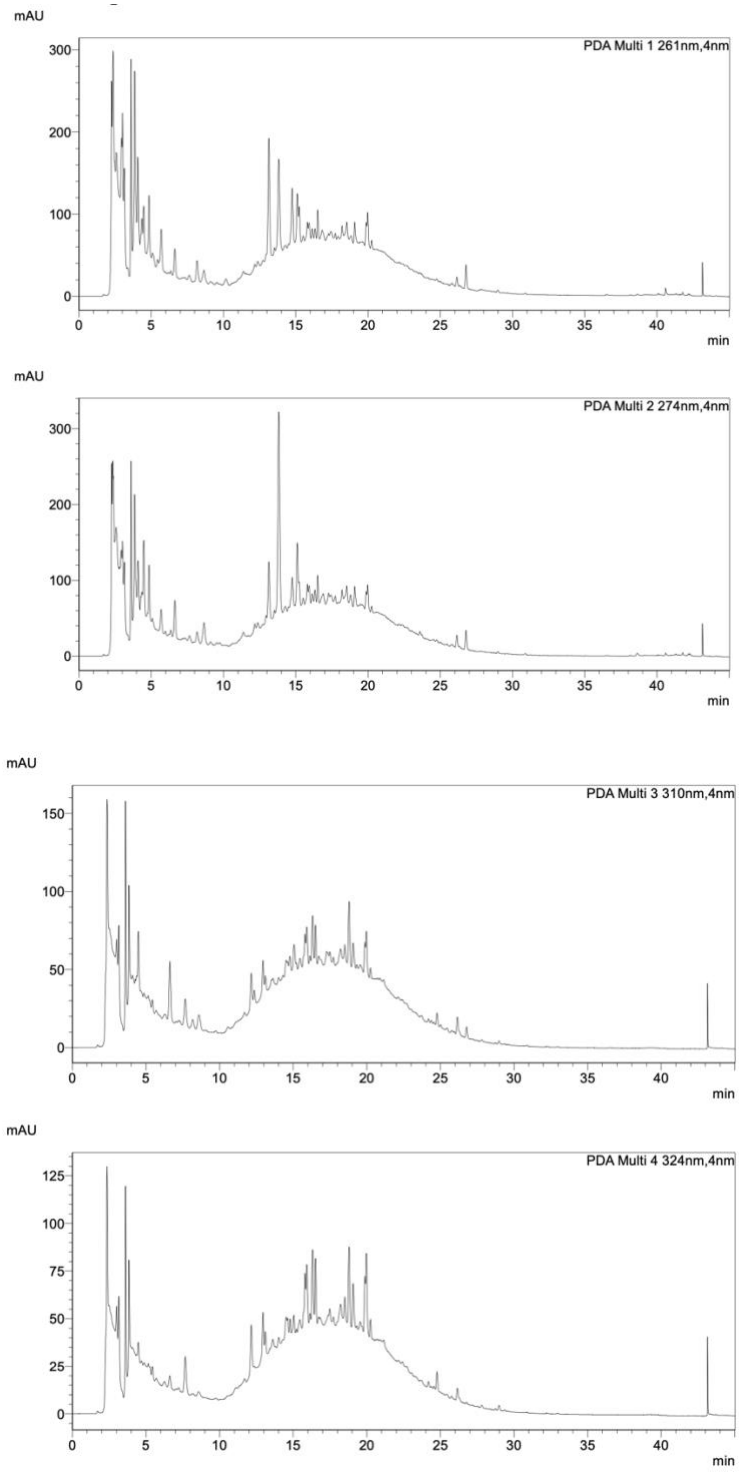

Figure 2.5. Total phytochemical profile of rum **vinasse** byproduct at different wavelengths of 261, 274, 310, and 324 nm used for detection of flavonoid and phenolic acid metabolites.

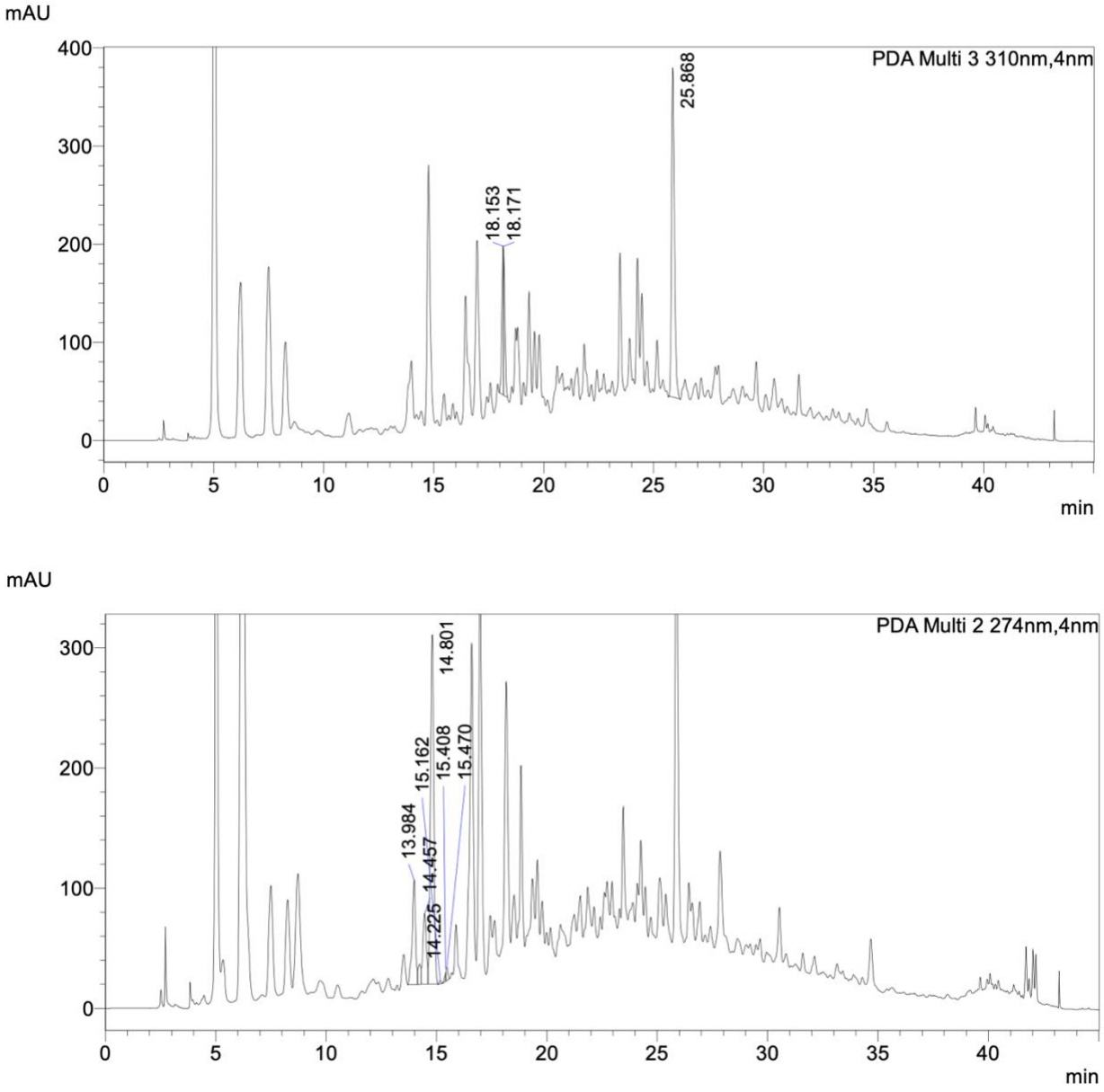

Figure 2.6. Phytochemical profile of DCM dichloromethane fraction of rum **molasses** at 310 nm for detection of flavonoids (top) and 274 nm for detection of phenolic acids (bottom).

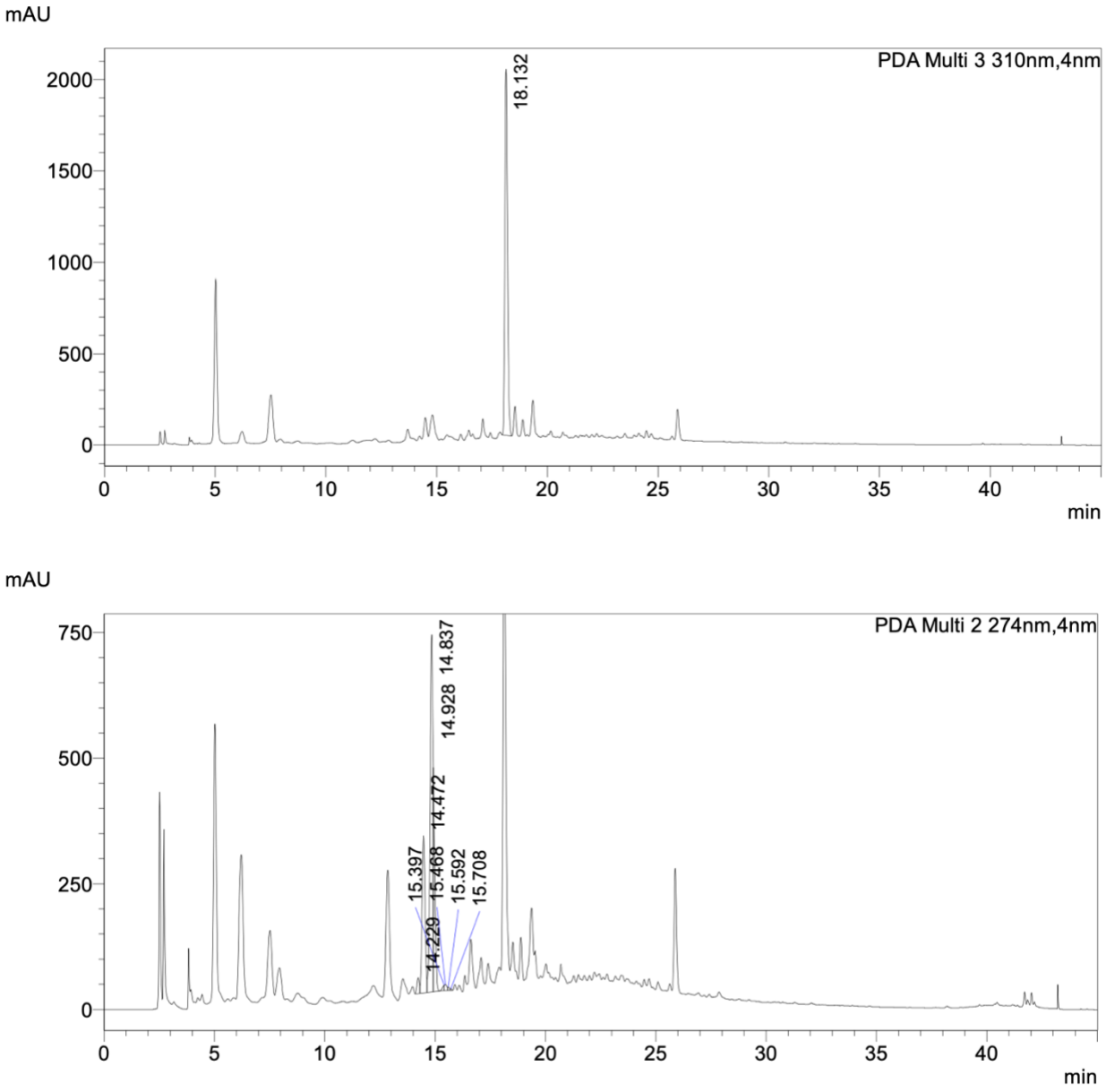

Figure 2.7. Phytochemical profile of EA ethyl acetate fraction of rum **molasses** at 310 nm for detection of flavonoids (top) and 274 nm for detection of phenolic acids (bottom).

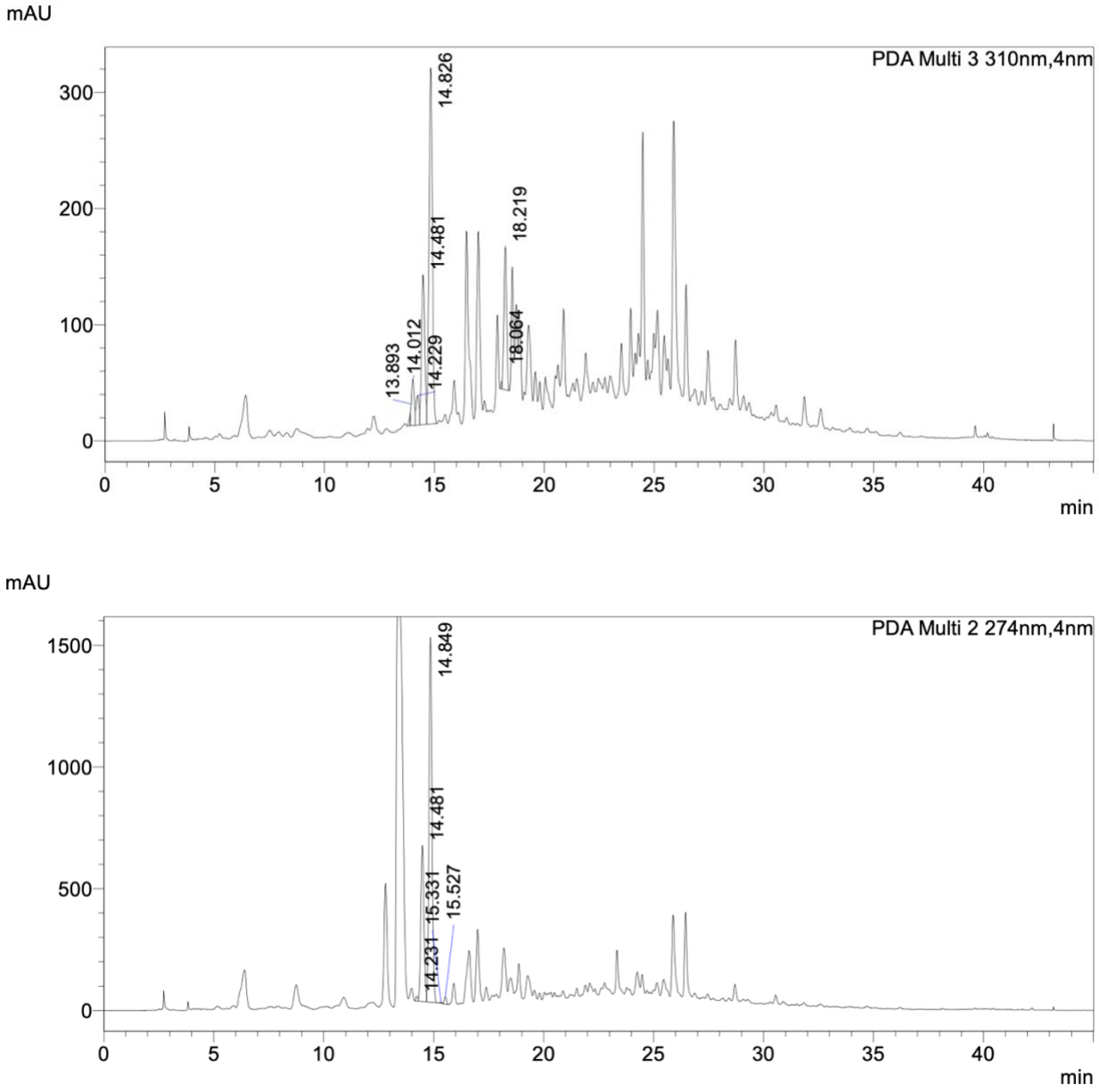

Figure 2.8. Phytochemical profile of DCM dichloromethane fraction of rum **vinasse** at 310 nm for detection of flavonoids (top) and 274 nm for detection of phenolic acids (bottom).

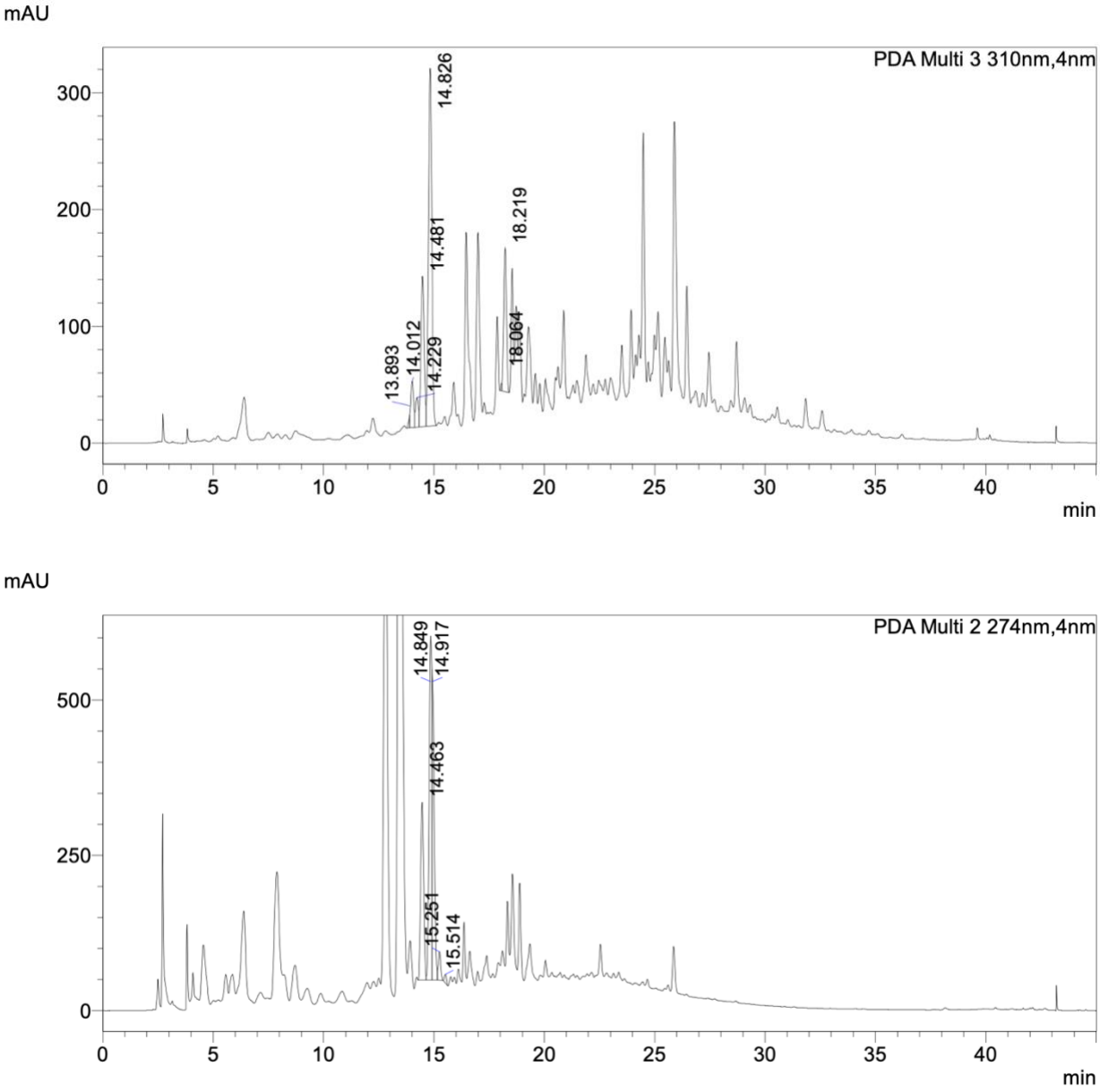

Figure 2.9. Phytochemical profile of EA ethyl acetate fraction of rum **vinasse** at 310 nm for detection of flavonoids (top) and 274 nm for detection of phenolic acids (bottom).

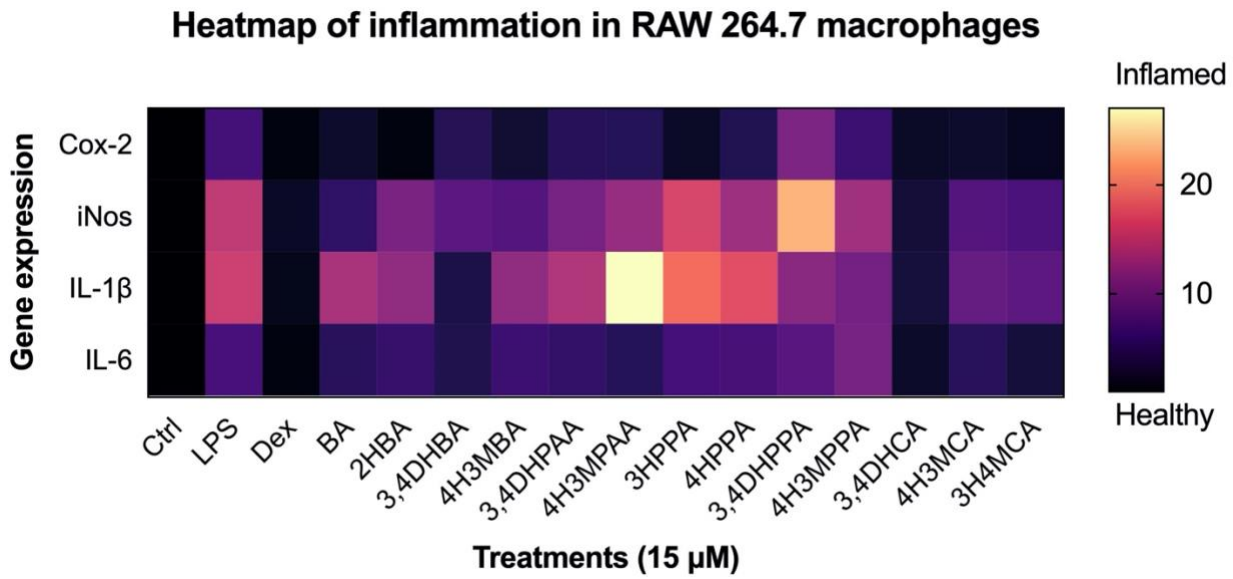

Figure 2.10. Heatmap of anti-inflammatory effects of phenolic acid metabolites based on qPCR gene expression profiles of key biomarkers of acute and chronic inflammation including cyclooxygenase-2 (Cox-2), inducible nitric oxide synthase (iNOS), interleukin-1β (IL-1β), and interleukin-6 (IL-6).

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CONSLUSIONS AND FUTURE DIRECTIONS

Humans, both actively and passively, ingest, absorb, and inhale a wide range of phenolic substances as a part of a regular diet. These phenolics undergo phase I (oxidation, reduction) and phase II (conjugation) xenobiotic metabolism, where they are further glucuronidated in the endoplasmic reticulum lumen, sulfated in the cytoplasm, methylated in both the cytosol or in the endoplasmic reticulum, or conjugated to amino acids in mitochondria. The degree of conjugation of a phenolic metabolite with a particular phase II pathway relates to its physiochemical properties, and different fragmentation patterns of phenolic catabolites originating from polyphenolic structures are expected to produce different metabolic signatures and physiological outcomes (Chapter 1).

Sugarcane (*Saccharum officinarum* L.) is the world's largest crop by production quantity, yet it is one of the lesser-known sources of polyphenols. After sugarcane juice or the concentrated product (molasses) is fermented, a significant quantity of sugarcane polyphenols ends up in the final, partially dehydrated, byproduct (vinasse). This study attempted to determine changes in phenolic metabolite profiles during the rum fermentation and distillation process that starts with the concentrated molasses (pre-fermentation) and ends with the vinasse (post-fermentation) waste byproduct. Different phenolic metabolites showed various degrees of stability and survival during the fermentation process that resulted in partial enrichment of vinasse products with small flavonoid and phenolic acid metabolites, in particular those that show increased hydrophobicity due to methylation. Phenolic acid metabolites found in vinasse reduced pro-inflammatory gene expression profiles in the LPS-stimulated macrophages, with benzoic and cinnamic acids showing maximum anti-inflammatory effects. This study lays the

foundation for future investigations evaluating the effects of phenolics present in sugarcane waste streams and their putative applications to managing immune health outcomes.

The hopes to apply these findings to tackle future nutritional interventions and public health outcomes rely in part on our ability to address the following critical gaps in the field: (1) develop new robust solutions and metabolomic pipelines to accelerate discovery and identification of all small phenolic metabolites; (2) attain high throughput microbial isolation and cultivation systems individually or as a consortium of a model microbiome to understand which microorganisms contribute to changes in the phenolic profiles; and (3) evaluate how subtle differences in metabolism and phenolic profiles can have direct effects on human health and wellbeing.