ELUCIDATION OF THE METABOLISM OF FRUIT TANNINS BY THE

INTESTINAL MICROBIOME

A Dissertation

by

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ABSTRACT

Tannins are polymers of polyphenols that are abundant in the diet. Due to their large size, tannins are not bioavailable unless extensively metabolized by the intestinal microbiome into phenolic acid metabolites. Despite the identification of several phenolic acid metabolites that are potentially derived from tannins, there are still discrepancies in the metabolism of tannins by the microbiome, especially for those that are rarer in nature, such as A-type proanthocyanidins.

To elucidate the metabolism of tannins by the intestinal microbiome, a static *ex vivo* fermentation model was utilized, whereas tannins were fermented with fecal microorganisms and the changes in their chemical structures and resulting metabolites were characterized and quantified via LC-ESI-MS/MS. Due to the importance of the microbiome in tannin metabolism, fecal microorganisms were collected also from donors who had a form of inflammatory bowel disease, ulcerative colitis, in order to better understand the composition of the microbiota's role in tannin metabolism. The metabolism of other polyphenolic classes were also evaluated in order to identify which metabolites were unique to tannins. Finally, a food processing method for the production of high-gallotannin mango juice with higher polyphenolic bioaccessibility was developed. Major differences occurred between the metabolism of a-type and b-type proanthocyanidins. B-type proanthocyanidins resulted in a larger number of phenolic acid metabolites that are bioavailable and have been identified in plasma and urine *in vivo*. A-type proanthocyanidins however resulted in predominantly epimers and microbially derived tannins that had undergone C-ring reduction. Both flavonoids and phenolic acids resulted in similar metabolites to tannins, however metabolite production from flavonoids was dependent on C-ring substitution and aromaticity. Tannase proved to successfully increase bioaccessibility of tannins

and can be utilized as a novel processing aid for mango juice. Tannin metabolism by the microbiome is unique to each class of tannin and is affected by degree of polymerization and type of bond that exists between monomers. Ultimately, these findings will help food processors optimize production of high-tannin functional foods.

DEDICATION

To my parents who gave me the world.

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Contributors

Funding Sources

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION

Tannins constitute one of the several classes of polyphenols, or secondary plant metabolites, that have been hypothesized to explain the inverse correlation of fruit and vegetable consumption and incidence of disease (Wang *et al*., 2014, Guasch-Ferre *et al*., 2017, Kotecha *et al*., 2016, Rienks *et al*., 2017, Pasinetti *et al*., 2015). Polyphenols, including tannins, are synthesized in plants through the shikimic acid and phenylpropanoid pathways to aid in their defense against excessive ultraviolet radiation, consumption by herbivores and insects due to their unpalatable bitterness and astringency, pathogenic bacterial infection and reactive oxygen species (Cires *et al.,* 2017, Sharma *et al*. 2012). An evolutionary example of this is the high concentration of tannins in seed coats that exist to protect seeds during the start of their lifecycle (Dixon, Xie, & Sharma, 2004). Anti-bacterial and radical scavenging activity of polyphenols are hypothesized to benefit not only plant health but also human health upon the consumption of fruits and vegetables. Originally, it was assumed that the higher the reducing capacity of polyphenols, the higher their biological activity. However, *in vivo* pharmacokinetic and *in vitro* caco-2 studies demonstrated that polyphenols have low bioavailability, especially tannins due to their large size, contradicting the hypothesis that their bioactivity benefits systemic biological systems and limiting their activity to the gastrointestinal tract (Duenas *et al.*, 2015, Manach *et al.*, 2005).

Recently, it has become well established that the human colon microbiome is an important site of tannin metabolism, resulting in metabolites that are more bioavailable and possibly bioactive than their parent compounds (Duenas *et al.*, 2015, Selma, Espin & Tomas-

Barberan, 2009, Tomas-Barberan *et al.*, 2016). Not only was it found that the microbiome modifies tannins through their catabolism, but also tannins modify microbiota composition by increasing and decreasing certain taxa. Modulation of the microbiome's bacterial composition is hypothesized to have important health implications as the microbiome has a large impact on the immune system and metabolic output of the host (Round & Mazmanian, 2014).

It is hypothesized that tannins, especially compared to other classes of polyphenols, can modulate the human colon microbiota due to their unique properties; the majority of tannins reach the colon upon consumption unchanged by gastric digestion and they are known to have anti-bacterial activity through multiple mechanisms (Smith, Zoetendal & Mackie, 2005, Monagas *et al.*, 2010). Further, tannins predominate in the diet as they have an estimated consumption rate of 0.5 g per day (Smeriglio *et al.*, 2017). For these reasons it is of interest to elucidate the metabolism of tannins by the microbiome and how in turn this impacts microbiota composition.

Specific objectives were:

1. Identify microbiome metabolites derived from cranberry proanthocyanins through targeted and untargeted metabolomics analysis via LC-ESI-MS/MS and determine how metabolite production differs between healthy individuals and those with ulcerative colitis.

2. Determine how cranberry proanthocyanidins modulate the microbiota composition of the microbiome through 16S rRNA marker gene metagenomics analysis.

3. Identify confounding metabolites produced from other polyphenols and which metabolites are genuine to tannins through fractionation and simulated microbiome metabolism of cranberry polyphenols.

4. Create food processing strategies that enhance the quality and bioaccessibility of tannin-rich juice.

CHAPTER II

LITERATURE REVIEW

Tannin Chemistry

Tannins are polymers of flavonoid and non-flavonoid polyphenols (Figure 1). Besides polymerization, one of the most distinctive aspects of tannin chemistry that separate them from other classes of polyphenols is their ability to form pH and concentration dependent associations with proteins. Tannins are especially well known to have high affinity for proline-rich proteins (e.g. saliva and gelatin) through hydrogen bonding and hydrophobic interactions between the aromatic rings of tannins and the pyrrolidine moiety of proline (Hagerman, Rice, & Ritchard, 1998). Additionally, tannins have multiple ortho-hydroxyl groups which chelate metal ions and can also interact with polysaccharides (Yoneda & Nakatsubo, 1998, Soares, Mateus & Freitas, 2012). Although they share several chemical properties, tannins are structurally diverse and are classified into 3 unique classes – condensed, hydrolysable and complex.
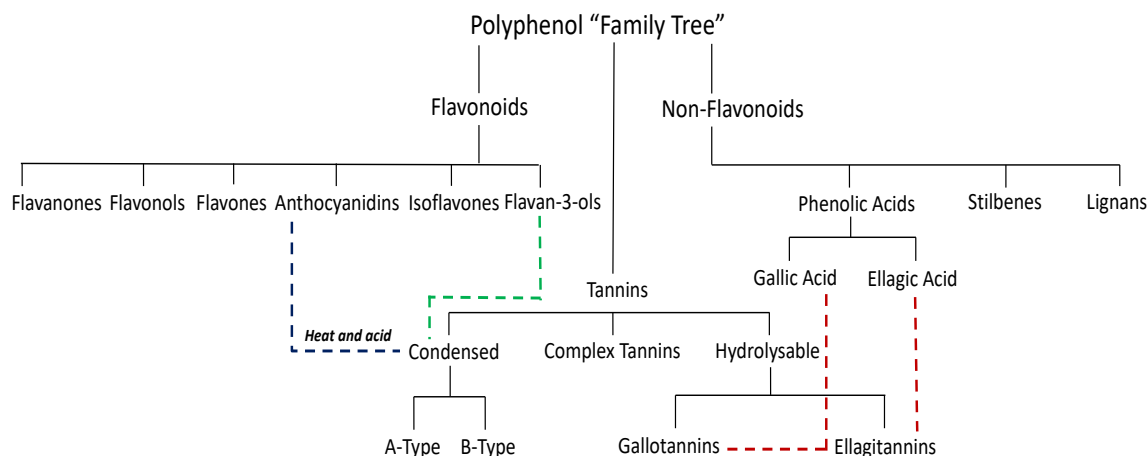
Figure 1 Polyphenol "family tree" or relationships between major classifications of polyphenols. Red and green dashed lines demonstrate how tannins are derived from both flavonoids and non-flavonoids.

Condensed Tannins

Condensed tannins, or proanthocyanidins, are polymers of flavan-3-ols. Flavan-3-ols are of the basic 3-ringed flavonoid structure, C6-C3-C6, with an additional hydroxyl group on C3 of the heterocyclic C-ring, denoting the origin of their name (Figure 2). Condensed tannins are structurally diverse due to the wide range of flavan-3-ol diastereomers in nature. The most common diastereomer monomers of condensed tannins are (-)-epicatechin and (+)-catechin. The B-ring and hydroxyl group on C3 of the C-ring of (-)-epicatechin are *cis* of one another while for (+)-catechin they are *trans*. Other common *cis/trans* diastereomer pairs are (+)-afzelechin and (-)-epiafzelechin, and (+)-gallocatechin and (-)-epigallocatechin (Nielson, O'Keefe & Bolling, 2016). There can additionally be substitution of a galloyl moiety on the hydroxyl group on the

C3 position of flavan-3-ols. However, this is more common to (-)-epigallocatechin and found in select foods such as grapes, tea and nuts (Gu *et al*., 2003).
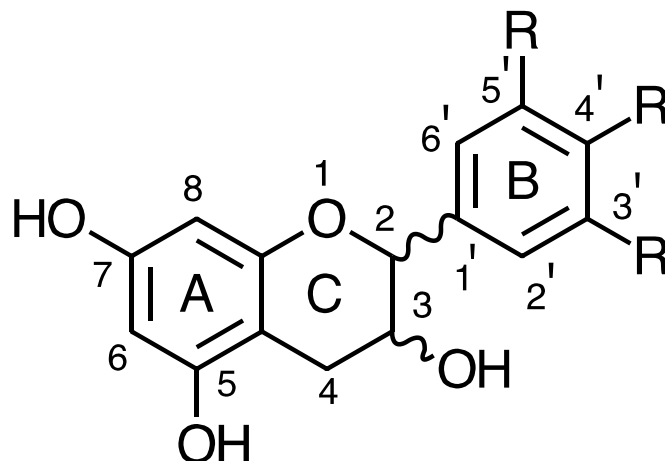
Figure 2 Basic structure and notation of flavan-3-ols.

Condensed tannins can also be distinguished by the substitution of the B-ring of flavan-3-ol monomers. The application of heat, butanol and acid hydrolyzes condensed tannins into their respective anthocyanidin aglycones, which is dependent on B-ring substitution (Schofield, Mbugua & Pell, 2001). For example, the most common condensed tannins, procyanidins, prodelphinidins, and propelargonidins with hydroxyl groups on C4', C3' and C4' and C3', C4' and C5', respectively, will hydrolyze into cyanidin, delphinidin and pelargonidin aglycones

(Nielson, O'Keefe & Bolling, 2016). This is why condensed tannins are interchangeably referred to as proanthocyanidins.

The substitution of the A-ring can also distinguish flavan-3-ol monomers. The A-ring can either have 1 hydroxyl group on C7, designating the monomer as 'resorcinol' type or 2 hydroxyl groups with an additional hydroxyl group on C5 designating the monomer as 'phloroglucinol' type. Procyanidins, prodelphinidins and propelargonidins are all phloroglucinol type condensed tannins (Mena *et al.,* 2015).

Flavan-3-ols are polymerized to form condensed tannins through interflavan bonds. The most common linkage extends from C4 of the extension unit monomer to C8 of the terminal unit monomer. C4 to C6 linkages also occur but to a lesser extent (Smeriglio, 2017, Nielson, O'Keefe & Bolling, 2016). This type of interflavan bond is classified as 'b-type' and is the most common bond between flavan-3-ol monomers (Figure 3). Accordingly, b-type proanthocyanidins are the most predominant class of tannin in the diet. Common dietary sources of b-type proanthocyanidins include cocoa, apples, berries, legumes, nuts, wine, tea, and several grains (Gu *et al*., 2003, Smeriglio *et al*., 2017). A rarer type of linkage in nature is the 'a-type' bond, where there is an additional ether bond between C2 and C7 (Figure 3). A-type condensed tannins typically have one a-type bond which could be between extension, terminal or in the middle of flavan-3-ol monomers, the remaining monomers are linked via b-type bonds (Sarnoski *et al.*, 2012). Dietary sources of a-type condensed tannins are cranberry, litchi, cinnamon, peanuts and avocado. Condensed tannins can exist as both homogenous and heterogenous mixtures of several flavan-3-ols, which further increases their structural diversity found in the diet. Condensed tannins can also vary in polymer size, or degree of polymerization (DP). Typically, condensed tannin DP ranges from 3-11, but a DP of 50 has been reported (Cires *et al*., 2017). In peanut

skins alone, Sarnoski *et al.* (2012) identified 45 unique a-type and b-type condensed tannins with

a DP ranging from 2-4.
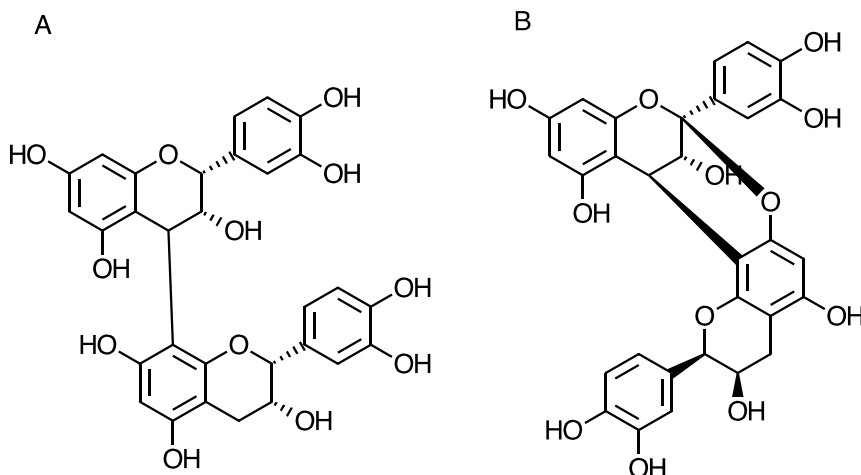
Figure 3 Chemical structures of procyanidin B2 (A) and procyanidin A2 (B). Both compounds contain two (-)-epicatechin units.

Hydrolysable Tannins

Hydrolysable tannins are polymers of phenolic acids (non-flavonoids) esterified to a

central polyol. Unique from condensed tannins and as suggested by their name, hydrolysable

tannins readily hydrolyze into their monomeric constituents in highly acidic or neutral to alkali

conditions (Mueller-Harvey, 2001). Depending on which free phenolic acids are generated post-

hydrolysis, hydrolysable tannins can be further classified into either gallotannins or ellagitannins.

Gallotannins

Gallotannins are polymers of strictly gallic acid typically esterified to beta-D-glucopyranose. Gallotannins have a DP of 5 or greater galloyl moieties. One of the simplest gallotannins is 1,2,3,4,6-pentagalloyl glucoside (PGG), where every hydroxyl group of beta-D-glucopyranose is esterified with a galloyl moiety. It has been found that PGG is synthesized through subsequent and highly specific esterification of galloyl moieties to beta-glucogallin (1-*O*-galloyl-beta-D-glucose), or monogalloyl glucoside, forming 1,6-*O*-digalloyl glucoside, 1,2,6-*O*-trigalloyl glucoside, and 1,2,3,6-*O*-tetragalloyl glucoside as intermediates (Niemetz & Gross, 2005). However, isomers of di, tri, tetra, and penta-galloyl glucoside have been identified via LC-MS in gallotannin containing fruits such as sumac, which indicates that there could be other pathways involved in gallotannin synthesis (Abu-Reidah *et al.*, 2016). PGG can increase in DP to form hexa, hepta, octa, nona and deca-galloyl glucosides through depside bonds between the *meta* or *para* hydroxyl group of an esterified gallic acid to beta-D-glucopyranose and the carboxyl group of another gallic acid. Several galloyltransferase isozymes that catalyze the depsidic addition of gallic acid to gallotannins have been identified to have a preferred substrate, resulting in several gallotannin isomers with a DP of 6 or greater (Niemetz & Gross, 2005). Although gallotannins can vary in the number and positioning of galloyl moieties, overall gallotannins are the least structurally diverse class of tannin.

Although, gallotannins are not ubiquitous in plants, they still have a high dietary intake. Gallotannins are found in one of the most globally popular fruits, mango, and also sumac - an important spice in many traditional Levantine dishes (Sivakumar *et al.*, 2011, Batal & Hunter, 2007).

Ellagitannins

Ellagitannins are polymers of hexahydroxydiphenic acid (HHDP), which when hydrolyzed spontaneously lactonizes into the phenolic acid, ellagic acid (Landete, 2011). Ellagitannins are formed from intramolecular oxidative coupling between two gallic acid residues in PGG, which results in a HHDP moiety. Ellagitannins with one, two or more HHDP moieties are referred to as monomeric, dimeric, or oligomeric ellagitannins, respectively. However, oxidative coupling reactions can also occur *inter*molecularly between two PGG molecules resulting in dimerized ellagitannins (Landete, 2011). Due to differences in the positions where oxidative coupling between galloyl residues can occur, number of galloyl moieties that are coupled together, and the ability to form intermolecular bonds, there have been over 500 ellagitannin compounds identified (Smeriglio *et al.*, 2017). Common dietary sources of ellagitannins include raspberries, pomegranates and strawberries (Smeriglio *et al.*, 2017).

The Human Colon Microbiome and Intestinal Health

The human colon microbiome is a large ecosystem of primarily bacteria estimated to include over 100 trillion bacterial cells, making the number of bacterial cells present in the body higher than that of human host cells (Possemiers *et al.*, 2011, Round & Mazmanian, 2014). The colon microbiota and the host are said to have a symbiotic relationship, where the host is benefitted by the microbiome through regulation of the gut immune system and increased metabolic output. Symbiosis is widely hypothesized to be dictated by the microbiota composition, which if not optimal can lead to health complications (Round & Mazmanian, 2014).

The composition of the microbiota becomes stable after 2-3 years of age and then is differentiated by ageing, genetics, the environment, antibiotic usage and diet making it unique to

each individual (Lozupone, *et al*., 2012, Duda-Chodak *et al.*, 2015). The composition is typically dominated by two phyla – Bacteroidetes and Firmicutes – which accounts for an estimated 90% of the bacterial population followed by Proteobacteria, Actinobacteria and Verrucomicrobia (Possemiers *et al.*, 2011, Duda-Chodak *et al.*, 2015, Manichanh *et al*., 2012). Although the composition of phyla are similar across individuals, at the species level the microbiota composition is highly variable, which is hypothesized to cause differences in inter-individual metabolic efficiency, including xenobiotics such as tannins (Manichanh *et al*., 2012, Marchesi *et al.*, 2016). Relevant to tannins, Tomas-Barberan *et al*. (2014) has classified three metabotypes grouped by the efficiency of the microbiome to metabolize ellagitannins. Interestingly, one of their identified metabotypes was associated with higher incidence of metabolic syndrome, colon cancer, and coronary heart disease (Gonzalez-Sarrias *et al*., 2017).

Several genera of bacteria are considered beneficial to host health and it is hypothesized that a lack of these bacteria as part of the microbiota composition can lead to disease (Marchesi *et al.*, 2016*). Lactobacillus, Bifidobacterium* and *Faecalibacterium spp.* have shown to downregulate the expression of inflammatory cytokines as well as promote the production of anti-inflammatory cytokine interleukin-10 (Manichanh *et al*., 2012). Further, *Bifidobacterium* and *Lactobacillus spp.* have shown to reduce cholesterol and *Bifidobacterium spp.* are involved in folic acid and short chain fatty acid production (Marchesi *et al.*, 2012, Touhy & Scott, 2015, Ventura *et al.,*2014). For these reasons several *Lactobacillus* and *Bifidobacterium spp*. have been classified as probiotics. When gut homeostasis is disturbed this is termed 'dysbiosis.' Dysbiosis is characterized by the overabundance of pro-inflammatory bacterial species and the lack of beneficial bacterial, which is hypothesized to cause the pathogenesis of intestinal diseases such as inflammatory bowel disease (IBD) (Possemiers *et al.*, 2011, Kostic, Xavier & Gevers, 2014).

11

It has been shown that individuals with ulcerative colitis, a form of IBD, have decreased *Bacteroides spp.*, *Bifidobacterium spp.*, *Lactobacillus spp.*, *Akkermansia muciniphila* and *Faecalibacterium prausnitzii,* alpha-diversity as well as increased adherent-invasive *E.coli spp.* (Costello *et al.*, 2017, Kostic, Xavier & Gevers, 2014).

The analysis of the microbiome has been made possible by metagenomics via sequencing instruments such as Illumina platforms opposed to traditional culturing methods which fail to capture the entirety of the population that is present, particularly obligate anaerobes (Rinttila *et al.*, 2004). Specifically, the highly conserved gene, 16S rRNA, has made it possible to optimize bacterial identification while limiting read length needed to differentiate between organisms (Jovel *et al.,* 2016). Targeted gene metagenomics has had limited use in evaluating how tannins modulate the microbiome and instead plating and qPCR have been utilized, not allowing for a comprehensive understanding of how these compounds impact microbial ecology (Vollmer *et al.*, 2018).

Impact of the Colon Microbiome on the Absorption, Distribution, Metabolism and Excretion (ADME) of Tannins

Condensed Tannins

The majority of condensed tannin metabolism occurs in the colon by microbiota metabolism. Without extensive microbial catabolism, proanthocyanidins are not absorbed through colon epithelial cells due to their large size. Although, it is debated whether dimers are small enough to be bioavailable (Monagas *et al.*, 2010, Feliciano *et al.*, 2016). Cocoa proanthocyanidins and procyanidin B2 standard have been utilized in several *in vitro* and *in vivo* studies to model the metabolic fate of condensed tannins (Stoupi *et al.*, 2010a, Stoupi *et al.*, 2010b, Baba *et al.*, 2002). It was first assumed that the intestinal microbiota had the capacity to

catabolize interflavan bonds of condensed tannins, releasing bioavailable (-)-epicatechin and (+)-catechin. *In vitro* digestion models have demonstrated that proanthocyanidins are stable to gastric digestion, preventing release of free (-)-epicatechin that could become available for absorption in the small intestine (Rios *et al.*, 2002). This suggests that (-)-epicatechin from condensed tannins first becomes bioaccessible in the colon by microbial catabolism of interflavan bonds. However, it has become well established that (-)-epicatechin can be metabolized into several other metabolites instead of being absorbed (Figure 4). Microbial metabolism of (-)-epicatechin begins with the production of 3,4-dihydroxyphenyl-gamma-valerolactone (34PV). 34PV is cleaved and dehydroxylated into 3,4-dihydroxyphenyl valeric acid which is then catabolized into 3,4-dihydroxyphenyl propionic acid (34PP) by beta-oxidation and subsequently into 3,4-dihydroxyphenyl acetic acid through alpha oxidation or 3,4-dihydroxy benzoic acid (protocatechuic acid) through beta oxidation of 34PP (Monagas *et al.*, 2010, Ou & Gu, 2014). The colon microbiota are known to have dehydroxylation activity, leading to monohydroxy phenyl valerates, propionates, and acetates which can also undergo beta and alpha-oxidation to form their corresponding monohydroxy metabolites. Dehydroxylation is favored in the C4' or para position of the phenyl substituent, leading to increased concentrations of metabolites with a hydroxyl group in the meta or C3' position. Free (+)-catechin was shown to epimerize into (-)-epicatechin during *in vitro* batch fermentation of fecal bacteria before metabolism, which suggests that the microbiota may prefer specific conformations of flavan-3-ols (Monagas *et al.*, 2010, Williamson & Clifford, 2010). Unlike condensed tannins, microbial metabolites of tannins are bioavailable. Catechol-*O*-methyltransferase (COMT), sulfotransferases (SULF), and UDP-glucuronyl transferase (UDT-GT) or phase II metabolites of (-)-epicatechin and its microbial metabolites have been found in both human plasma and urine

(Feliciano *et al.*, 2016). (-)-Epicatechin metabolism requires the action of multiple bacterial species. *Eubacterium spp.* and *Lactobacillus planatarum* were only capable of forming the intermediate of 34PV, 3',4'-dihdyroxyphenyl-2"4"6"-trihydroxyphenyl-propan-2-ol (DTPO), but were not able to form 34PV itself (Monagas *et al.*, 2010). However, *Flavonfactor plautii* was found to form 34PV, propionates and acetates using DTPO as substrate (Takagaki & Nanjo, 2015).
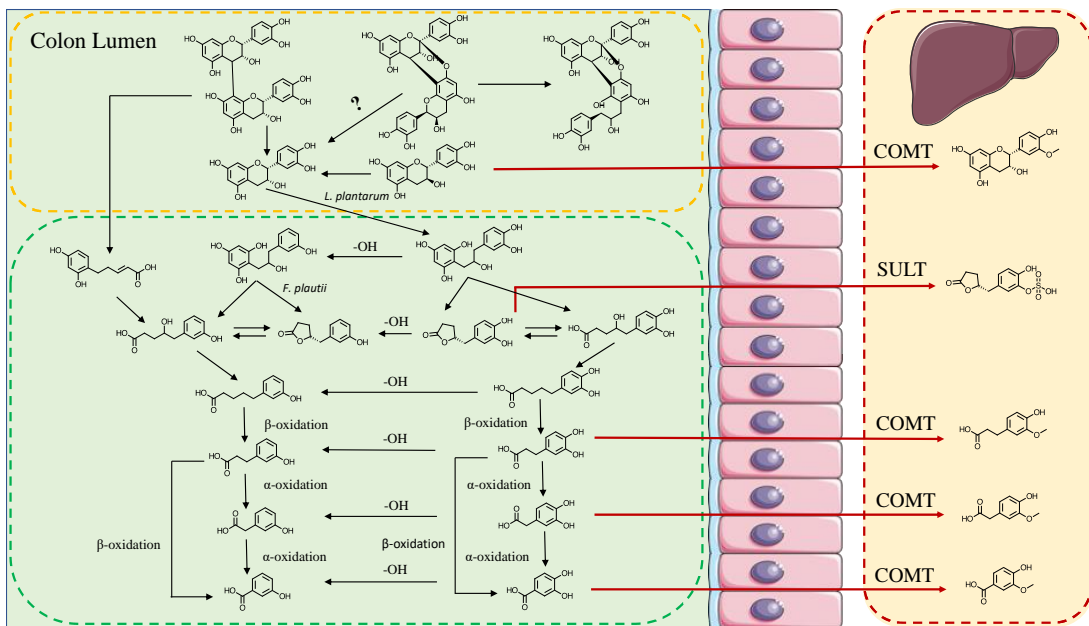
Figure 4 Hypothesized microbial and hepatic metabolism of a-type and b-type condensed tannins. Adapted from Ou & Gu, 2014, Engemann *et al.*, 2012, Stoupi *et al.*, 2010a, Stoupi *et al.*, 2010b and Monagas *et al.*, 2010.

Although the metabolism of (-)-epicatechin is well characterized, the metabolism of condensed tannins is still not completely understood as the release of free (-)-epicatechin through breakage of interflavan bonds is now believed to contribute to only 10% of condensed tannin metabolism (Appeldoorn *et al.*, 2009). Alternative mechanisms were proposed by Stoupi *et al.*, (2010) when several high molecular weight metabolites were produced from procyanidin B2 standard (Figure 4). In accordance with this Engemann *et al.*, (2012) identified high molecular weight catabolites derived from a-type condensed tannins isolated from litchi, procyanidin A2 and cinnamtannin B1. The structures of these high molecular weight catabolites were tentatively characterized as having undergone C-ring fission in the terminal flavan-3-ol unit. However, it is still not well understood how propionates and acetates are further derived from these newly identified metabolites.

Although many microbial metabolites derived from flavonoids have been elucidated, there is still confounding evidence on whether certain metabolites are actually derived from condensed tannins and the kinetics of their metabolite production. Most batch and continuous fermentations of fecal bacteria were carried out utilizing complex fruit extracts that contained multiple classes of polyphenols and not solely condensed tannins. Propionates, acetates and benzoates have also been identified as microbial metabolites of flavonols, such as quercetin and its glycosides, hydroxycinnamates, such as chlorogenic acid, and flavanone glycosides, such as naringin (Selma, Espin & Tomas-Barberan, 2009). Further, few studies have reported the metabolic fate of a-type condensed tannins. It is still unknown if 34PV is a metabolite of procyanidin A2 and a-type proanthocyanidins, even though 34PV is a unique metabolite of its flavan-3-ol monomer, (-)-epicatechin (Ou & Gu, 2014).

15

Hydrolysable Tannins

Hydrolysable tannins do not require extensive colonic metabolism for their monomers gallic acid or ellagic acid to become bioavailable due to their hydrolysis at physiological conditions and single bacterial enzymes that can catalyze their hydrolysis. Mosele *et al.,* (2016) demonstrated a 772% increase in gallic acid during *in vitro* gastric digestion due to acid hydrolysis of gallotannins, which would lead to absorption of gallic acid in the small intestine. Contradictory to this Krook & Hagerman, (2012) and Sirven, Negrete & Talcott, (2018) have shown minor to no acid hydrolysis of gallotannins during gastric digestion. This demonstrated that much of hydrolysable tannins consumed traverse to the colon. In the colon, gallotannins can be metabolized by *Lactobacillus plantarum* or *Streptococcus galyticus* into gallic acid by tannase and decarboxylated into pyrogallol through pyrogallol decarboxylase expressed by the same bacteria (Jimenez *et al.*, 2013). Gallotannins can also hydrolyze at the neutral pH of the colon into free gallic acid which can also be subsequently metabolized into pyrogallol. Both gallic acid and pyrogallol methyl, sulfate and glucuronide phase II metabolites have been found in rat urine and plasma (Ma *et al.,* 2018). Ellagitannins can also auto-hydrolyze into HHDP during gastric digestion and the neutral pH of the colon. Free ellagic acid that is produced is metabolized by the colon microbiome by *Gordonibacter spp.* forming several metabolites – urolithin a, b, c and d. Urolithin a, urolithin b, ellagic acid and their UDP-GT derivatives are the most predominant metabolites found in human plasma and urine *in vivo* post consumption of high-ellagitannin containing fruits, such as pomegranate (Landete, 2011, Romo-Vaquero *et al.*, 2015).

Modulation of the Colon Microbiome by Tannins

Diet has been claimed to be one of the most important factors that impact the composition of the colon microbiota and dietary tannins are believed to modulate the microbiota

by acting as selective agents through their anti-bacterial activity (Monagas *et al.*, 2010, Tuohy & Scott, 2015). This anti-bacterial activity includes substrate depletion, metal ion chelation, and disruption of cell wall and membrane functions. Tannins are able to complex with proteins through hydrophobic interactions and hydrogen bonding, making proteins unavailable for bacterial metabolism. Tannins negatively impact bacterial membrane functionality by complexing with membrane proteins and disrupting cell wall synthesis (Smith, Zoetendal & Mackie, 2005). Tannins can also chelate nutritionally important metals for bacteria such as iron. *Ex vivo* batch culture fermentation studies have shown that *Lactobacillus and Bifidobacterium spp.* increase when in the presence of proanthocyanidins and flavan-3-ol monomers (Fogliano *et al.*, 2011, Tzounis *et al*., 2008, Duenas *et al.*, 2015, Cueva *et al.*, 2012, Etxeberria *et al*., 2013). It is hypothesized that tannins can support the growth of these genera because iron is not vital for their metabolism (Smith, Zoetendal & Mackie, 2005). Bacteria are characterized as tannin-resistant or tannin-tolerant if they have mechanisms to overcome toxicity by tannins or can grow in the presence of tannins without being harmed, respectively (Smith, Zoetendal & Mackie, 2005 & Monagas *et al.*, 2010). *Enterobacteriaceae* and *Bacteroides* have been reported to increase with tannin consumption and this was hypothesized to occur because they are tannin-resistant bacteria (Smith & Mackie, 2003, Cueva *et al.*, 2012).

It has been shown that tannins can increase the abundance of bacterial species in the colon that are associated with intestinal health. Condensed tannins increased intestinal *Akkermansia municipila* in mice 30% (Anhe *et al.*, 2015). This bacterium is a mucin-consuming bacterium that can decrease intestinal permeability leading to less inflammation caused by leakage and its overall presence is inversely correlated to intestinal health (Anhe *et al.*, 2015). It has also been shown that tannins can promote the growth of the *Clostridium coccoides-*

eubacterium rectale group. This group of bacteria are associated with improved intestinal health as they are butyric acid producers (Duenas *et al.*, 2015).

There have been contradictory results over the effect of tannins on the growth of *E.coli spp*. Smith et al., (2003) and Tzounis et al., (2008) found that *E.coli spp.* increase in the presence of tannins but tannins were also found to prevent adherent-invasive *E.coli* adhesion to epithelial cells by agglutination of their fimbriae (Polewski *et al.*, 2016). A-type proanthocyanidins from cranberry have long been associated with the prevention of urinary tract infections by the prevention of adhesion of pathogenic *E.coli spp.* to the urinary tract (Howell *et al.*, 2007). Further, some *E.coli spp.* are not considered pathogenic and have been classified as probiotics. Proanthocyanidins may then promote the growth of probiotic *E.coli* species but have specific anti-bacterial effects against pathogenic adherent-invasive *E.coli*.

There is still inconclusive evidence that distinguishes the bacterial modulatory effects of tannins from other polyphenols. Conclusions on the modulatory effects of proanthocyanidins were derived from fermentation models using complex plant extracts containing several classes of polyphenols (Table 1). Other studies utilized high concentrations of analytical standards that do not represent the structural diversity of tannins that would actually be consumed by the host (Stoupi *et al.*, 2010a). Further, a modulatory effect may be misrepresented from the high concentrations utilized that may actually not be seen in the diet.

Table 1 Batch and continuous fermentations showing modulatory effects of tannin-rich extracts and analytical standards on fecal bacterial communities.

Tannin Source	Tannin Classification	Concentration	Study Type	Bacteria Quantification method	Bacteria Modulation	Reference
Cranberry Extract	A-type and B-type proanthocyanidins	500 ppm	SHIME	qPCR	*Enterobacteriaceae* decrease	Sanchez-Patan, *et al.* 2015
Grape Seed Extract	B-type proanthocyanidins	500 ppm	SHIME	qPCR	*Enterobacteriaceae* decrease	Sanchez-Patan, *et al.* 2015
Grape Seed Extract	B-type proanthocyanidins	600 ppm	Batch Fermentation	FISH	*Clostridium histolyticum* decrease, *Lactobacillus* increase, *Enterococci* increase	Cueva *et al.*, 2012
Analytical standards	(-)-Epicatechin, (+)-Catechin	150 ppm, 1000 ppm	Batch Fermentation	FISH	*C. coccoides-Eubacterium rectale* group increase	Tzounis, 2008
Water Insoluble Cocoa Extract	B-type proanthocyanidins	1 g	Continuous Fermentation	FISH	*Bifidobacterium*, *Lactobacillus* increase	Fogliano et al., 2011

CHAPTER III

ULCERATIVE COLITIS RESULTS IN DIFFERENTIAL METABOLISM OF CRANBERRY

PROANTHOCYANIDINS BY THE COLON MICROBIOME *EX VIVO*

Introduction

Cranberries (*Vaccinium macrocarpon*) have long been associated with the prevention of bacterial infections in the urinary tract through an anti-adhesion mechanism against pathogenic *E. coli* (Jepson, Williams & Craig, 2012). More recently cranberries have become of interest because of their anti-inflammatory activity and modulatory effects on the bacteria in the gut that can promote improved intestinal health (Zhao, Liu & Gu, 2018). This bioactivity is thought to be attributed to the abundance of heterogenous polyphenols naturally present in cranberry.

Cranberries contain several classes of polyphenols, including proanthocyanidins (PACS), flavonols, anthocyanins and phenolic acids. PACS are among the most predominant compounds present and consist of polymers of flavan-3-ols polymerized through interflavan bonds between C4 of the extension unit and C8 or C6 of the terminal unit, in what is referred to as a 'b-type' linkage (Mena *et al*., 2015). Cranberries also contain another class of PACS with 1 or more 'a-type' linkages that consists of a second ether bond between C2 and C7 (Gu *et al*., 2003, Mena *et al*., 2015). Both a and b-type PACS have demonstrated anti-bacterial activity because of their ability to precipitate proteins and chelate iron (Smith, Erwin & Mackie, 2005). PACS with a-type linkages have additionally been found to have anti-adhesion activity against p-fimbriated *E.coli,* which may be a result of the conformation of the polymers due to the a-type linkage (Howell *et al*., 2007). By contrast, PACS have also shown potential prebiotic activity, enhancing the growth of symbiotic bacteria such as *Bifidobacterium spp., Lactobacillus spp.,* and *Akkermansia*

muciniphila (Cires *et al*. 2017, Zhao, Liu & Gu, 2018, Anhe *et al*. 2015*).* Other polyphenols found in cranberry such as anthocyanins, flavonols, and phenolic acids have also shown to have modulatory effects on the microbiome (Faria *et al*., 2014, Etxeberria *et. al*., 2013, Tomas-Barberan *et. al*, 2013). Therefore, cranberry polyphenols may modulate the colon microbiota composition and as a result may serve to promote a healthy gut.

The microbiome of the colon is likely the most important site of polyphenol metabolism due to their low absorption in the small intestine. It is estimated that as much as 90% of dietary polyphenols traverse the gastrointestinal tract to the large intestine where they are then metabolized into the bioavailable metabolites 3,4-dihydroxyphenyl propionic acid, 3,4-dihydroxyphenyl acetic acid, 3,4-dihydroxy benzoic acid and their monohydroxylated derivatives, as well as the flavan-3-ol specific derivative, 3,4-dihydroxyphenyl-gamma-valerolactone (Etxeberria *et al*., 2013). Metabolism can vary amongst individuals, whereas inter-individual variability in 3,4-dihydroxyphenyl-gamma-valerolactone production by the microbiome after walnut PAC consumption has been described (Cortes-Martin *et al*., 2019). Tomas-Barberan *et al*., (2014) has also identified 3 'metabotypes' of pomegranate ellagitannin colonic metabolism that correlated with subject health status, their response to long-term ellagitannin consumption and colon microbiota composition. Microbial metabolites of polyphenols could then potentially be utilized to assess health status, which may be dependent on the efficiency of the colon microbiome to metabolize polyphenols.

Ulcerative colitis (UC) is a form of inflammatory bowel disease (IBD) characterized by a 'dysbiotic' microbiome, or a disbalance within the microbiota composition which causes negative immunological responses in the host (Kostic, Xavier & Gevers., 2014). The etiology of UC is hypothesized to come about not only from genetic susceptibility and environmental factors

but also UC may be caused by compositional differences of microbiota present in the colon in comparison to healthy individuals. Differences found in UC microbiota compositions compared to healthy individuals includes decreased Bacteroides, Firmicutes, Clostridia, *Ruminococcaceae, Bifidobacterium spp., Lactobacillus spp., Faecalibacterium spp.*, increased Gammaproteobacteria, and the presence of adherent-invasive *E.coli* (Kostic, Xavier & Gevers, 2014, Duranti *et al*., 2016). UC is also associated with decreased microbial metabolic output in the colon, including decreased short chain fatty acid production, amino acid synthesis, and carbohydrate metabolism. It has also been hypothesized that decreased metabolism of xenobiotics, such as polyphenols, may result from microbial dysbiosis although there are few studies that show differential metabolism of these compounds in humans (Trelau, Sowada & Luch, 2015).

To assess if gut bacterial dysbiosis impacts the metabolism of cranberry polyphenols, the metabolism of cranberry polyphenols between healthy fecal donors and fecal donors medically diagnosed with moderate to severe UC was compared. It was hypothesized that differing microbiota composition from donors with UC would produce lower concentrations of polyphenol fecal metabolites compared to healthy individuals. Further, because polyphenols may impart modulatory effects on the microbiota composition, diversity of microorganisms between healthy and UC subjects was also evaluated after fermentation with cranberry polyphenols.

Materials and Methods

Chemicals

LC-MS standards of 3-hydroxyphenylacetic acid (MPA), 3,4-dihydroxyphenylacetic acid (34PA), 3-(3,4-dihydroxyphenyl)propionic acid (34PP) and hippuric acid were acquired from Sigma Aldrich (St. Louis, MO, USA). Standards of 3-(3-hydroxyphenyl)propionic acid (MPP),

3,4-dihydroxybenzoic acid (34BA), 3-hydroxybenzoic acid (MBA) and benzoic acid (BA) were acquired from Alfa Aesar (Tewksbury, MA, USA). R-3,4-dihydroxyphenyl-gamma-valerolactone (34PV) was purchased from Toronto Chemicals (North York, ON, Canada). LC-MS grade mobile phases were purchased from Sigma Aldrich and formic acid was purchased from Fisher Scientific (Hampton, NH, USA).

Cranberry Polyphenol Extract Preparation and Characterization

To prepare a polyphenol extract, 0.5 g of cranberry powder (~60% w/w PACS) provided by Ocean Spray Cranberries, Inc. was dissolved in 50 mL of 0.01% (v/v) formic acid and polyphenols were isolated utilizing a 10 g C18 solid-phase extraction (SPE) column (35cc, 55-105 µm; Waters, Milford, MA, USA). The SPE column was first conditioned with one column volume (CV) of 100% methanol and 1.5 CV of 0.01% (v/v) formic acid. The cranberry solution was then loaded onto the column and washed with 1 CV of 0.01% (v/v) formic acid. Cranberry polyphenols were eluted with 0.01% (v/v) formic acid in methanol and then evaporated under reduced pressure. This procedure was repeated 10-times and all repetitions were combined into a concentrated aqueous stock extract and total polyphenolics were determined by the Folin-Ciocalteu assay (Singleton, Orthofer & Lamuela-Raventos, 1999). The stock extract was aliquoted into 15-mL falcon tubes and frozen at -20°C until utilized in a fecal fermentation. Total PACS were determined by the 4-dimethylaminocinnamaldehyde (DMAC) assay (Prior *et al*., 2010) while individual polyphenolics present in the extract were characterized and quantified via LC-ESI-MS/MS according to the methods outlined in chapter IV and V (Table 2).

Table 2 Concentrations of individual cranberry polyphenols identified in the cranberry extract utilized in fecal fermentations for each recruited subject. Concentrations are expressed as μM ± SEM.
*m/z in positive ionization mode.

Compound	[M-H]-	MS$_2$	Concentration (μM)
Anthocyanins	-	-	-
Cyanidin-3-hexose	449*	287*	439.49 ± 34.62
Cyanidin-3-pentose	419*	287*	785.97 ± 38.57
Peonidin-3-hexose	463*	301*	1023.21 ± 40.84
Peonidin-3-pentose	433*	301*	699.96 ± 31.84
Tannins	-	-	10,052 ± 942.17
Flavonols	-	-	-
Quercetin aglycone	301	179, 151	2,566.97 ± 323.21
Quercetin Pentose 1	433	301	307.63 ± 15.26
Quercetin Pentose 2	433	301	179.38 ± 24.25
Quercetin Pentose 3	433	301	110.57 ± 14.71
Quercitrin	447	301	660.46 ± 24.86
Quercetin Hexose	463	301	2,584 ± 104.23
Phenolic Acids	-	-	-
Caffeic acid	179	135	487.52 ± 45.83
Caffeic acid hexose	341	179	3,790.07 ± 256.70
Chlorogenic acid	353	191	1,106.24 ± 68.14
p-Coumaryl hexose 1	325	163	3,472.01 ± 248.76

Table 2 Continued

Compound	[M-H]-	MS₂	Concentration (μM)
p-Coumaryl hexose 2	325	163	2,807.71 ± 145.05
Protocatechuic acid	153	109	601.14 ± 57.42

Recruitment of Subjects

This clinical protocol was approved by the Texas A&M University Institutional Review Board (TAMU IRB# 2017-0568D). Healthy participants and individuals with moderate to severe UC were recruited according to specific inclusion and exclusion criteria. Healthy subjects qualified if they were 18-65 years of age and had no history of chronic diseases. Subjects were excluded from the healthy group if they had a history of alcohol or substance abuse, had recurrent hospitalizations, have had seizures, or have taken antibiotics in the last 6 months. Additionally, subjects were excluded if they were lactose intolerant, gluten sensitive/had celiac disease, smoked more than one pack of cigarettes a week, had renal or liver dysfunction, and if female were currently pregnant or lactating. The same inclusion and exclusion criteria applied to UC subjects, however UC subjects additionally reported the severity of their disease as diagnosed by a physician. UC subjects also completed two questionnaires during screening, the Simple Clinical Colitis Activity Index (SCCAI) and the Short Inflammatory Bowel Disease Questionnaire (SIBDQ) to assess disease activity (Jowett *et al*., 2001).

Ex Vivo *Colon Microbiome Metabolism of Cranberry Polyphenols*

Stool samples were collected from subjects within 2 hr of defecation and further processed inside an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI, USA) held at 37°C and regulated with nitrogen, hydrogen (5%), and carbon dioxide (5%). Resazurin strips (Sigma Aldrich, St. Louis, MO, USA) were utilized to confirm anaerobic conditions inside the chamber during fermentations. A fecal slurry was produced by mixing 5 g of feces with 50 mL of pre-reduced and sterile (PRAS) pH 7.5 phosphate buffered saline (PSB) with added sodium thioglycolate and L-cysteine (Anaerobe Systems, Morgan Hill, CA, USA). The prepared cranberry polyphenol extract was diluted to 6,000 mg L_{-1} GAE soluble polyphenols in PRAS fecal fermentation media formulated as described by Tzounis *et al.*, (2008) (2.0 g peptone water, 0.5 g bile salts, 2.0 g yeast extract, 0.5 g L-cysteine, 0.05 g haemin, 0.01 mL vitamin K, 0.001 g resazurin, 0.01 g $CaCl_2$ * 6 H_20, 0.01.g $MgSO_4$ * 7 H_20, 0.04 g KH_2PO_4, 0.04 g K_2HPO_4, 0.10 g NaCl, 2.0 g $NaHCO_3$, 2.0 mL tween 80 per 1 L) and produced by Anaerobe Systems (Morgan Hill, CA, USA). To simulate microbiome metabolism of cranberry polyphenols, 1 mL of cranberry extract in media was mixed with 8 mL of PRAS fecal fermentation media and 1 mL of fecal slurry to achieve a final concentration of 600 mg L_{-1} GAE of total cranberry polyphenols. 9 mL of media and 1 mL of fecal slurry was prepared to serve as a control. Samples were fermented for 48 hr and fermentation vessels designated for each time point were collected at 0, 6, 12, 24 and 48 hr. 1 mL aliquots designated for chemical analysis of metabolites were acidified with 5 µL of formic acid and all aliquots were stored at -80ºC until analysis for metabolite production, short/branched chain fatty acids, and bacteria composition. Fermentation vessels were prepared in triplicate for each time point.

ELISA Quantification of Calprotectin

A sub-sample of stool from each subject was collected and frozen on receipt at −80°C. After thawing, 100 mg stool was suspended in 5 mL of PBS (pH 7.0), vortexed for 40 s, and mixed for 30 min on an automated shaker. Afterwards, 1 mL of homogenate was centrifuged for 20 min at 10,000 x g and the supernatant was used for the assay after a 50-fold dilution in PBS. Fecal calprotectin levels were determined using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Biomatik Corp., Cambridge, ON, Canada) according to the manufacturer's instructions.

16S rRNA Sequencing and Analysis

Bacterial DNA was extracted by centrifuging 3 mL of the fermentation aliquot at 3000 x g for 5 min at room temperature. The supernatant was discarded while the pellet was extracted utilizing the QIAmp PowerFecal DNA kit (Qiagen, Hilden, Germany) following the instructions of the manufacturer. DNA was quantified in ng μL_{-1} utilizing a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). The library was prepared by following Illumina's 16S Metagenomic Sequencing Library Preparation guide (Illumina, 2013). The V3 and V4 region of the gene was amplified using the forward primer 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3', and the reverse primer 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3' (IDT, Coralville, Iowa, USA). The library was sequenced on the Illumina MiSeq platform at the Texas A&M Agrilife Genomics and Bioinformatics Service center. The paired-end sequences were then demultiplexed, quality filtered, and removed of chimeras via Qiime2 software. Taxonomy was assigned by clustering sequences into Operational Taxonomic Units (OTUs) utilizing the Greengenes version 13.8 database in Qiime2. Taxa was collapsed on the phyla, family and genus level. Diversity metrics

were sampled based on the minimum reads per sample. Beta-diversity was represented as the

principle coordinates of Bray-Curtis dissimilarity between healthy and UC microbiomes at each

hr, as calculated by Qiime2. Alpha-diversity, represented as the Shannon index and Pielou's

evenness, were also calculated by Qiime2.

LC-ESI-QQQ-MS/MS Analysis of Cranberry Polyphenol Microbial Metabolites

Fermentation aliquots were prepared for chemical analysis by extracting 350 µL of

ferment and 50 µL of 50 mg L-1 ethyl gallate as internal standard with 1400 µL MeOH. The

solution was vortexed and then centrifuged at 10,000 rpm for 10 min at 4ºC and 1 mL of

supernatant was acidified with 1 µL of formic acid. Quantification of cranberry polyphenol

metabolites produced during fecal fermentations was performed using an Ultimate3000 UPLC

equipped to a Thermo Scientific TSQ Quantiva mass spectrometer with an ESI source.

Separations were carried out on a Synergi Fusion RP column (150 x 2 mm, 4 µm) with 0.1%

(v/v) formic acid as mobile phase A and 0.1% (v/v) formic acid in methanol as mobile phase B.

The flow rate was 400 µL min-1 and gradient elution began with 10% B, which increased to 40%

B after 5 min, and then increased to 95% B after 7 minutes. After 8 min, the column was re-

equilibrated with 10% B until 13 min elapsed. Mass spectrometer was run in the negative

ionization mode and parameters consisted of a 2300 V spray voltage, a sheath gas of 50,

auxiliary gas of 15, and a sweep gas of 1, ion transfer tube temperature set at 350 °C and

vaporizer temperature set at 400 °C. Scheduled single reaction monitoring (SRM) was used to

identify and quantify the metabolites based on optimized conditions and transitions of their

respective standards. Transitions were the following: 34PV 207/163; 34BA 153/109; 34PA

167/123; 34PP 181/137; MPA 151/107; MBA 136/93; ethyl gallate 197/124; MPP 165/121; BA

121/77; hippuric acid 178/134. Each compound additionally contained identifying qualifying

ions and all compounds were quantified utilizing a standard curve prepared with authentic standards. In order to control for matrix effects, standard solutions were prepared in matrix that was prepared from 350 µL of control ferment (media and fecal slurry without polyphenols) that had been fermented for 24 hr, 50 µL of 0.1% formic acid and 1400 µL of MeOH. The matrix was then centrifuged for 10 min at 10,000 rpm at 4ºC and 1 µL of formic acid was added to 1 mL of supernatant.

GC-MS Analysis of Short and Branched Chain Fatty Acids

Short chain fatty acids (SCFA) and branched chain fatty acids (BCFA) were analyzed by combining 50 µL of fermentation aliquot with 800 µL of 30 mM HCl containing 0.1 mM d7-butyric acid as internal standard. The solution was then vortexed and centrifuged at 15,000 x g for 10 min at 4ºC. 400 µL of supernatant was extracted with 400 µL of ethyl acetate, vortexed and centrifuged again at 15000 x g for 1 min at 4ºC. 150 µL of supernatant was stored at -20ºC until analysis by GC-MS. The entirety of the extraction was performed on ice. SCFAs and BCFAs were detected and quantified on a gas chromatography triple quadrupole mass spectrometer (TSQ EVO 8000, Thermo Scientific, Waltham, MA). Chromatographic separation was achieved on a ZB WAX Plus column (30m x 0.25mm x 0.25um) (Phenomenex, Torrance, CA, USA). 1 µl of the extracted sample was injected with a split ratio of 20:1. The ionization was carried out in the electron impact (EI) mode at 70 eV. The injector, MS transfer line and ion source were maintained at 230°C, 240°C and 240°C, respectively. The flow rate of helium carrier gas was kept at 1 mL min-1. The MS data and retention times were acquired in positive polarity and full scan mode from *m/z* 40-500 for the individual target compounds and compared with authentic standards. The target compounds were quantified in the selected ion monitoring (SIM) mode using the following product ions for each compound: acetic acid 43, 45, 60;

propionic acid 43, 73, 74; butyric acid 42, 60, 73; valeric acid 41, 60, 73; isobutyric 41, 43, 73; isovaleric 42, 60, 87; d7-buytric acid 45, 63, 77.

<p style="text-align:center;">*Statistics*</p>

Data was analyzed utilizing JMP software (Cary, NC, USA). Concentrations of polyphenol metabolites between healthy and UC groups were compared at each hour utilizing the two-sample Mann-Whitney U test. Bacteria abundances were analyzed using two-way ANOVA, where hour of fermentation (0 or 48) was the first factor and health status (healthy or UC) was the second factor. The interaction between both time and health status was also assessed. To analyze for inter-individual differences a two-way ANOVA was run between each subject within groups where hour of fermentation and subject were the two factors. SCFA and BCFA were analyzed as a full factorial of 3 factors that included hour (0 or 48), whether or not polyphenols were present (polyphenols or control) and health status. Fecal calprotectin was compared using a two-sample t-test. Significance was designated if the P value was < 0.05.

<p style="text-align:center;">**Results**</p>

<p style="text-align:center;">*Subject Characteristics*</p>

Demographic information was collected from all subjects as well as non-invasive measures of UC disease activity to characterize the fecal donors at time of collection. 5 subjects were recruited as a fecal donor in the healthy group with a median (range) age of 25 (24-25). 3 subjects classified their race as Caucasian and 2 designated their race as other. 2 of the subjects were of Hispanic ethnicity. 4 participants were recruited to the UC group. The median age of the UC fecal donors was 32 (21-63) and all subjects were Caucasian and not of Hispanic ethnicity. The average SIBDQ and SCCAI scores for the UC group were 4.55 ± 0.76 and 7.67 ± 3.18,

respectively (Table 3). Fecal calprotectin for the UC group was 7.86 ± 3.93 µg g^{-1} and

significantly higher (P<0.05) than the healthy group with 3.00 ± 1.34 µg g^{-1} (Table 4).

Table 3 Results of questionnaires designed to assess disease activity of IBD, given to those recruited in the UC group.

Questionnaire	Score (mean ± SEM)
SIBDQ	4.55 ± 0.76
SCCAI	7.67 ± 3.18

Table 4 Comparison of fecal calprotectin content between healthy and UC stool donors at time of collection.

Fecal Calprotectin (mean ± SEM µg g^{-1})	Group
3.00 ± 1.34 b	Healthy
7.67 ± 3.18 a	UC

16S rRNA Analysis 0 and 48 Hr After Cranberry Polyphenol Fermentation with Fecal

Microorganisms

The microbiota composition of healthy and UC fecal samples were compared utilizing

16S rRNA targeted gene metagenomics after 0 and 48 hr of fermentation with cranberry

polyphenols. There were no significant differences in beta-diversity or alpha-diversity, but differences could be observed between groups comparing abundances of different OTU's (Figure 5 & 7). The most abundant phyla present in both healthy and UC microbiomes were Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria and Verrucomicrobia, which is typical for the human gut microbiome (Figure 6). On the phyla level, there were no differences in the abundances of Bacteroidetes, Verrucomicrobia and Actinobacteria between healthy and UC microbiomes before and after fermentation with cranberry polyphenols.
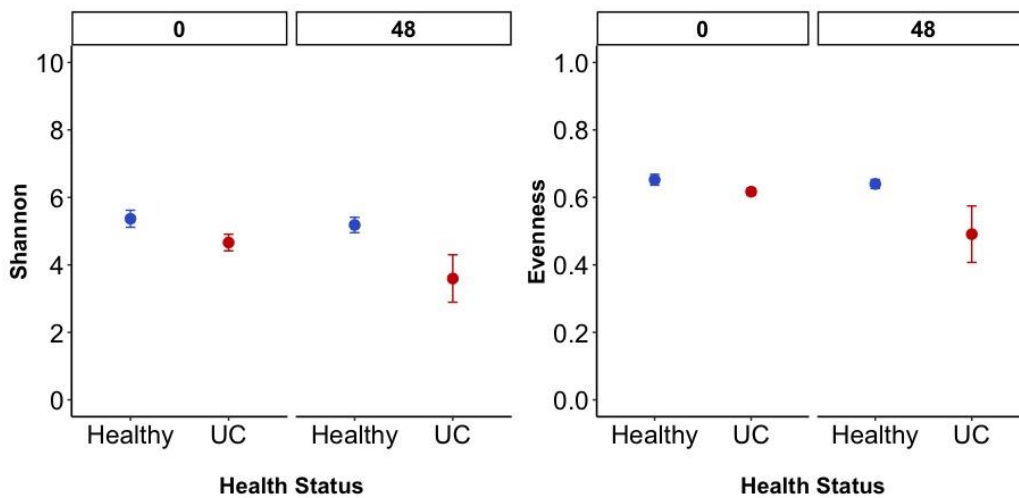
Figure 5 Comparison of alpha-diversity between healthy (blue) and UC (red) microbiomes. Alpha-diversity was measured using the Shannon Index (left) and Pielou's Evenness (right).
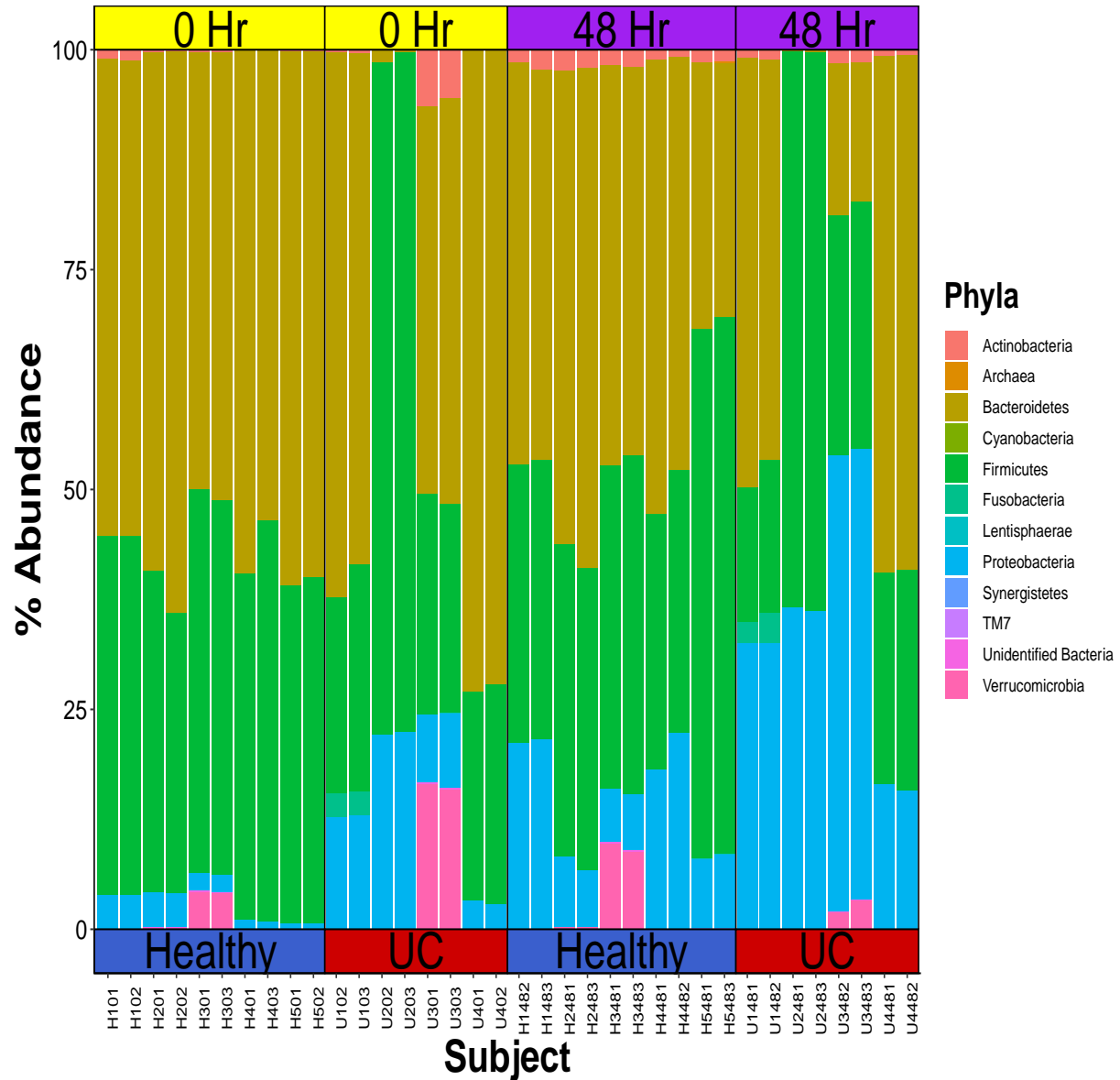
Figure 6 Comparison between percent abundance of each phyla identified in healthy (blue) and UC (red) fecal microbiomes before (0 hr) and after (48 hr) fermentation with cranberry polyphenols, as determined by 16S rRNA targeted metagenomics. Each subject is presented in duplicate as analyzed.
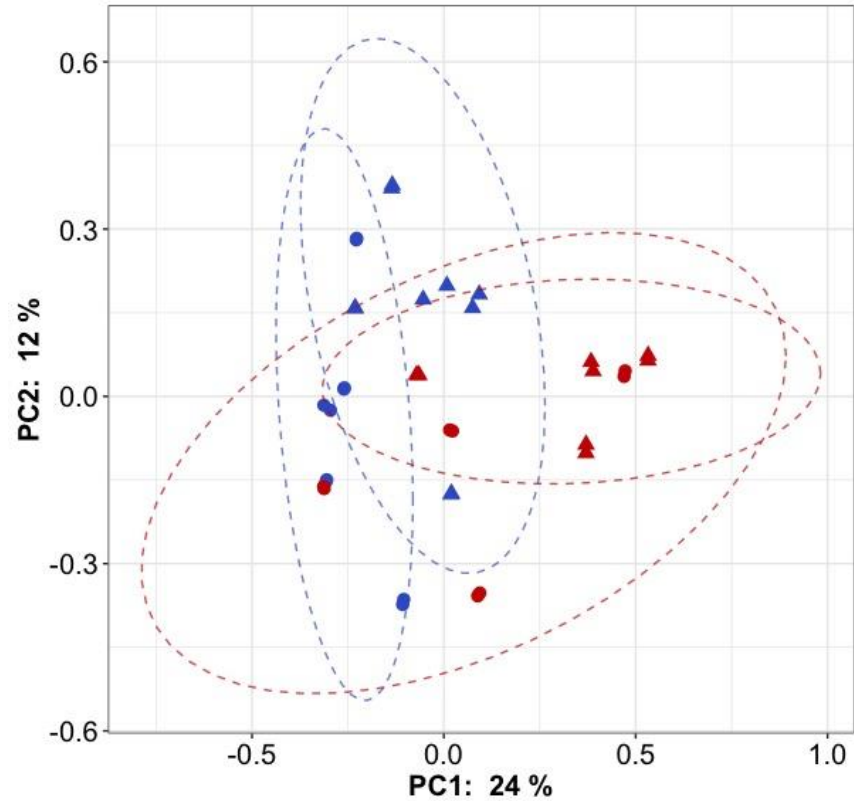
Figure 7 Beta-diversity represented as principle coordinates of Bray-Curtis dissimilarity between UC (red) and healthy (blue) microbiomes after 0 (circle) and 48 (triangle) hours of fermentation with cranberry polyphenols.

However, within Actinobacteria, the family *Coriobacteriacea* significantly increased (P=0.002) in both the healthy and UC microbiomes after 48 hr to an abundance of $1.23 \pm 0.24\%$ and $0.59 \pm 0.21\%$, respectively (Figure 8). On the genus level, the increase in *Coriobacteriacea* corresponded with the significant increase (P=0.007) in *Erggerthella* in both groups after 48 hr. *Bifidobacteriaceae*, also within the phyla Actinobacteria, was not significantly different between groups after both 0 and 48 hr and was also higher in the UC group after 0 hr at $1.31 \pm 1.22\%$ (Figure 9). Within Verrucromicobia, the genus Akkermansia was also higher in UC microbiomes

34

at both 0 and 48 hr, but not significant (Figure 10). The phylum proteobacteria was significantly

higher (P=0.001) in UC microbiomes at 0 hr with an abundance of $11.59 \pm 4.10\%$ in comparison

to healthy microbiomes with $2.29 \pm 0.70\%$ (Figure 6). Within Proteobacteria,

Enterobacteriaceae significantly increased in both the healthy and UC microbiomes after 48 hr

(P=0.011) to $10.32 \pm 4.45\%$ and $31.50 \pm 9.33\%$, respectively (Figure 11). The abundance of

Firmicutes, although not significant, was higher in the healthy group in comparison to the UC

group at 0 hr with $39.90 \pm 1.60\%$ and $37.43 \pm 13.13\%$, respectively (Figure 6). Within

Firmicutes, *Ruminococcaceae* were significantly higher (P= 0.003) in the healthy group in

comparison to UC with abundances at 0 hr of $16.31 \pm 2.83\%$ and $4.20 \pm 2.25\%$ (Figure 12).

Accordingly, on the genus level *Faecalibacterium* was significantly higher (P=0.005) in the

healthy group after 0 hr. Unexpectantly, there were no significant differences in symbiotic

bacteria between either group before and after fermentation with cranberry polyphenols and main

differences occurred within *Enterobacteriaceae* and *Ruminococcacea*.
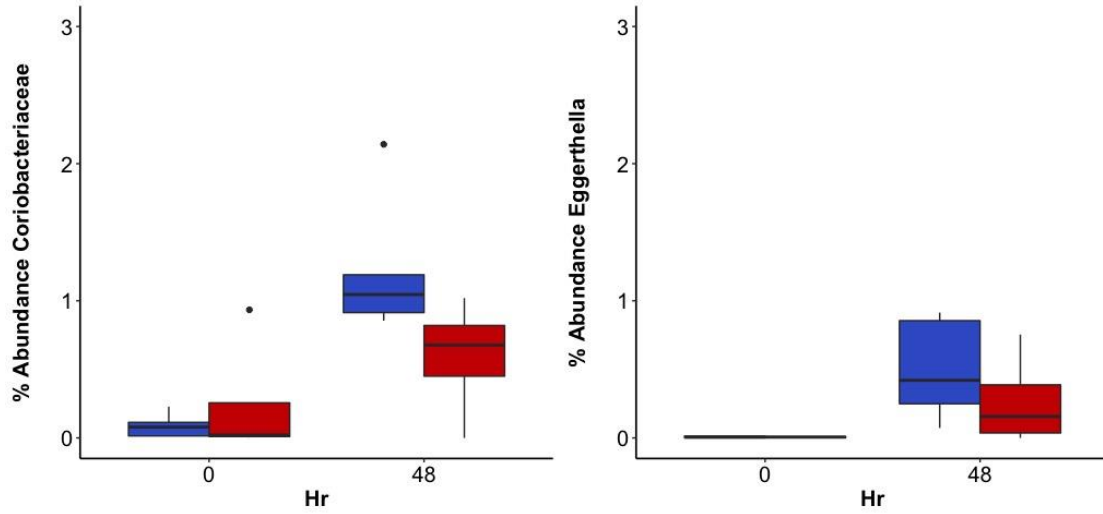
Figure 8 Percent abundance of the family *Coriobacteriaceae* (left) and the genus *Eggerthella* (right) which is within the family *Coriobacteriaceae*, compared between healthy (blue) and UC (red) fecal microbiomes. Hour after fermentation with cranberry polyphenols was a significant factor influencing the abundance of both *Coriobacteriaceae* (P=0.002) and *Eggerthella* (P=0.007).
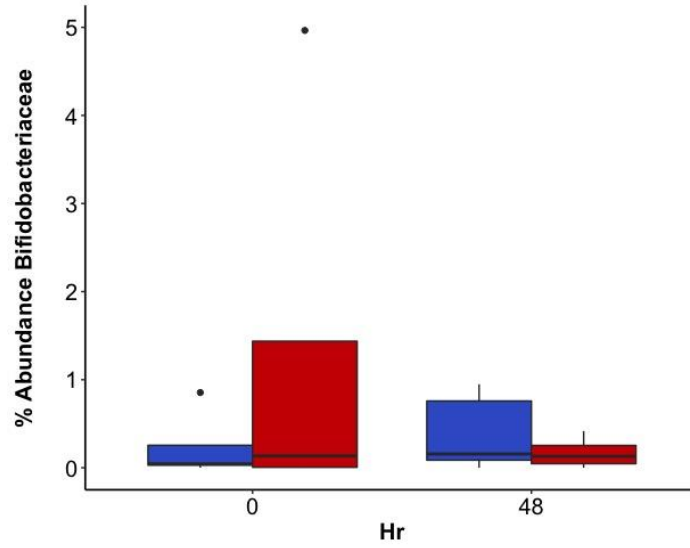
Figure 9 Percent abundance of the family *Bifidobacteriaceae* compared between healthy (blue) and UC (red) fecal microbiomes. Hour, health status and the interaction between hour and health status were not significant factors (P<0.05) influencing the abundance of *Bifidobacteriaceae*.
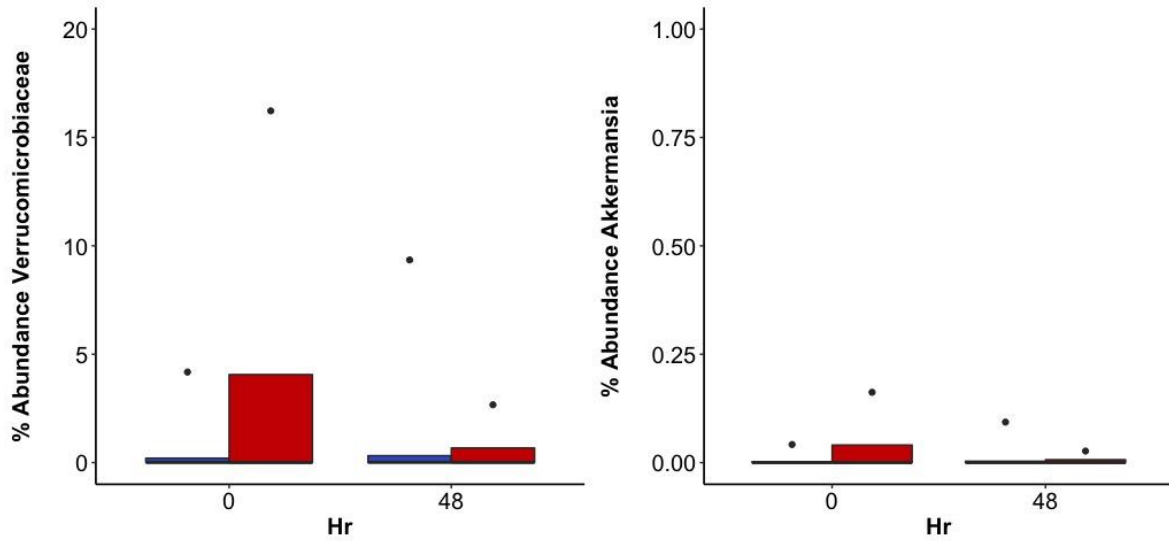
Figure 10 Percent abundance of *Verrucomicrobiaceae* (left) and the genus *Akkermansia* (right), which is within the family *Verrucomicrobiaceae*, compared between healthy (blue) and UC (red) fecal microbiomes. Hour, health status and the interaction between hour and health status were not significant factors (P<0.05) influencing the abundance of *Verrucomicrobiaceae* and *Akkermansia*.
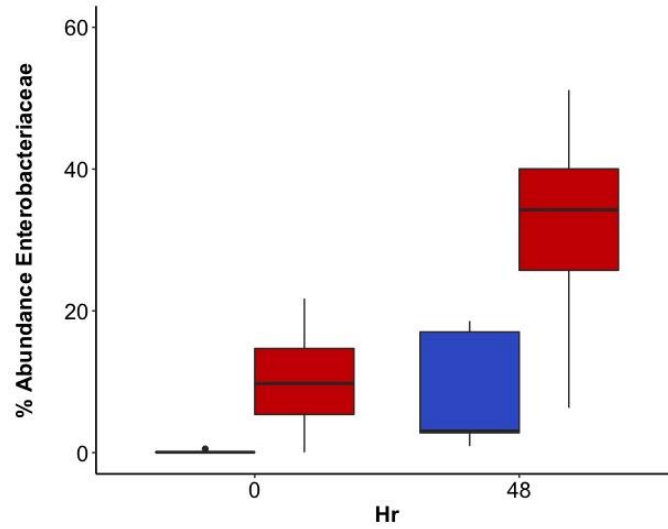
Figure 11 Percent abundance of the family *Enterobacteriaceae* compared between healthy (blue) and UC (red) fecal microbiomes. Hour after fermentation with cranberry polyphenols was a significant factor influencing the abundance of *Enterobacteriaceae* (P=0.011).
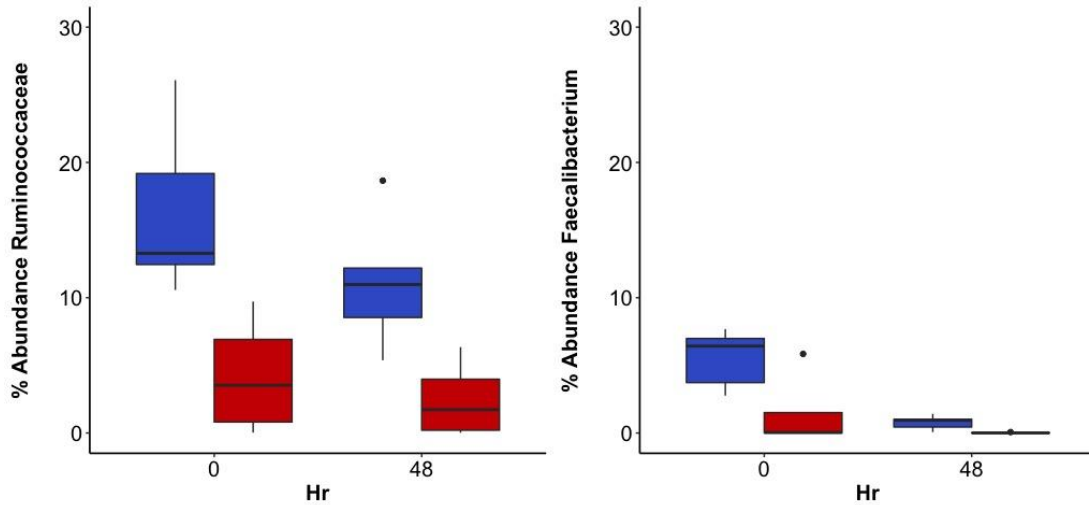
Figure 12 Percent abundance of *Ruminococcaceae* (left) and the genus *Faecalibacterium* (right), which is within the family *Ruminococcaceae*, compared between healthy (blue) and UC (red) fecal microbiomes. Health status was a significant factor influencing the abundance of *Ruminococcaceae* (P=0.003) and *Faecalibacterium* (P=0.005).

Comparison of the Microbiome Metabolism of Cranberry Polyphenols between UC and Healthy

Fecal Donors

Fecal microorganisms were cultivated anaerobically in the presence of cranberry polyphenols to compare metabolism between the colon microbiomes of healthy and UC fecal donors. Overall, the healthy group's fecal microorganisms produced higher concentrations of polyphenol metabolites than UC fecal microorganisms. 34PV was produced within the first 6 hr for both healthy and UC groups (Figure 13). After 6 hr, healthy subjects produced higher (P=0.062) concentrations of 34PV with 28.96 ± 6.82 μM which was 5.25-fold higher than that produced by UC individuals at 5.52 ± 5.52 μM. Throughout the course of the fermentation 34PV was produced in higher concentrations in healthy subject fermentations with a maximum

40

concentration reached at 12 hr at 42.99 ± 7.53 μM while the UC maximum concentration was

significantly lower (P=0.020) at 16.69 ± 5.62 μM, also after 12 hr. Concentrations of 34PV

started to decline after 12 hr and was less than 6 μM for both groups after 48 hr. For healthy

subjects, 34PP was higher (P=0.176) after 12 hrs of fermentation at 42.26 ± 41.93 μM compared

to 22.74 ± 14.72 μM for the UC group (Figure 14). Although not statistically significant,

maximum concentrations of 34PP was reached after 6 hr and was 46.86 ± 27.22 μM for the

healthy group and 25.56 ± 12.05 μM for the UC group, a 1.83-fold difference. The concentration

of 34PP plateaued in the UC group fermentations from 12-48 hr. By contrast, 34PP was rapidly

metabolized by the healthy group and was no longer detectable after 48 hr. The

monohydroxylated derivative of 34PP, MPP, was also not significantly different between healthy

and UC microbiomes (Figure 15) with maximum concentrations produced after 48 hr for healthy

and UC groups at 183.67 ± 44.81 μM and 126.89 ± 104.56 μM, respectively. Over the first 6-12

hrs of fermentation 34PA was higher in the healthy group in comparison to the UC group,

although the concentrations were not significantly different (Figure 16). The peak concentration

of 34PA was produced after 12 hr (P=0.111) for the healthy group with 180.72 ± 19.78 μM and

for the UC group after 24 hr with 112.49 ± 38.55 μM. The healthy group started to produce MPA

after 6 hr and significantly higher (P<0.05) concentrations of this metabolite from 12-24 hr as the

UC group only produced MPA after 48 hr (Figure 17). There were no significant differences

between the UC and healthy groups in the production of 34BA, commonly known as

protocatechuic acid (Figure 18). Maximum concentrations of 34BA occurred after 12 hr at 12.38

± 2.62 μM and 13.69 ± 1.28 μM for the healthy and UC groups, respectively. Likewise, no

differences were observed for MBA between groups (Figure 19) and concentrations were

41

considerably low at <1 μM in both healthy and UC fermentations. Overall, those diagnosed with

UC metabolized cranberry polyphenols to a lesser extent resulting in reduced production of

polyphenol microbial metabolites in comparison to healthy individuals who had no history of

chronic disease.
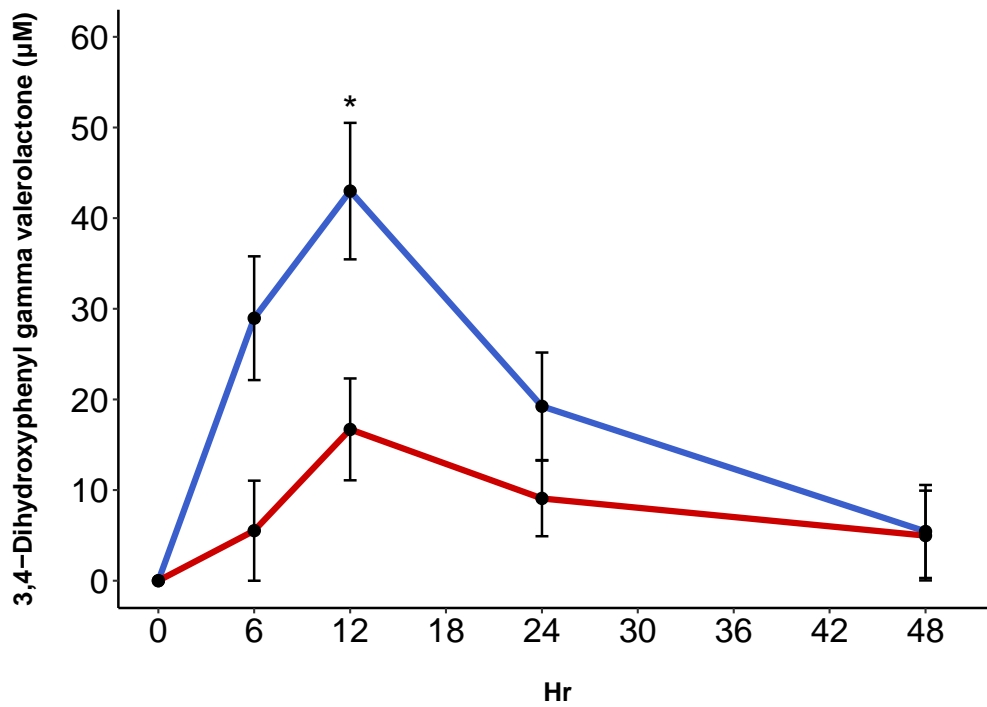
Figure 13 Comparison of the production of 3,4-dihydroxyphenyl-gamma-valerolactone (34PV) between healthy (blue) and UC (red) fecal microbiomes over 48 hours of fermentation with cranberry polyphenols. Data is presented as mean ± SEM μM. *Significant difference (P<0.05) between healthy and UC microbiome produced concentrations of 34PV after 12 hours.
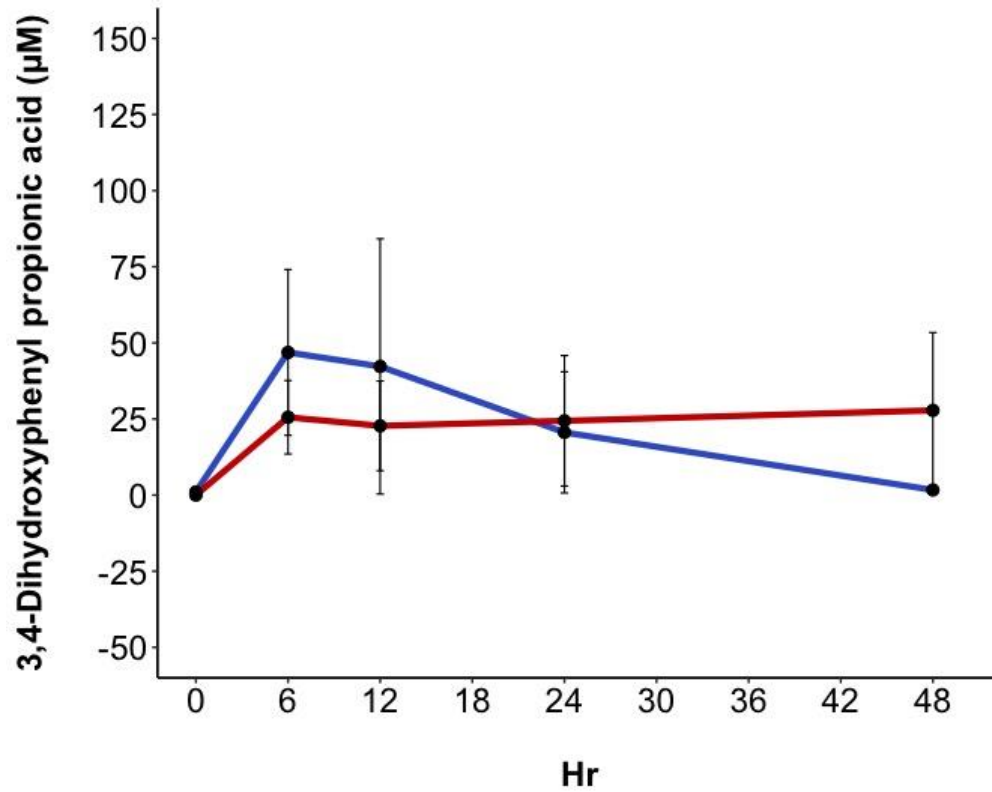
Figure 14 Comparison of the production of 3,4-dihydroxyphenyl propionic acid (34PP) between healthy (blue) and UC (red) fecal microbiomes over 48 hours of fermentation with cranberry polyphenols. Data is presented as mean ± SEM μM. There were no significant differences (P<0.05) in the concentration of 34PP between groups at any hour.
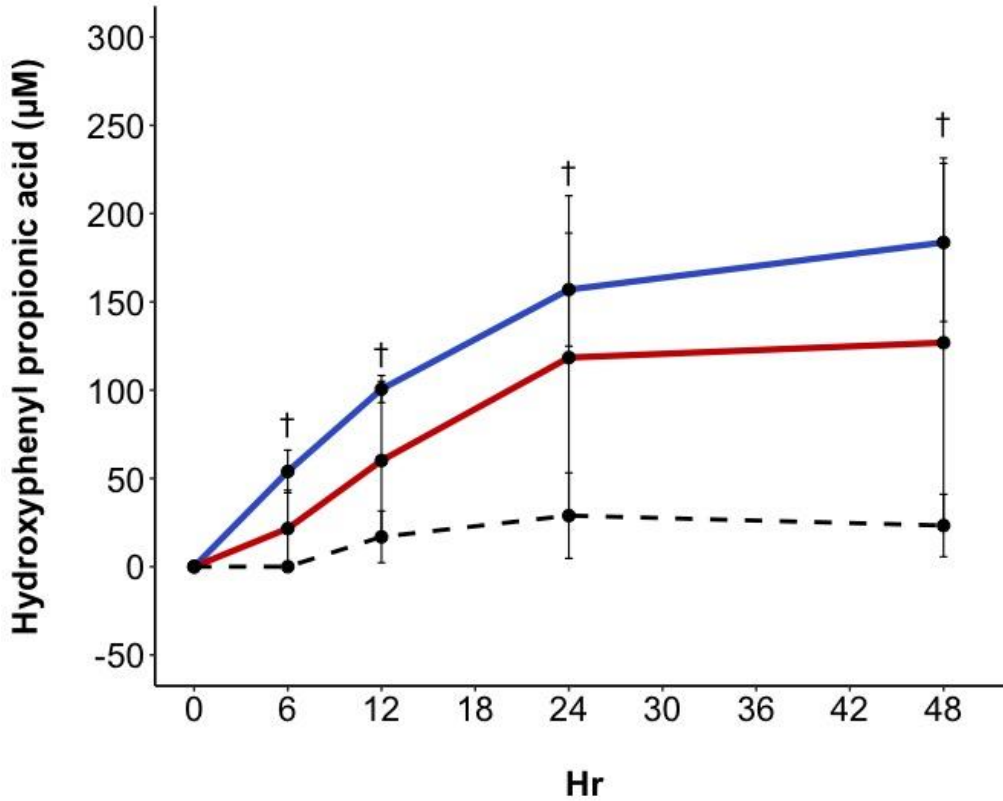
Figure 15 Comparison of the production of hydroxyphenyl propionic acid (MPP) between healthy (blue) and UC (red) fecal microbiomes over 48 hours of fermentation with cranberry polyphenols. Data is presented as mean ± SEM μM. There were no significant differences (P<0.05) in the concentration of MPP between groups at any hour. †Significant differences (P<0.05) between healthy and UC microbiomes at 6-48 hours when UC outlier is removed.
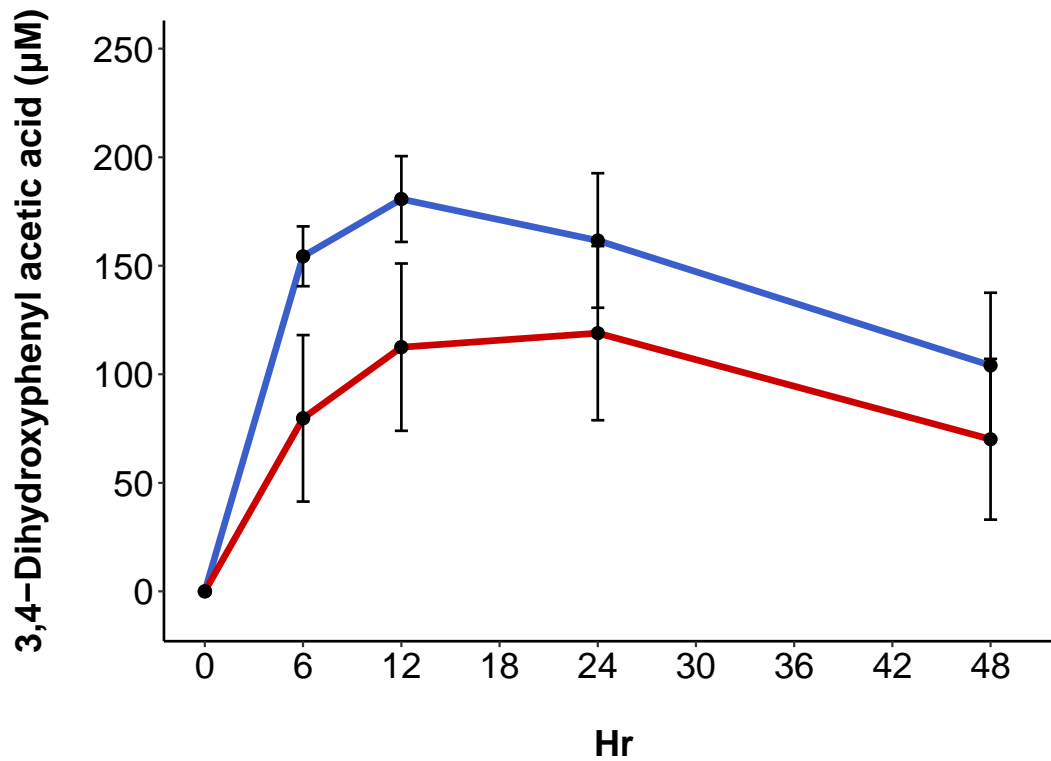
Figure 16 Comparison of the production of 3,4-dihydroxyphenyl acetic acid (34PA) between healthy (blue) and UC (red) fecal microbiomes over 48 hours of fermentation with cranberry polyphenols. Data is presented as mean ± SEM μM. There were no significant differences (P<0.05) in the concentration of 34PA between groups at any hour.
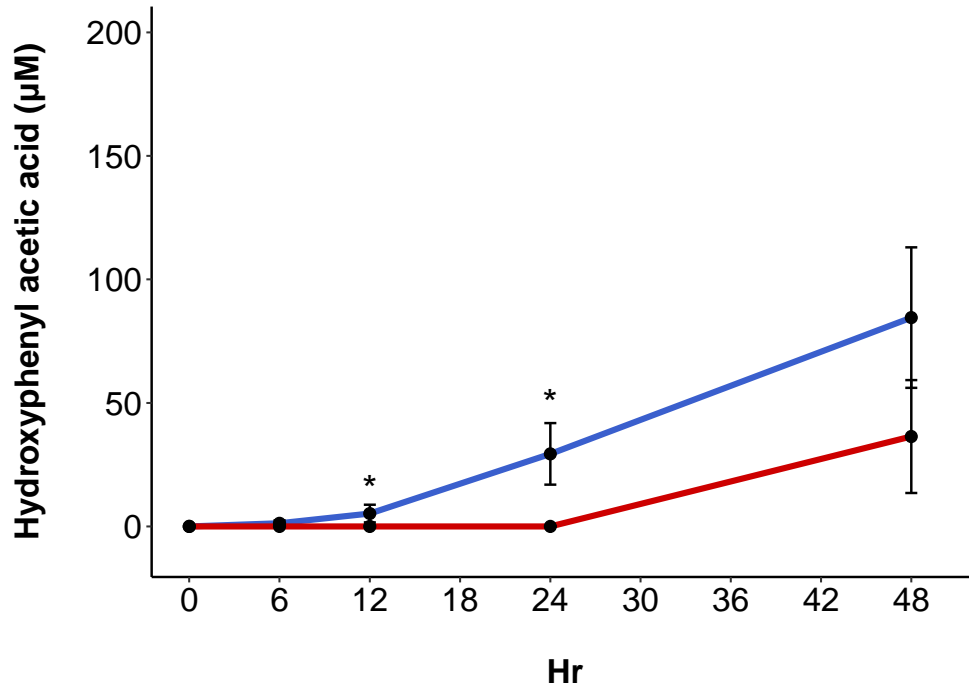
Figure 17 Comparison of the production of hydroxyphenyl acetic acid (MPA) between healthy (blue) and UC (red) fecal microbiomes over 48 hours of fermentation with cranberry polyphenols. Data is presented as mean ± SEM μM. *Significant difference (P<0.05) between healthy and UC microbiome produced concentrations of MPA after 12 and 24 hours.
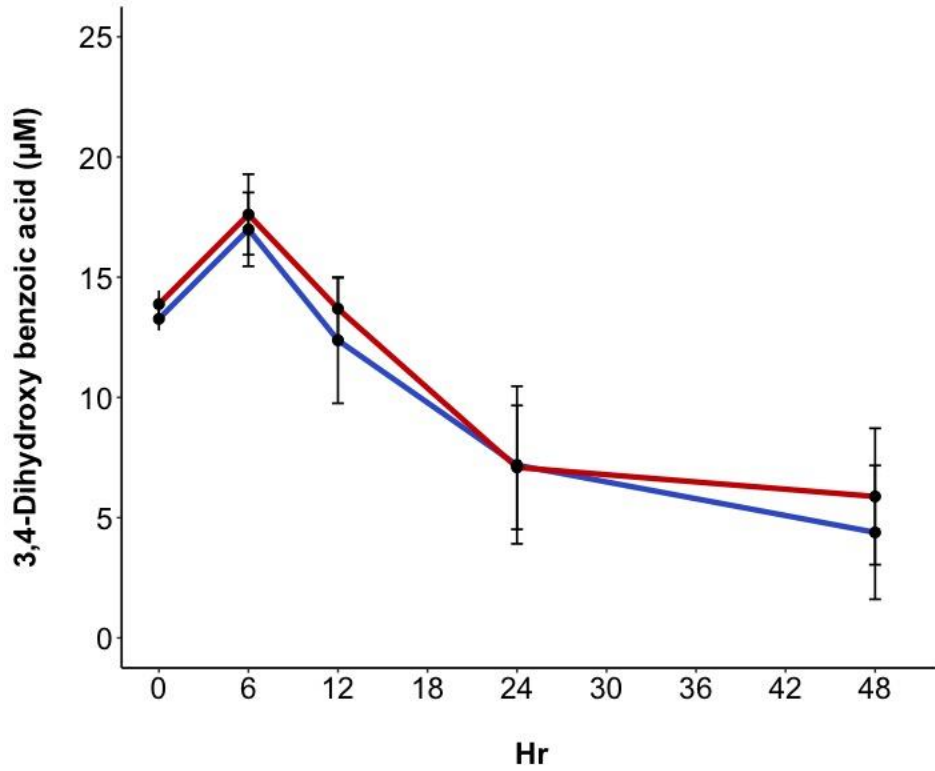
Figure 18 Comparison of the production of 3,4-dihydroxy benzoic acid (34BA), or protocatechuic acid, between healthy (blue) and UC (red) fecal microbiomes over 48 hours of fermentation with cranberry polyphenols. Data is presented as mean ± SEM μM. There were no significant differences (P<0.05) in the concentration of 34BA between groups at any hour.
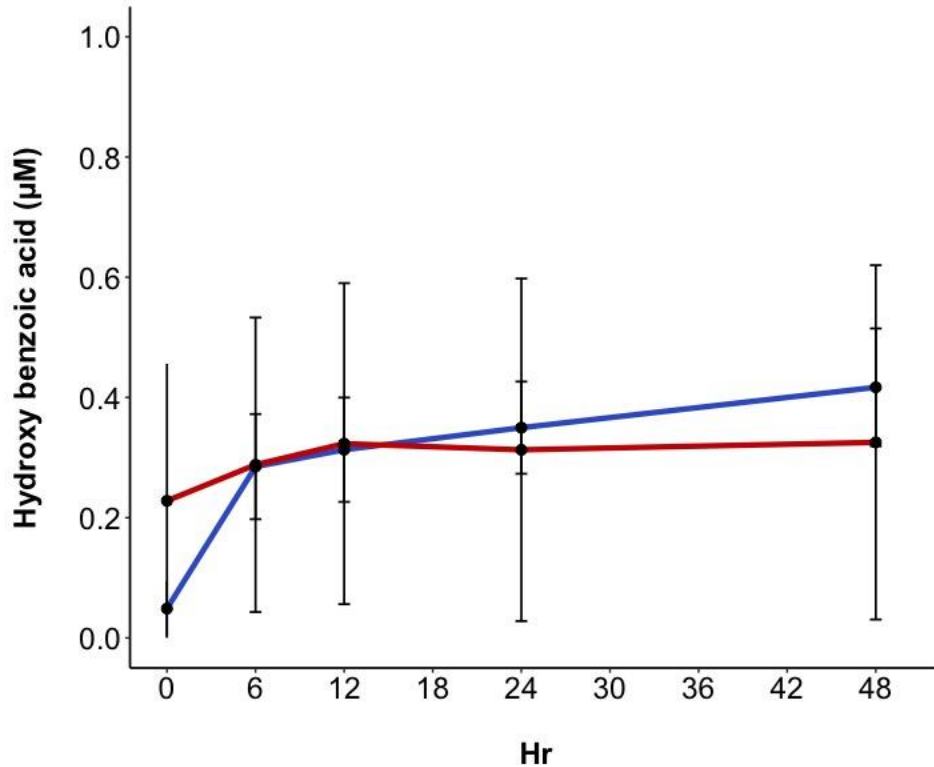
Figure 19 Comparison of the production of hydroxy benzoic acid (MBA) between healthy (blue) and UC (red) fecal microbiomes over 48 hours of fermentation with cranberry polyphenols. Data is presented as mean ± SEM μM. There were no significant differences (P<0.05) in the concentration of MBA between groups at any hour.

Inter-Individual Differences of Cranberry Polyphenol Metabolism Within Groups

Large inter-individual differences in polyphenol metabolism are common and believed to come about from differences in the composition and function of the microbiome. Therefore, differences between cranberry polyphenol metabolism were compared within the healthy and UC groups. Time of fermentation and individual subject were significant factors (P<0.001) for the production of 34PP, MPP, 34PA and 34PV within the healthy group, whereas 2 subjects produced significantly higher concentrations of these metabolites in comparison to the other 3

48

healthy subjects. Time of fermentation and individual subject were also significant factors (P<0.001) for the production of 34PP, MPP, 34PA, MPA and 34PV within the UC group, whereas 2 subjects produced higher concentrations of these metabolites in comparison to the other 2 subjects and maximum concentrations were produced at different hrs. In particular, 1 UC subject produced significantly higher concentrations (P<0.05) of MPP than the other 3 UC subjects from 6-48 hr of fermentation with cranberry polyphenols (Figure 15). Additionally, when this subject was removed from the UC group, there was significantly lower (P<0.05) concentrations of MPP produced by UC microbiomes in comparison to healthy microbiomes. Larger inter-individual differences occurred between the UC group which could be a consequence of the larger variation within the microbiome of the UC group.

Short Chain and Branched Chain Fatty Acid Production

SCFA and BCFA were quantified to assess differences in microbial saccharolysis and proteolysis activity between healthy and UC microbiomes. The SCFAs acetic acid, propionic acid and butyric acid are markers of metabolites produced from non-digestible carbohydrates, while the BCFAs isovaleric and isobutyric acid are derived from amino acid metabolism (Windey, De Preter & Verbeke, 2012). Time was the only significant factor impacting the concentration of acetic acid for both groups (P<0.001), as the concentration of acetic acid was significantly higher after 48 at 313.75 ± 21.75 µM (Figure 20). Only negligible amounts of acetic acid was present at 0 hr for both UC and healthy microbiomes, with or without cranberry polyphenols. Concentrations of propionic acid were also only dependent on time (P<0.001) and after 48 hr, all groups produced an average of 154.39 ± 20.32 µM (Figure 21). By contrast, butyric acid concentrations were dependent on the interaction of whether microbiomes were from a UC or Healthy fecal donor and time (P<0.001) (Figure 22). After 48 hr, healthy subjects

49

produced significantly higher concentrations of butyric acid in comparison to UC subjects with

$302.39 \pm 14.15\ \mu M$ and $193.50 \pm 36.67\ \mu M$, respectively. Fermentations that contained cranberry

also had higher but not significant concentrations of butyric acid after 48 hr with an average

value of 273.73 ± 25.37 and 234.26 ± 25.27 from samples with cranberry polyphenols and non-

polyphenol containing control samples, respectively. Likewise, valeric acid concentrations were

dependent on the interaction of whether the fecal donor had UC or was healthy and time

$(P=0.008)$, where healthy microbiomes also produced higher concentrations of valeric acid after

48 hr in comparison to UC at $65.88 \pm 5.98\ \mu M$ and $35.80 \pm 13.41\ \mu M$, respectively (Figure 23).

Concentrations of isobutyric and isovaleric acid after 48 hr were dependent on time and whether

or not cranberry polyphenols were present in the fermentation $(P<0.050)$ (Figure 24 & 25). When

cranberry polyphenols were present, isobutryic acid was significantly lower after 48 hr in

comparison to the non-polyphenol containing control at $34.22 \pm 6.69\ \mu M$ and $53.95 \pm 5.51\ \mu M$,

respectively. This was also true for isovaleric acid whereas after 48 hr, when cranberry

polyphenols were present, concentrations were lower at $31.56 \pm 6.31\ \mu M$ in comparison to the

control at $49.79 \pm 5.33\ \mu M$. Overall, SCFA production was dependent on health status and not

on whether cranberry polyphenols were present, with the exception of butyric acid. For BCFA,

the presence of cranberry polyphenols influenced their production opposed to the health status of

the fecal donor.
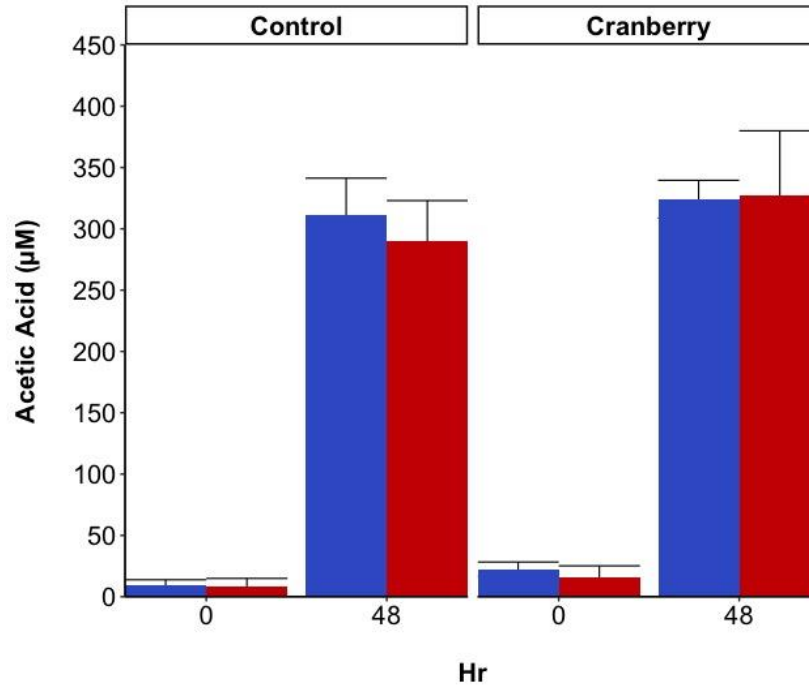
Figure 20 Comparison of the concentration of the SCFA, acetic acid, produced by healthy (blue) and UC (red) microbiomes after 0 and 48 hours of fermentation with or without (control) cranberry polyphenols. Data is reported as mean ± SEM μM. Acetic acid was significantly higher (P<0.05) after 48 hours of fermentation.
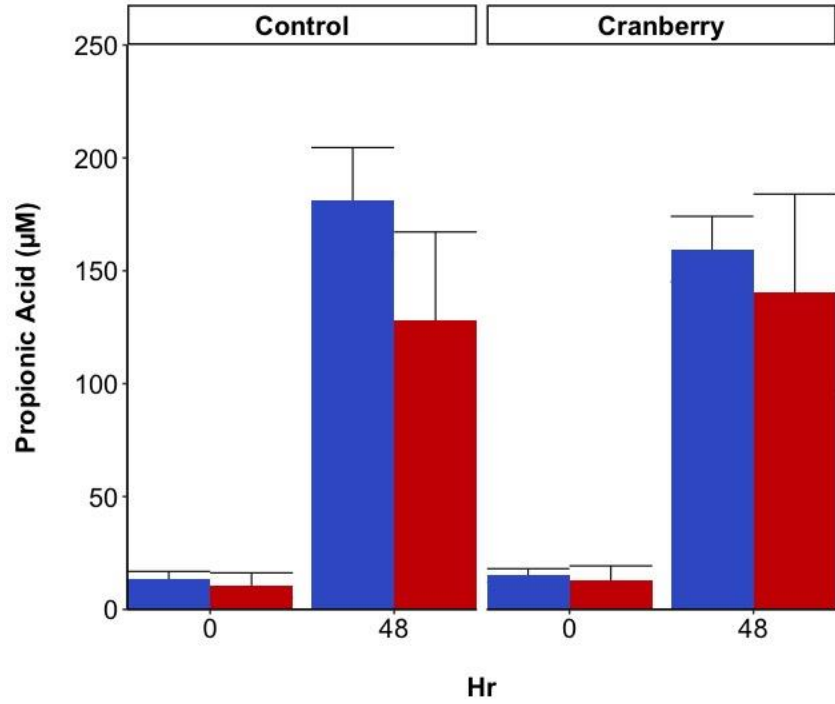
Figure 21 Comparison of the concentration of the SCFA, propionic acid, produced by healthy (blue) and UC (red) microbiomes after 0 and 48 hours of fermentation with or without (control) cranberry polyphenols. Data is reported as mean ± SEM µM. Propionic acid was significantly higher (P<0.05) after 48 hours of fermentation.
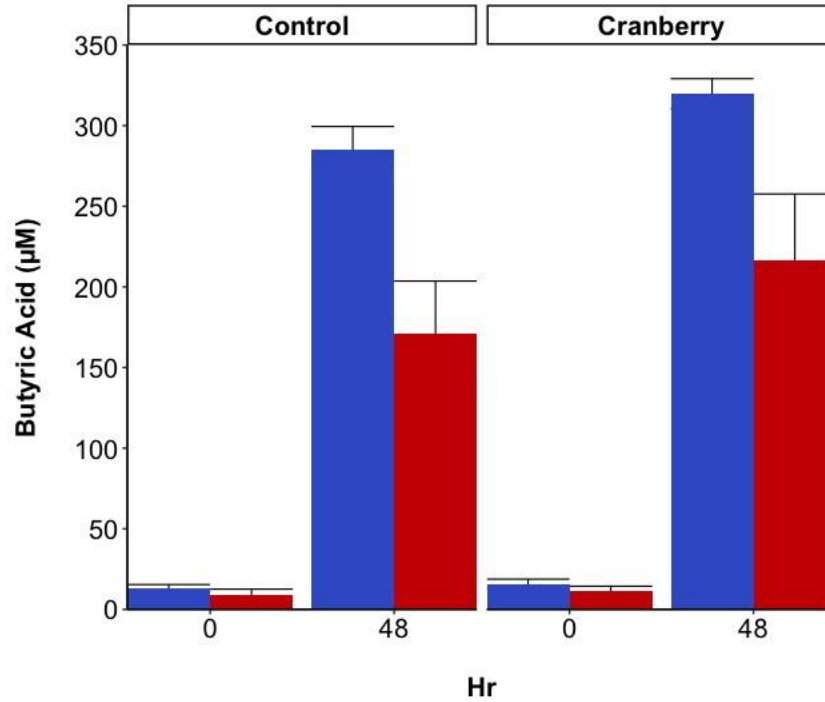
Figure 22 Comparison of the concentration of the SCFA, butyric acid, produced by healthy (blue) and UC (red) microbiomes after 0 and 48 hours of fermentation with or without (control) cranberry polyphenols. Data is reported as mean ± SEM µM. The interaction of time and health status was significant (P<0.001), whereas healthy microbiomes produced significantly higher (P<0.05) concentrations of butyric acid after 48 hours.
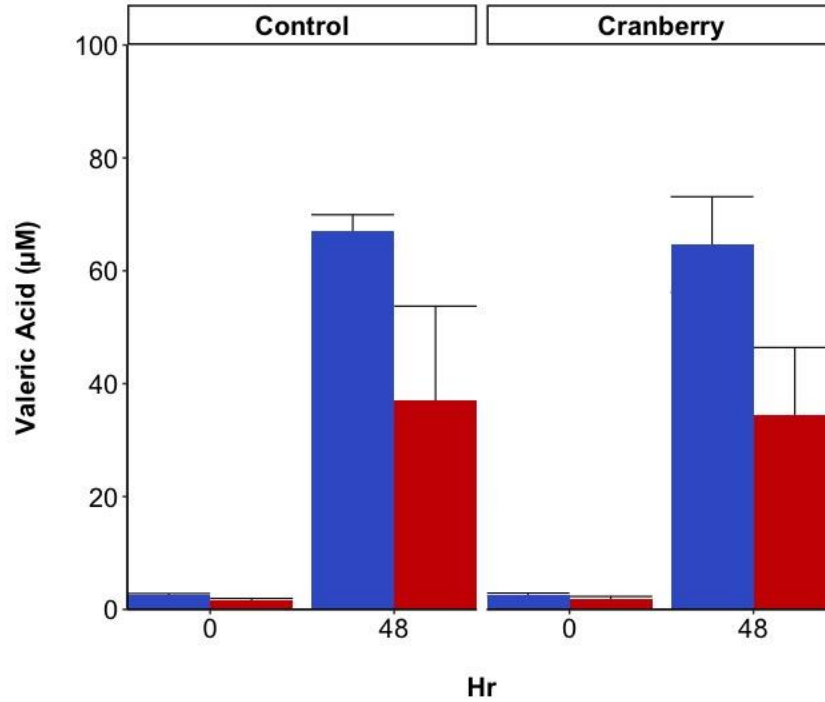
Figure 23 Comparison of the concentration of the SCFA, valeric acid, produced by healthy (blue) and UC (red) microbiomes after 0 and 48 hours of fermentation with or without (control) cranberry polyphenols. Data is reported as mean ± SEM μM. The interaction of time and health status was significantly (P=0.008), whereas healthy microbiomes produced significantly higher (P<0.05) concentrations of valeric acid after 48 hours.
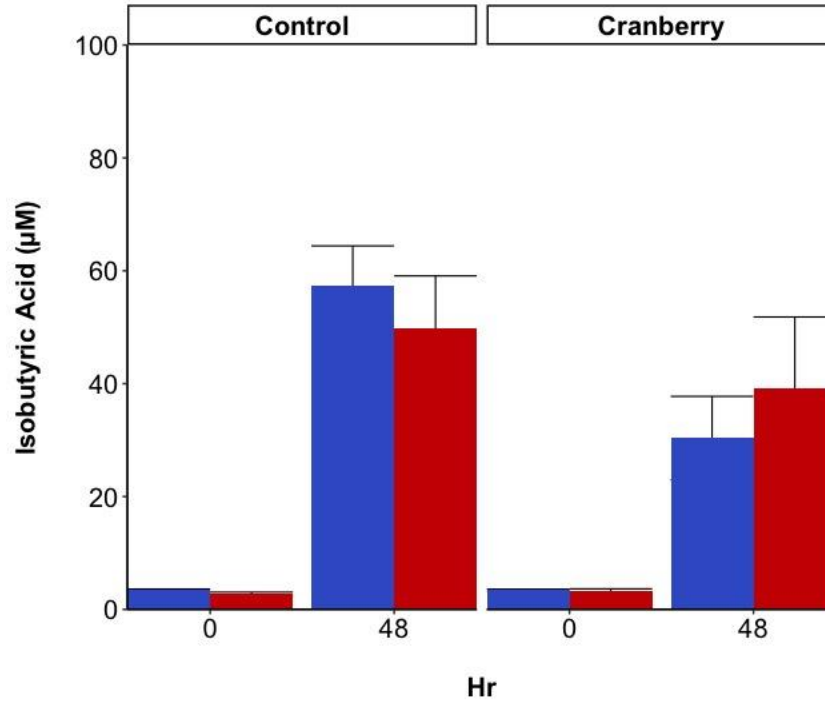
Figure 24 Comparison of the concentration of the BCFA, isobutyric acid, produced by healthy (blue) and UC (red) microbiomes after 0 and 48 hours of fermentation with or without (control) cranberry polyphenols. Data is reported as mean ± SEM μM. The interaction of time and presence of cranberry polyphenols was significant (P<0.05), whereas fermentations containing cranberry polyphenols produced significantly lower (P<0.05) concentrations of isobutyric acid after 48 hours.
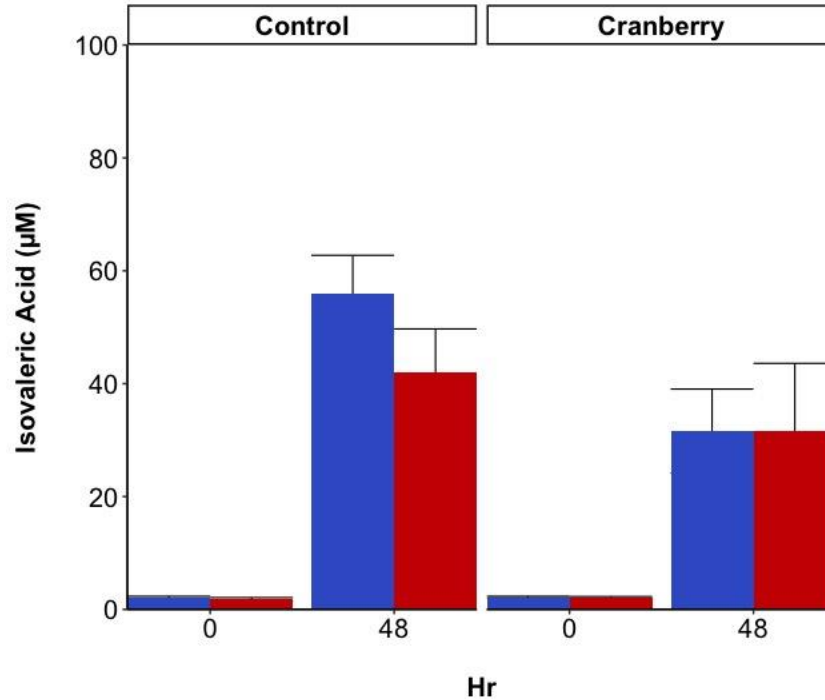
Figure 25 Comparison of the concentration of the BCFA, isovaleric acid, produced by healthy (blue) and UC (red) microbiomes after 0 and 48 hours of fermentation with or without (control) cranberry polyphenols. Data is reported as mean ± SEM μM. The interaction of time and presence of cranberry polyphenols produced significantly lower (P<0.05) concentrations of isovaleric acid after 48 hours.

Discussion

Subject Characteristics

The SIBDQ and SCCAI questionnaires and fecal calprotectin were utilized as non-invasive measures of UC disease activity. The SIBDQ consists of 10 questions where subjects evaluate their quality of life by selecting a response on a 7-point scale, 1 being of the worst health and 7 of the best health. The 10 questions are then averaged to obtain a score. The average score of the UC group was comparable to what has been found previously in those diagnosed

with active UC (Voiosu *et al.*, 2014). Jowett *et al.* (2001) was able to differentiate severity of disease with the SIBDQ score of UC patients. According to those findings, the UC group disease activity ranges from mild-severe. The SCCAI is inversely correlated with the SIBDQ and is a 19-point scale, where 0 indicates no disease activity and 19 indicates highest disease activity. The SCCAI scores indicated that all subjects had active UC. Fecal calprotectin was also assessed as it is a marker of inflammation and also with SIBDQ score is correlated with extent of UC disease activity (Voiosu *et al.*, 2014). Fecal calprotectin in the UC group was double that of the healthy group, which together with the results of the SIBDQ and SCCAI, confirmed disease activity in the UC group.

Microbiota Composition of Healthy and UC Fecal Microorganisms

Microbiome dysbiosis in UC refers to characteristic alterations in microbiota composition and alpha-diversity, which may or may not be a cause or consequence of the disease. The microbiota composition of UC microbiomes deviates from healthy microbiomes by having lower abundances of *Ruminococcaceae*, Bacteroides, *Bifidobacterium* and *Lactobacillus* spp. and overall alpha-diversity (Kostic, Xavier & Gevers, 2014). Further, UC microbiomes tend to have higher abundances of Gammaproteobacteria and Fusobacteriaceae, which may play a role in the pathogenesis of UC through pro-inflammatory mechanisms (Mukhopadhya *et al.*, 2012, Chen *et al.*, 2019). Accordingly, the UC microbiome composition agreed with past findings that characterized dysbiosis in UC, as the UC group had decreased alpha-diversity and a significant bloom of *Enterobacteriaceae* in comparison to the healthy group. The lack of butyrate-producing bacteria *Faecalibacterium prausnitzii* and *Roseburia hominis* have also been identified as markers of dysbiosis in UC (Machiels *et al.*, 2014). However, only abundances of *Faecalibacterium* and not *Roseburia* (data not shown) was lower in UC microbiomes. Dysbiosis

57

was also evident at 0 hr within the phylum Firmicutes. Specifically, the UC microbiomes in comparison to healthy microbiomes contained decreased abundance of *Ruminococcaceae*. *Ruminococcaceae* contains bacteria that are capable of metabolizing flavonoids, such as *Flavonfactor plautii,* which can catalyze the reaction of flavan-3-ols to 34PV through C-ring cleavage (Kutschera *et al*., 2011, Branue & Blaut, 2016). C-ring cleavage is the initial reaction involved in flavonoid metabolism, and the decreased abundance of these bacteria due to dysbiosis can explain the inefficiency of metabolism of cranberry polyphenols by UC microbiomes.

Polyphenols and the colon microbiome have an interdependent relationship, whereas the microbiome metabolizes polyphenols into metabolites with increased bioavailability and polyphenols in turn can modulate the bacterial composition of the microbiome. Polyphenols modulate microbiome composition by selecting for the growth of symbionts and have even been classified as prebiotics, as several authors have shown that *in vivo* consumption and *ex vivo* fermentation of polyphenols by fecal bacteria increases the abundance of *Bifidobacterium* and *Lactobacillus* spp. (Tzounis *et al*., 2008, Vendrame *et al*., 2011). However, this modulatory effect did not occur after *ex vivo* fermentation of cranberry polyphenols. Further, *Bifidobacterium* and *Lactobacillus spp.*, supposed symbiotic bacteria, have decreased abundances in UC microbiomes and have been identified as markers of dysbiosis in UC (Duranti *et al*., 2016). By contrast, *Bifidobacterium* and Lactobacillales (data not shown) were more prevalent in the UC group at 0 hr and Wang *et al*., (2014) have also found that these taxa were increased in UC microbiomes. Additionally, cranberry polyphenols did not show prebiotic effects on the growth of *Bifidobacterium* or *Lactobacillales.* The abundance of the mucin-degrading bacteria *Akkermansia muciniphila* in the microbiome is inversely correlated with

intestinal health, and cranberry polyphenols have shown to have prebiotic effects on the growth

of *Akkermansia spp.* (Anhe *et al.*, 2015, Anhe *et al.*, 2016). However, although not significant,

Akkermansia was also more prevalent in the UC group at 0 hr in comparison to the healthy group

and was not increased after *ex vivo* fermentation of cranberry polyphenols. There was evidence

of modulation of microbiome composition by cranberry polyphenols for other taxa. Specifically,

Eggerthella increased in both healthy and UC microbiomes after 48 hr. *Eggerthella lenta* can

metabolize flavan-3-ols by C-ring fission and also dehydroxylate the phenyl substituent (Braune

& Blaut, 2016). The increase in this taxon could have come about from its ability to metabolize

the large concentration of flavan-3-ols and PACS present in the cranberry extract.

Enterobacteriaceae also markedly increased in both the healthy and UC microbiomes, which has

also occurred *in vivo* after long-term consumption of high-PAC foods (Holscher *et al.*, 2018,

Smith *et al.*, 2004). *Enterobacteriaceae* have been classified as PAC-resistant, whereas they can

overcome the anti-bacterial activity of PACS by metabolizing PACs or by complexing PACs

with extracellular glycoproteins and/or carbohydrates (Smith *et al.*, 2005, Braune & Blaut,

2016). This is contradictory to reports of the specific, anti-bacterial activity of a-type PACS

against adherent-invasive *E.coli* (*Enterobacteriaceae*) in the urinary tract, although the specific

species within *Enterobacteriaceae* that increased after cranberry polyphenol fermentation could

not be identified with 16S rRNA targeted gene sequencing. Taken together, evidence suggests

that cranberry polyphenols may modulate bacteria in the microbiome by selecting for taxa that

have polyphenol-resistant mechanisms and/or the ability to metabolize polyphenols.

Effect of UC on Metabolism of Cranberry Polyphenols by the Microbiome

The colon microbiome is important for several metabolic processes including the

synthesis of vitamins and the metabolism of amino acids, bile acids and non-digestible

carbohydrates. More recently, the importance of the metabolism of polyphenols by the colon microbiome into bioavailable metabolites has also been recognized (Lozupone *et al.*, 2012, Neis, Dejong, & Rensen, 2015, Jones *et al.*, 2008, Biesalski, 2016, Possemiers *et al.*, 2011, Rowland *et al.*, 2018). Polyphenols found in cranberry can be metabolized by the microbiome into phenyl propionates and/or phenyl acetates through several mechanisms that are dependent on polyphenol molecular weight and aglycone chemical structure (Selma *et al.*, 2009). Flavan-3-ols, a major class of polyphenol found in cranberry, are metabolized into unique phenyl valerolactone derivatives before subsequent alpha and beta-oxidation reactions to form phenyl propionate and/or phenyl acetates (Stoupi *et al.*, 2010a). These mechanisms have been elucidated by the isolation of polyphenol metabolizing bacteria from feces and also *ex vivo* static batch fermentations of polyphenols from healthy fecal donors (Jin & Hattori, 2012, Takagaki & Nanjo, 2015). Identified microbial polyphenol metabolites and their corresponding hepatic phase II derivatives have also been confirmed *in vivo* in urine and plasma after human consumption of cranberry juice (Feliciano *et al.*, 2016).

Although the role of the colon microbiome in cranberry polyphenol metabolism has been elucidated, few studies report on the effect of an individual's disease state on polyphenolic metabolism, especially those diseases that affect the function or composition of the microbiome, such as UC and obesity. It was hypothesized that the microbiome of individuals with UC would not be able to produce the same metabolites from cranberry polyphenols or metabolize cranberry polyphenols to the same extent as healthy individuals, due to microbiota compositional differences. Accordingly, in this study comparing healthy to dysbiotic UC fecal donors, is was found that the total action of fecal microorganisms of UC subjects produced significantly lower concentrations of targeted polyphenol metabolites, including phenyl valerolactones, propionates

and acetates. The kinetics of the production of these metabolites were also altered in UC in comparison to fecal microorganisms from healthy subjects. Several authors have reported that active UC can cause altered metabolism of macronutrients and micronutrients in comparison to healthy controls, evident by increased or decreased levels of metabolites in serum and feces including, SCFA, BCFA, bile acids, amino acids, and polyamines (Gall *et al*., 2011, Scoville *et al*., 2018). These alterations in metabolism were hypothesized to be due to bacterial dysbiosis in the gut of those with UC, which was shown in this study to also impact cranberry polyphenol metabolism. More relevant to polyphenol metabolism, obese individuals and those with metabolic syndrome and colorectal cancer were more likely to show alterations in ellagitannin metabolism into microbial derived urolithins, which was also explained by an increased incidence of gut dysbiosis in these groups (Selma *et al*., 2016, Tomas-Barberan *et al*., 2014).

Notably, there were several targeted metabolites that were not significantly different between UC and healthy fecal donors, including MBA and 34BA. However, because negligible concentrations of these compounds were produced by both healthy and UC fecal donors, these may not be suitable targeted metabolites related to cranberry polyphenol metabolism. Further, hippuric acid has also been reported as a major metabolite indicative of polyphenol metabolism but it was not detected in any fermentations in this study. However, these metabolites have been suggested by others to be relevant to cranberry polyphenol metabolism *in vivo,* as they have been detected in μM concentrations in plasma and urine (Roowi *et al*., 2010).

This study uniquely highlights how disease state impacted by bacterial dysbiosis can alter polyphenol metabolism and future *in vivo* studies should consider including metabolomics in their experimental designs to improve understanding of causal relationships between polyphenol metabolism and their therapeutic effects.

Inter-Individual Differences in Cranberry Polyphenol Metabolism Within Groups

Inter-individual differences in polyphenol metabolism refers to the large variation in polyphenol pharmacokinetic parameters that occurs within a population as a result of differing microbiome composition, age, gender, genetics and diet. There have been two distinct manners in which inter-individual differences have been characterized. It has been found that populations can be stratified into metabotypes depending on whether or not they can produce urolithins or equol, or binary inter-individual metabolism (Hazim *et al*., 2016). Inter-individual differences in metabolism can also be quantitative, where all subjects produce every major microbial metabolite but to a higher or lower extent relative to one another (Cortes-Martin *et al.*, 2019). Inter-individual differences in the metabolism of flavan-3-ols and PACs, major classes of polyphenols in cranberry, have been found to differ *in vivo* quantitatively and recruited populations have not been able to be stratified into metabotypes (Cortes-Martin *et al*., 2019). However, Gil-Sanchez *et al*., (2017) found that a third of healthy volunteers did not produce 34PV from grape pomace extract fermented *ex vivo,* which could suggest that identification of PAC metabotypes are possible. By contrast, in this study all healthy subjects produced all targeted metabolites, although at differing rates resulting in large quantitative inter-individual differences. Large inter-individual differences were also present within the UC group specifically with monohydroxylated derivates. High inter-individual differences may also come about from confounding metabolites produced from amino acids, as these compounds are isomers of several polyphenol metabolites (Windey, De Preter & Verbeke, 2012). Overall, large inter-individual quantitative differences in cranberry polyphenol metabolism occurs, and is enhanced in populations with colon disease.

Effect of Health Status and Cranberry Polyphenols on SCFA and BCFA Production

SCFA and BCFA are important biochemical markers of microbiome metabolism and intestinal health. SCFA, mainly acetic, butyric and propionic acid, are produced by the microbiome from non-digestible carbohydrates (Morrison & Preston, 2016). SCFA serve many beneficial functions in the gut including the maintenance of tight junctions and colonocyte energy balance and the regulation of immune responses (Morrison & Preston, 2016). By contrast, the BCFAs isobutyric and isovaleric acid are produced by the microbiome from the amino acids valine and leucine, respectively. BCFA are increased in those with high-protein diets and are indirect markers of the presence of amino acid derived metabolites that are produced by the microbiome and toxic to humans, but they have also been directly linked with insulin resistance and obesity (Russell *et al*., 2011, Newgard *et al*., 2009, Van Nuenen *et al*., 2004). Lower concentrations of SCFA and higher concentrations of BCFA are correlated with UC dysbiosis (Van Nuenen *et al*., 2004, Huda-Faujan *et al*., 2010). In accordance, UC microbiomes resulted in lower concentrations of SCFA, which was not dependent on the presence of cranberry polyphenols. It has been suggested that SCFA can be directly chemically derived from polyphenols, however the non-polyphenol containing controls resulted in the same concentrations of SCFA (Mena *et al*., 2015). An exception was butyric acid, whereas the presence of cranberry polyphenols increased concentrations of butyric acid in both UC and healthy microbiomes. It has also been suggested that polyphenols promote the production of SCFA from non-digestible carbohydrates indirectly by acting as prebiotics, and have shown to promote the growth of the butyrate-producing bacteria, *Faecalibacterium prausnitzii* (Moreno-Indias *et al*., 2016). By contrast, Bazzocco *et al*., 2008 found that apple PACS with a DP > 7 suppressed SCFA production through PAC inhibition of SCFA-producing microbial enzymes.

Although this did not occur with SCFA, cranberry polyphenols did decrease concentrations of BCFA produced by both healthy and UC microbiomes which could be due to the large DP PACs present in the cranberry extract. PACs, especially those with a large DP, form complexes with proteins, which can potentially prevent their metabolism by the microbiome (Zeller *et al*., 2015). Taken together, cranberry polyphenols can influence metabolism within the gut by indirectly promoting the production of butyric acid and decreasing amino acid metabolism in dysbiotic microbiomes.

Native Polyphenols v. Polyphenol Metabolites – Which are Bioactive?

A question that remains in the field today is whether it is the native polyphenol compounds or their metabolites that are bioactive, or instead if polyphenol metabolites are biomarkers of a healthy gut. (Tomas Barberan, Selma & Espin, 2018). Cranberry polyphenols have shown to down-regulate inflammation, prevent bacterial translocation and improve physio-chemical properties of mucins (Narayansingh & Hurta, 2008, Georgiades *et al*., 2014) in *in vitro* colonocyte models. However, these results are not representative of physiological conditions because native compounds undergo metabolism by the microbiome, resulting in a mixture of both native polyphenols and their metabolites, depending on the microbiome's efficiency of polyphenol metabolism. Many *in vivo* studies with polyphenol interventions do not include metabolomics data, making it even more difficult to draw comparisons between *in vivo* and *in vitro* studies because it is unknown to what extent the native polyphenols have been metabolized by the microbiome and how this correlates with the observed therapeutic benefit (Anhe *et al*., 2015). This issue has been highlighted in human clinical trials as several *in vivo* studies have identified relationships between efficiency of polyphenol metabolism by the microbiome and extent of polyphenol bioactivity (Gonzalez-Sarrias *et al*., 2016). Significant differences between

groups were not apparent until subjects were stratified by their microbiome's efficiency to metabolize polyphenols, and those who were more efficient at metabolizing polyphenols saw a more positive response to interventions (Gonzalez-Sarrias *et al*., 2016). Further, supplementation with polyphenol metabolites does not result in a more pronounced therapeutic benefit, which suggests that polyphenol metabolites may only serve as biomarkers (Hazim *et al*., 2016). This study highlighted how the extent of polyphenol metabolism is influenced by microbiome composition and future studies should include metabolomics data so it could be better understood how microbiome metabolism influences polyphenol bioactivity.

Conclusion

The effect of microbiome dysbiosis on the metabolism of polyphenols was evaluated by static *ex vivo* fermentation of cranberry polyphenols by fecal microorganism from healthy and UC fecal donors. Microbiome dysbiosis in UC was characterized by a decreased abundance of *Ruminococcaceae* and increased *Enterobacteriaceae*, which resulted in decreased production of cranberry polyphenol microbial metabolites in comparison to healthy microbiomes. Cranberry polyphenols also influence amino acid metabolism, as BCFA production was decreased. Future studies should include metabolomics data in experimental designs so that the importance of inter-individual differences in the efficiency of polyphenol metabolism can be realized.

CHAPTER IV

PROCYANIDIN DIMER METABOLISM BY THE COLON MICROBIOME IS ALTERED

BY DYSBIOSIS IN ULCERATIVE COLITIS *EX VIVO*

Introduction

Proanthocyanidins (PACs), also known as condensed tannins, are an ubiquitous class of polyphenols found in many popular global food commodities. Their estimated dietary consumption is 0.1-0.5 g per day and common dietary sources include apples, cranberries, cacao, peanuts, cinnamon, avocados, wine and tea (Smeriglio *et al*., 2017). Despite the abundance of PACs in the diet, PAC metabolism and physiological effects are still poorly understood. PACs are polymers of the flavonoid diastereomers, flavan-3-ols, and are formed through the condensation of flavan-3-ols through interflavan bonds. These bonds are typically between C4 and C8 of two unique monomer units but are also less commonly found between C4 and C6. PACs with this type of bond are referred to as 'b-type,' which is the most predominant PAC bond type found in fruits and botanicals. By contrast, foods such as cranberry, peanuts and cinnamon contain the less common 'a-type' PACs which are differentiated by a second ether bond between C2 and C7 of two unique flavan-3-ol monomers. A-type PACs can contain 1 or more a-type linkages and both a-type and b-type linkages can occur in the same polymer (Gu *et al*., 2003).

Cranberries contain both a and b-type PACs, which through thiolysis have been identified as polymers of mainly (-)-epicatechin, but also (+)-catechin and (-)-epigallocatechin (Gu *et al*, 2003). A-type PACs from cranberry are speculated to mitigate urinary tract infections through anti-adhesion mechanisms against p-fimbriated, adherent-invasive *E. coli* (Howell *et al*., 2007).

However, due to their large size it is widely debated whether PACs have sufficient bioavailability to localize and exhibit efficacy in the urinary tract (Ou *et al*., 2012). Recently, due to their low absorption in the small intestine and subsequent localization in the large intestine, more focus has been put on the ability of PACs and their colon microbiome metabolites to promote intestinal health by stimulating the growth of symbionts, maintaining the structure and promoting the production of mucins and by down-regulating intestinal inflammation (Anhe *et al*., 2015).

Ulcerative colitis (UC) is a form of inflammatory bowel disease characterized by inflammation and ulcerations in the distal colon (Kostic, Xavier & Gevers , 2014). A repercussion of UC is bacterial dysbiosis within the colon, or a microbiota composition that deviates from that of healthy individuals. Common trends in bacterial dysbiosis of those with UC have been observed, and this includes decreased numbers of *Bifidobacterium* and *Lactobacillus spp.*, decreased alpha-diversity, increased Proteobacteria and the presence of adherent-invasive *E.coli* (Kostic, Xavier & Gevers, 2014). These bacterial deviations have shown to negatively impact metabolism by decreasing the metabolomic output of UC microbiomes (Van Nuenen *et al*., 2004).

Static *ex vivo* fermentation models have been utilized to elucidate metabolites that are derived from polyphenol metabolism, and not those produced by the host. Although several authors have been able to identify novel metabolites of PACs and outlined a tentative pathway for their catabolism, few studies have identified changes in the parent or precursor PACs throughout their metabolism (Sanchez-Patan *et al*., 2015, Roowi *et al*., 2010). Further, studies describing the metabolism of a-type PACs such as those by Engemann *et al*., (2012) and Ou *et al*., (2014) are limited. In chapter III it was demonstrated that polyphenol metabolism is

dependent on health status and microbial dysbiosis in UC significantly decreased polyphenol microbial metabolite production by the microbiome. For these reasons, the comparison of the metabolism of cranberry a and b-type PACS between healthy and UC microbiomes was evaluated, with a focus on parent PAC metabolism.

Materials and Methods

Chemicals

LC-MS standards of (-)-epicatechin, procyanidin B2, 3-hydroxyphenylacetic acid (MPA), 3,4-dihydroxyphenylacetic acid (34PA) and 3-(3,4-dihydroxyphenyl)propionic acid (34PP) were acquired from Sigma Aldrich (St. Louis, MO, USA). Standards of 3-(3-hydroxyphenyl)propionic acid (MPP), 3,4-dihydroxybenzoic acid (34BA) and 3-hydroxybenzoic acid (MBA) were acquired from Alfa Aesar (Tewksbury, MA, USA). R-3,4-dihydroxyphenyl-gamma-valerolactone (34PV) was purchased from Toronto Chemicals (North York, ON, Canada). Procyanidin A2 was purchased from Extrasynthese (Lyon, France). LC-MS grade mobile phases were also purchased from Sigma Aldrich and formic acid was purchased from Fisher Scientific (Hampton, NH, USA).

Recruitment of Subjects

This clinical protocol was approved by the Texas A&M University Institutional Review Board (TAMU IRB# 2017-0568D) and is the same protocol from chapter III. Healthy subjects qualified if they were 18-65 years of age and had no history of chronic diseases. Subjects were excluded from the healthy group if they had a history of alcohol or substance abuse, had recurrent hospitalizations, have had seizures, taken antibiotics in the last 6 months, were lactose intolerant, gluten sensitive/had celiac disease, smoked more than one pack of cigarettes a week, had renal or liver dysfunction, and if female were currently pregnant or lactating. The same

inclusion and exclusion criteria applied to UC subjects, however UC subjects additionally reported the severity of their disease as diagnosed by a physician.

Fermentation of Procyanidin A2 and B2

A stool sample was collected from a recruited healthy subject and within 2 hr of defecation the fecal sample was further processed inside an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI, USA) held at 37°C and regulated with nitrogen, hydrogen (5%), and carbon dioxide (5%). Resazurin strips (Sigma Aldrich, St. Louis, MO, USA) were utilized to confirm anaerobic conditions inside the chamber during fermentations. A fecal slurry was produced by mixing 5 g of feces with 50 mL of pre-reduced and sterile (PRAS) pH 7.5 phosphate buffered saline with added sodium thioglycolate and L-cysteine (Anaerobe Systems, Morgan Hill, CA, USA). 5 mg of either procyanidin A2 or B2 were solubilized in 18 mL fecal fermentation media formulated as described by Tzounis *et al.*, 2008 (2.0 g peptone water, 0.5 g bile salts, 2.0 g yeast extract, 0.5 g L-cysteine, 0.05 g haemin, 0.01 mL vitamin K, 0.001 g resazurin, 0.01 g $CaCl_2 * 6 H_2O$, 0.01.g $MgSO_4 * 7 H_2O$, 0.04 g KH_2PO_4, 0.04 g K_2HPO_4, 0.10 g NaCl, 2.0 g $NaHCO_3$, 2.0 mL tween 80 per 1 L) and produced by Anaerobe Systems (Morgan Hill, CA, USA). To simulate microbiome metabolism of procyanidin dimers, 2 mL of fecal slurry was added to the fermentation vessels. 18 mL of media and 2 mL of fecal slurry was prepared to serve as a control. Samples were fermented for 48 hr and 1 mL aliquots were taken after 0, 6, 12, 24 and 48 hr and acidified with 5 µL of formic acid. All aliquots were stored at -80ºC until analysis for small and large molecular weight metabolites and metabolism of parent compounds.

Preparation of High-Tannin Cranberry Extract

Cranberry extract was prepared according to the extraction method outlined in chapter III. Total soluble polyphenolics was determined by the Folin-Ciocalteu assay and total PACs were determined by the 4-dimethylaminocinnamaldehyde (DMAC) assay (Singleton, Orthofer & Lamuela-Raventos, 1999, Prior *et al*. 2010).

Fermentation of High-Tannin Cranberry Extract

The high-PAC cranberry extract was fermented with fecal microorganisms to simulate microbiome metabolism of PACs according to the method outlined in chapter III. A control fermentation where there was no polyphenols added was ran in parallel. Aliquots of ferment were removed after 0, 6, 12, 24 and 48 hr and acidified with 5 µL of formic acid to quantify changes in the concentration of PACs via LC-ESI-MS/MS and to identify novel large-molecular weight metabolites.

Preparation of Fermentation Aliquots for LC-ESI-MS/MS Analysis

Acidified aliquots (250 µL) from each time point in the fermentation was mixed with 50 µL of 25 mg L_{-1} of ethyl gallate as an internal standard. The solution was then diluted with 300 µL 0.1% formic acid. A 200 mg C18 column (Waters) was conditioned with 1 mL of MeOH and 1.5 mL of 0.1% formic acid before the solution was loaded onto the column. The cartridge was washed with 1.5 mL of 0.1% formic acid and eluted with 750 µL of 0.1% formic acid in MeOH. Each fermentation aliquot was extracted in triplicate for each time point. The control fermentation samples that did not contain polyphenols were extracted in the same way, except the internal standard was replaced with 0.1% formic acid, in order to control for matrix effects during LC-ESI-MS/MS analysis.

LC-ESI-MS/MS of Procyanidin A2 and B2 Metabolites

Quantification of procyanidin A2 and B2 metabolites produced during fecal fermentations was analyzed via an Ultimate3000 UPLC equipped to a Thermo Scientific TSQ Quantiva mass spectrometer with an ESI source as described in chapter III.

Analysis of Proanthocyanidins and Large Molecular Weight Proanthocyanidin Metabolites

Cranberry PACs were analyzed via a Thermofinnigan LCQ Deca LC-MS equipped with an ESI source and an ion trap as the mass analyzer. Compounds were separated on a Kinetex C18 column (2.6 µM, 4.6 x150 mm) with 0.1% formic acid as mobile phase A and 0.1% formic acid in methanol as mobile phase B. The flow rate was 450 µL min^{-1} and gradient elution began with 90% A and 10% B for 5 min, decreased to 70% A after 40 min, decreased to 10% A after 45 min, increased to 90% after 50 min and the column was equilibrated for an additional 5 min with isocratic conditions before injection of another sample. MS was run in the negative ionization mode with a capillary temperature of 320°C, source temperature of 350°C and sheath gas and auxiliary gas of 30 and 10 arbitrary units, respectively. Source parameters and collision energy were optimized utilizing an A-type PAC trimer in cranberry extract. The mass range was set from *m/z* 150-1500. Due to the lack of available standards, PACs were quantified in (-)-epicatechin equivalents (Robbins *et al.*, 2009).

Statistics

Metabolites of procyanidin A2 and B2 were compared utilizing a two-way ANOVA, where time of fermentation, type of procyanidin and their interaction were factors. Cranberry PAC metabolism was compared between healthy and UC microbiomes at each hr of fermentation using the two-sample Mann Whitney U test. Significance was designated if $P < 0.05$.

Comparison of the Metabolism of Procyanidin A2 and B2 by Fecal Microorganisms

Procyanidin A2 and B2 are both PAC dimers of (-)-epicatechin found in cranberry, and procyanidin A2 only differs structurally by an additional ether bond between C2 and C7 of the two (-)-epicatechin monomers. However, this additional bond dramatically changed PAC dimer metabolism by the microbiome into both phenolic acid metabolites and metabolites that have a molecular weight >290 amu, referred to by others as dimeric metabolites (Stoupi *et al.*, 2010b) (Figure 26). Fermentation of procyanidin B2 with human fecal microorganisms resulted in a larger number of targeted metabolites over the 48-hour fermentation period in comparison to procyanidin A2. After 6 hr, procyanidin B2 resulted in the production of 55.26 ± 1.34 µM of 34PV, 10.96 ± 0.46 µM of MPP, 84.69 ± 4.78 µM of 34PA, and 13.69 ± 0.36 µM of MBA. Benzoic acid was the only targeted metabolite detected after 6 hr of fermentation of procyanidin A2 with 0.10 ± 0.07 µM detected (Table 5).

34PV is a unique microbial metabolite of flavan-3-ols and PAC dimers, as it has not been identified as a metabolite of any other flavonoid or phenolic acid post-*ex vivo* fermentation with fecal microorganisms. 34PV was produced in significantly higher ($P<0.05$) concentrations from procyanidin B2 after 6 hr. After 12 hr, 34PV was still detected from fermentation of procyanidin B2 with 30.33 ± 0.81 µM, but was not detected again thereafter (Table 5). 34PV was not detected after fermentation of procyanidin A2 from 0-48 hr, which is in accordance with the findings of Engemann et al., (2012) who incubated procyanidin A2 isolated from lychee with porcine cecum microorganisms and did not find 34PV. 34PV has been postulated to be derived from (-)-epicatechin through reduction of the C-ring into 1-(3',4'-dihydroxy phenyl)-3-(2",4",6"-trihydroxy phenyl)-propan-2-ol and subsequent lactonization (Stoupi *et al.*, 2010a). It has been

suggested that 34PV could be derived from procyanidin B2 through breakage of the interflavan

bond and production of (-)-epicatechin monomer, estimated to account for ~10% of procyanidin
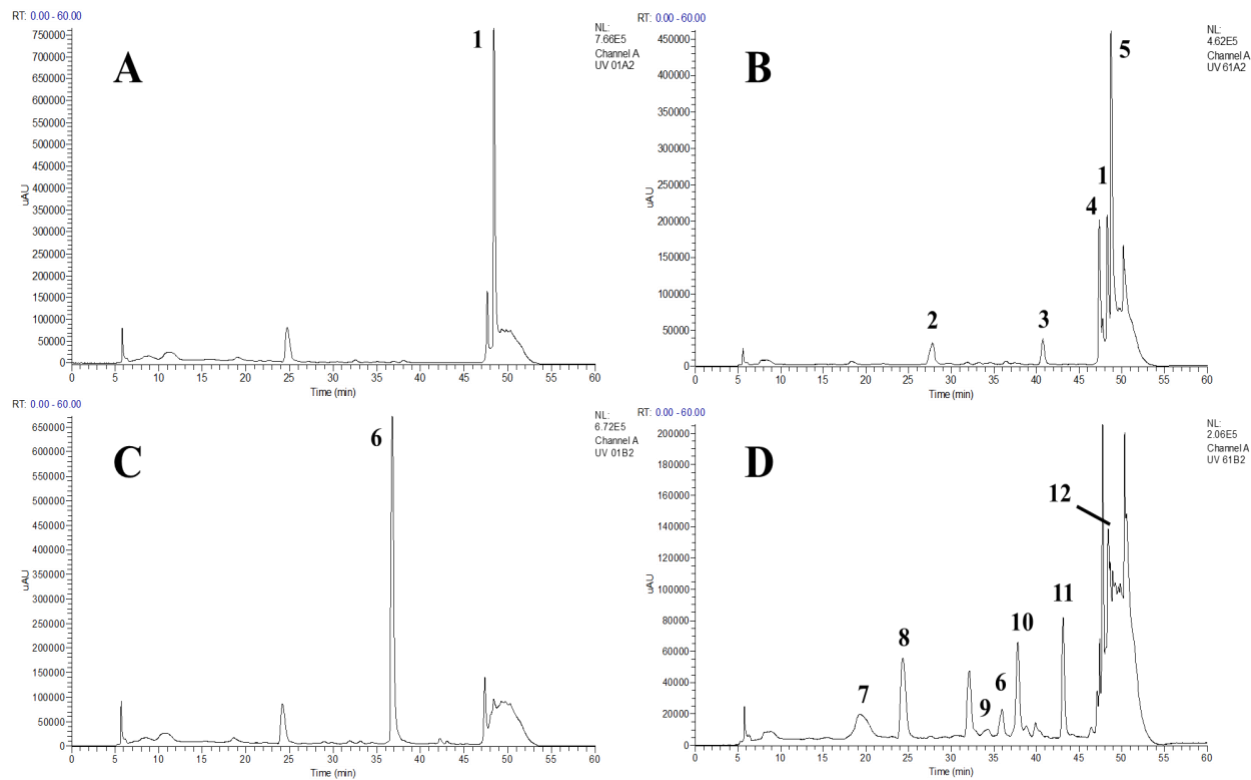
Figure 26 Comparison of the metabolism of procyanidin A2 (A&B) and B2 (C&D) after 0 (A&C) and 6 hours (B&D) of fermentation with fecal microorganisms.
(1) Procyanidin A2, (2) Procyanidin A2 epimer 1, (3) Procyanidin A2 epimer 2, (4) Procyanidin A2 epimer 3, (5) Procyanidin A2 microbial dimer, (6) Procyanidin B2, (7) (+)-catechin, (8) 34PP, (9) Procyanidin B2 epimer, (10) 34PV, (11) 3,4-dihydroxyphenyl-trihydroxy phenyl propan-2-ol, (12) Procyanidin B2 microbial dimer.

Table 5 Concentrations of microbial metabolites produced from procyanidin A2 (A2) and procyanidin B2 (B2) authentic standards after 48 hours of fermentation with fecal microorganisms. Data is reported as mean ± SEM µM and differing letters indicate significant differences between concentrations of metabolites at each hour and whether the metabolite was derived from A2 or B2. ND=Not detected.

Microbiome Metabolite	Concentration (µM)									
	0 hr		6 hr		12 hr		24 hr		48 hr	
	A2	B2	A2	B2	A2	B2	A2	B2	A2	B2
-	964.45 ± 115.91	1,172.45 ± 52.33	102.82 ± 19.04	30.02 ± 4.71	32.69 ± 3.39	ND	ND	ND	ND	ND
34PV	ND	ND	ND	55.26 ± 1.34 a	ND	30.33 ± 0.81 b	ND	ND	ND	ND
34PP	ND	ND	ND	ND	ND	ND	4.77 ± 0.24 a	ND	ND	ND
MPP	ND	ND	ND	10.96 ± 0.46 c	3.63 ± 0.08 d	11.58 ± 0.49 c	120.26 ± 2.04 a	ND	19.69 ± 0.28 b	2.38 ± 0.17 d

Table 5 Continued

Microbiome Metabolite	Concentration (µM)									
	0 hr		6 hr		12 hr		24 hr		48 hr	
	A2	B2	A2	B2	A2	B2	A2	B2	A2	B2
34PA	0.64 ± 0.50 c	ND	ND	84.69 ± 4.78 b	ND	105.04 ± 2.01 a	ND	0.74 ± 0.57 c	0.51 ± 0.39 c	2.61 ± 0.06 c
MPA	ND	ND	ND	13.69 ± 0.36 d	ND	54.39 ± 1.76 c	ND	140.08 ± 1.20 b	ND	157.16 ± 3.76 a
34BA	0.13 ± 0.10 a	ND	ND	ND	ND	ND	ND	ND	ND	ND
MBA	0.70 ± 0.55 a	ND	ND	ND	ND	ND	ND	ND	ND	ND
BA	ND	ND	0.10 ± 0.07 ns	ND	ND	ND	0.70 ± 0.38 ns	ND	0.02 ± 0.01 ns	ND

B2 metabolism by the microbiome (Stoupi *et al*., 2010a, Appeldorn *et al*., 2009). Stoupi *et al*., (2010a) was able to identify free (-)-epicatechin as a metabolite of procyanidin dimers. However, others have not been able to detect produced (-)-epicatechin. Instead of (-)-epicatechin, its diastereomer (+)-catechin was identified after 6 hr of fermentation of procyanidin B2. The a-type linkage can therefore hinder the conversion of procyanidin A2 into free (-)-epicatechin and its subsequent metabolism into 34PV.

MPP was present after fermentation of both procyanidin A2 and B2 after 12 hr with 3.63 $\pm$ 0.08 and 11.58 $\pm$ 0.49 µM produced, respectively (Table 5). MPP reached a maximum concentration after 24 hr for procyanidin A2 with 120.26 $\pm$ 2.04 µM, which was significantly higher ($P<0.05$) than the concentration at any other time point and after fermentation of procyanidin B2. By contrast, the maximum concentration of procyanidin B2 was reached after 12 hr with 11.58 $\pm$ 0.49 µM. MPP has been hypothesized to be a dehydroxylated derivative of 34PP (Monagas *et al*., 2012), which was only found after 24 hr of fermentation of procyanidin A2 and was never detected in procyanidin B2 ferments. Stoupi *et al*., (2010a) detected 34PP 24-48 hr after fermentation of procyanidin B2 and (-)-epicatechin and MPP after 6-9 hr of incubation of procyanidin B2 and (-)-epicatechin. By contrast, Appeldorn *et al*., (2009) proposed that MPP is derived from dehydroxylated 34PV, as 34PP was not detected after fermentation of isolated grape seed PAC dimers and trimers. Further, MPP has also been identified as a metabolite of the amino acid tyrosine, which could be present in the proteins contained in the media (Windey, De Preter & Verbeke, 2012). This could suggest that 34PP is difficult to detect due to its rapid metabolism into MPP and subsequent metabolites or rather that MPP may be produced from an alternative pathway such as through dehydroxylation of 34PV or amino acid metabolism.

Phenyl acetates have been identified as major metabolites of flavan-3-ol monomers and procyanidin b-type dimers and their production was compared between procyanidin A2 and B2. 34PA reached its maximum concentration after 12 hr of fermentation of procyanidin B2 with 105.04 ± 2.01 µM produced (Table 5). 34PA was produced in significantly lower ($P<0.05$) concentrations after fermentation of procyanidin A2 reaching a maximum concentration after 48 hr of 0.51 ± 0.39 µM. 34PA has been hypothesized to be derived from the B-ring of the extension unit of procyanidin B2 or through alpha-oxidation of 34PP (Appeldoorn *et al.*, 2009, Ou *et al.*, 2014). The former mechanism is more plausible considering the lack of production of 34PP produced from procyanidin B2, and the additional ether bond between C2 and C7 between (-)-epicatechin units in procyanidin A2 may prevent production of 34PA from the B-ring and explains the lower concentrations detected after fermentation of procyanidin A2. The dehydroxylated derivative of 34PA, MPA reached its maximum concentration after fermentation of procyanidin B2 after 48 hr with 157.16 ± 3.76 µM. This could suggest that MPA is derived from 34PA considering that the concentrations of MPA produced from procyanidin B2 are comparable on a molar basis to that of 34PA and that 34PA was absent after fermentation of procyanidin A2.

Protocatechuic acid and 3-hydroxy benzoic acid have been proposed as alpha or beta-oxidation derivatives of phenyl acetates and propionates, respectively (Monagas *et al.*, 2012, Roowi *et al.*, 2010). However, neither compound was detectable in either procyanidin A2 or procyanidin B2 fermentations, suggesting that these are unlikely metabolites of these compounds and may be derived from other polyphenols (Table 5) (Sanchez-Patan *et al.*, 2015).

Two dimeric microbial metabolites were also identified that were unique derivatives of both procyanidin A2 and B2. An ion with a *m/z* of 577 and MS2 fragments of 451 and 291 reached a maximum concentration of 2,058.94 ± 73.81 µM after 6 hr and then decreased from 12-24 hr until the compound was not detectable after 48 hr (Figure 27 & 28). This compound was unique to procyanidin A2 and was not detected after fermentation of procyanidin B2. It has previously been identified by Engemann *et al*. (2012) as procyanidin A2 that has undergone C-ring fission in the terminal (-)-epicatechin unit, indicated by the fragment ion of *m/z* 291. Additionally, 3 procyanidin A2 epimers that also had a *m/z* of 575 were identified and were present in the fermentation from 6-24 hr (Figure 29). At physiological pH, PACs undergo conformational changes into epimers of the same polymer size (Lu *et al*., 2011). By contrast, no epimers of procyanidin B2 could be identified that had a signal to noise ratio greater than 5. There were 20 dimeric metabolites previously identified after fermentation of procyanidin B2 with fecal microorganisms, but only 1 was identified in this study (Stoupi *et al*. 2010b). A dimeric metabolite with a *m/z* of 579 and MS2 fragments of *m/z* 453, 409 and 291 was detected after fermentation of procyanidin B2 and was not detected in procyanidin A2 fermentations (Figure 30). This compound reached its maximum concentration of 58.20 ± 6.77 µM after 6 hr and was not detectable thereafter (Figure 31). This dimeric metabolite has been identified as a metabolite of B2 that has undergone C-ring fission in the extension unit (*m/z* 291), although the location of the reduced C-ring has not been confirmed (Stoupi *et al*., 2010b). Fragment ions of *m/z* 453 and 209 are also characteristic of PAC dimers that have fragmented through the Retro-Diehl's Alder reaction (*m/z* 409) and loss of phloroglucinol (*m/z* 453). It is debated whether or not procyanidin dimers are bioavailable, as there is contradictory evidence of their absorption through colonocytes *in vitro* and identification in plasma and urine *in vivo* (Shoji *et al*., 2006,

Baba *et al*., 2002). Dimeric metabolites not only differentiate the microbiome metabolism between procyanidin A2 versus B2, but also could explain why procyanidin dimers are not found in urine and plasma *in vivo*. More likely, PAC dimers may be rapidly metabolized into phenolic acid metabolites, especially procyanidin B2, or in the case of procyanidin A2, rapidly metabolized into dimeric, large molecular weight metabolites or undergo epimerization.
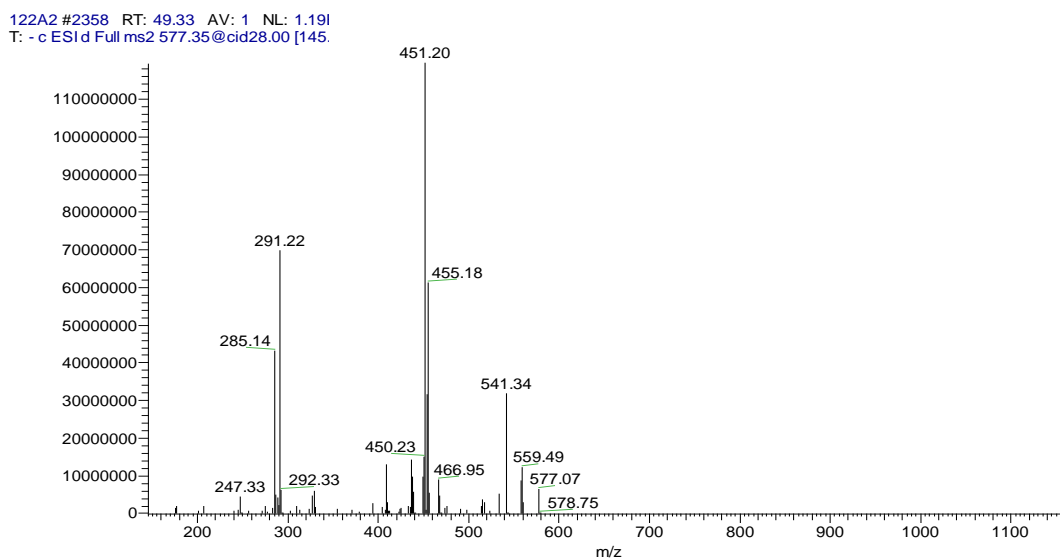
Figure 27 MS₂ fragmentation of an ion peak with m/z 577 produced after fermentation of procyanidin A2 with fecal microorganisms.
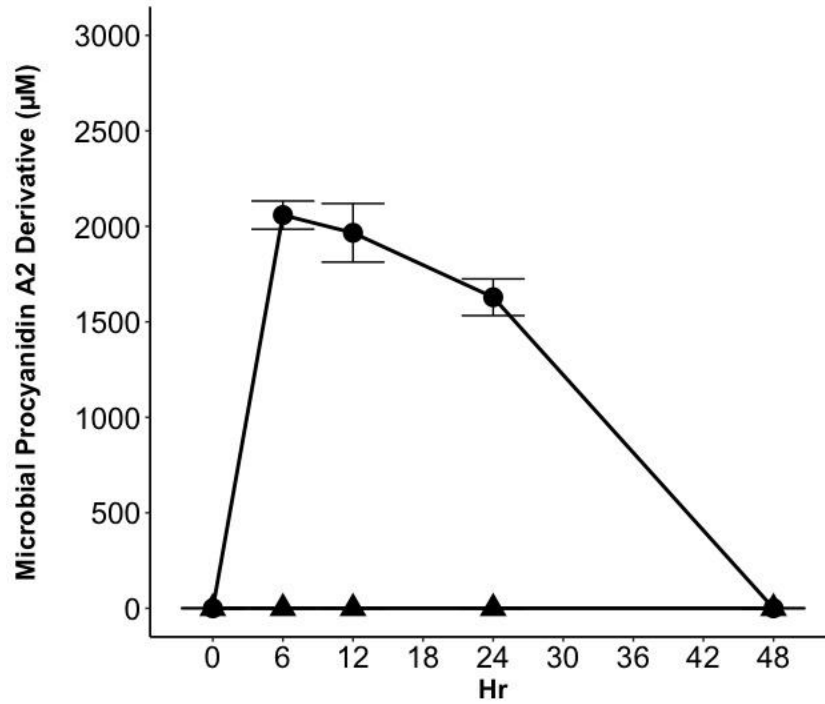
Figure 28 Concentrations of dimeric microbial metabolite with an ion peak of m/z 577 produced after fermentation of procyanidin A2 (circle) and procyanidin B2 (triangle) with fecal microorganisms. Data is presented as mean ± SEM µM and concentrations are expressed in (-)-epicatechin equivalents.
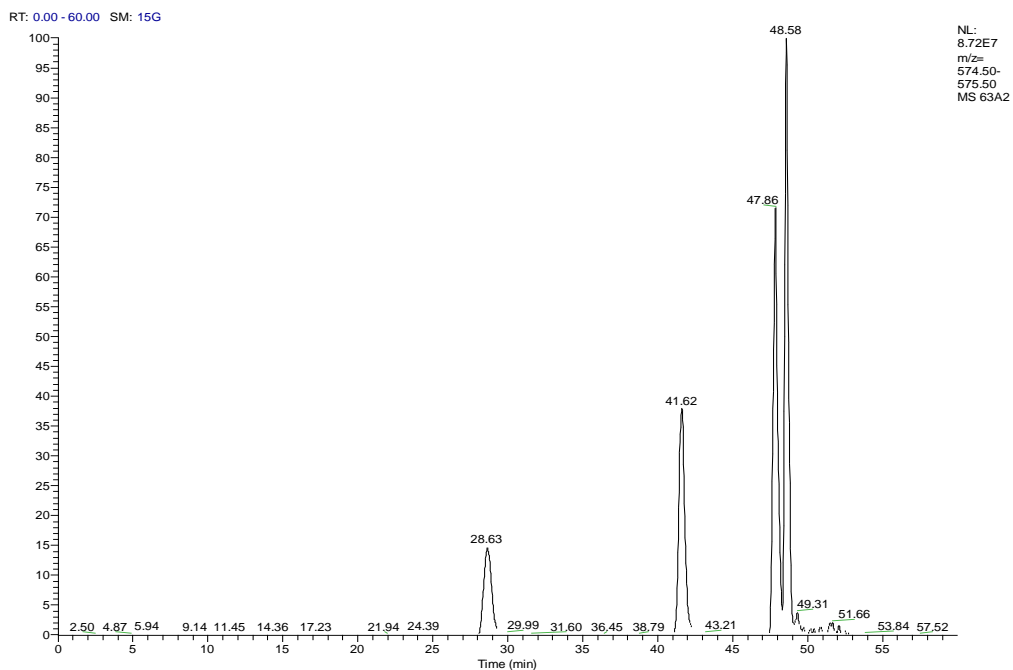
Figure 29 Extracted ion chromatogram of compounds that have an ion of m/z 575. Peaks with retention times of 28.63, 41.62 and 47.86 min are epimers of procyanidin A2 (48.58 min).
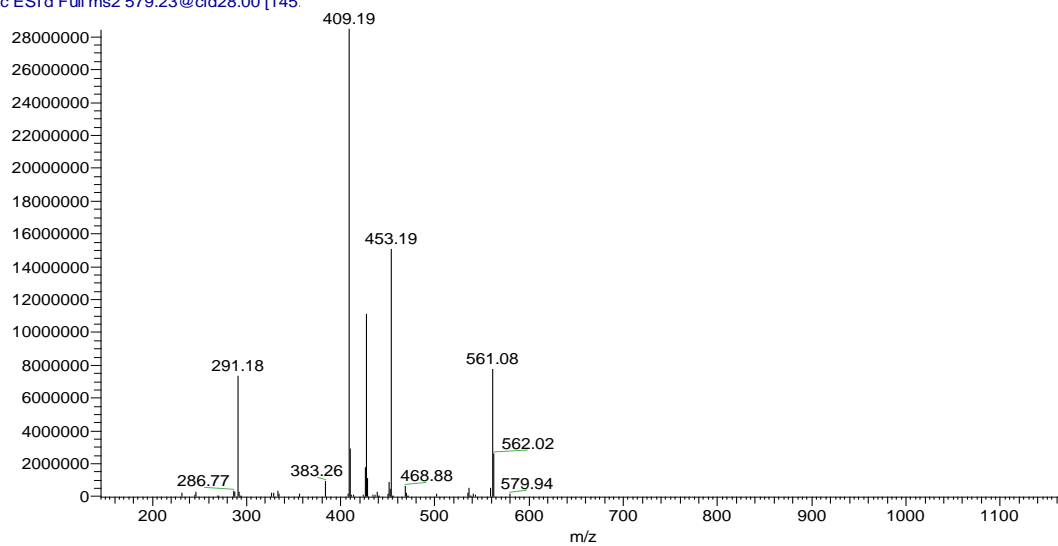
Figure 30 MS₂ fragmentation of an ion peak with m/z 579 produced after fermentation of procyanidin B2 with fecal microorganisms.
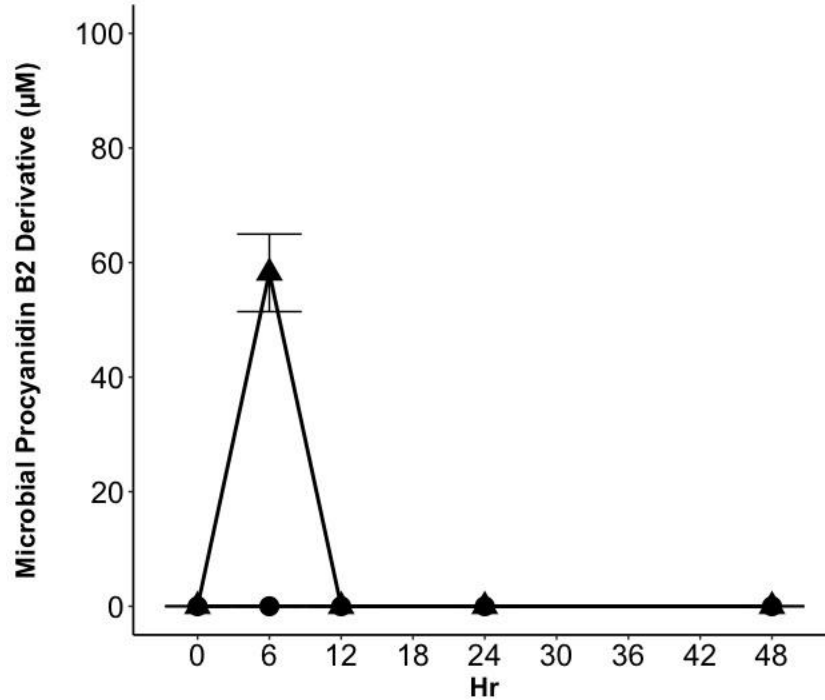
Figure 31 Concentrations of dimeric microbial metabolite with an ion peak of m/z 579 produced after fermentation of procyanidin A2 (circle) and procyanidin B2 (triangle) with fecal microorganisms. Data is presented as mean ± SEM μM and concentrations are expressed in (-)-epicatechin equivalents.

Comparison of the Metabolism of Cranberry Proanthocyanidins between Healthy and IBD

Microbiomes

A high-PAC cranberry extract was fermented with human fecal microorganisms of either healthy or UC donors to elucidate how bacterial dysbiosis alters PAC metabolism. Identification and characterization of each PAC in the cranberry extract is presented in Table 6. Neither procyanidin A2 nor procyanidin B2 was detected after 6 hr of fermentation with fecal microorganisms from healthy individuals (Figure 32 & 33). However, procyanidin A2 and B2 both persisted in fermentations with fecal microorganisms from individuals with UC and both

compounds were still detectable after 48 hr. It has been found that procyanidin B2 and A2 require up to 24 hr to be metabolized by healthy donors (Ou *et al*., 2014, Stoupi *et al.* 2010a), but these results indicate a faster catabolic response. The majority of procyanidin A2 and B2 were metabolized by healthy donors within the first 6 hr both in the cranberry extract and as authentic standards. The faster metabolism in the cranberry extract may be due to the lower concentration of procyanidin A2 and B2 present in the fermentation (62.59-66.58 µM) in comparison to the authentic standards (964.45 – 1172.45 µM). (-)-Epicatechin present in the cranberry extract at 0 hr was also not detected in the healthy group after 6 hr, but was still present in the UC group with 4.94 ± 4.94 µM remaining after 48 hr (Figure 34). These results are in accordance with Ou *et al.* 2014 who found that (-)-epicatechin was not detectable after 6 hr of fermentation. Overall, microbial dysbiosis results in decreased or inefficient metabolism of procyanidin dimers and monomers.

Table 6 LC-ESI-MS/MS identification and characterization of proanthocyanidins (PACs) in cranberry extract fermented with fecal microorganisms from healthy and UC fecal donors.

Proanthocyanidin	Degree of Polymerization	[M-H]-	MS$_2$
(-)-Epicatechin	1	289	245
Procyanidin A2	2	575	449, 423, 289
Procyanidin B2	2	577	451, 425, 407, 289
A-Type PAC Trimer 1 (Epi-A-Epi-Epi)	3	863	711, 573, 451, 411, 289
A-Type PAC Trimer 2 (Epi-Epi-A-Epi)	3	863	711, 575
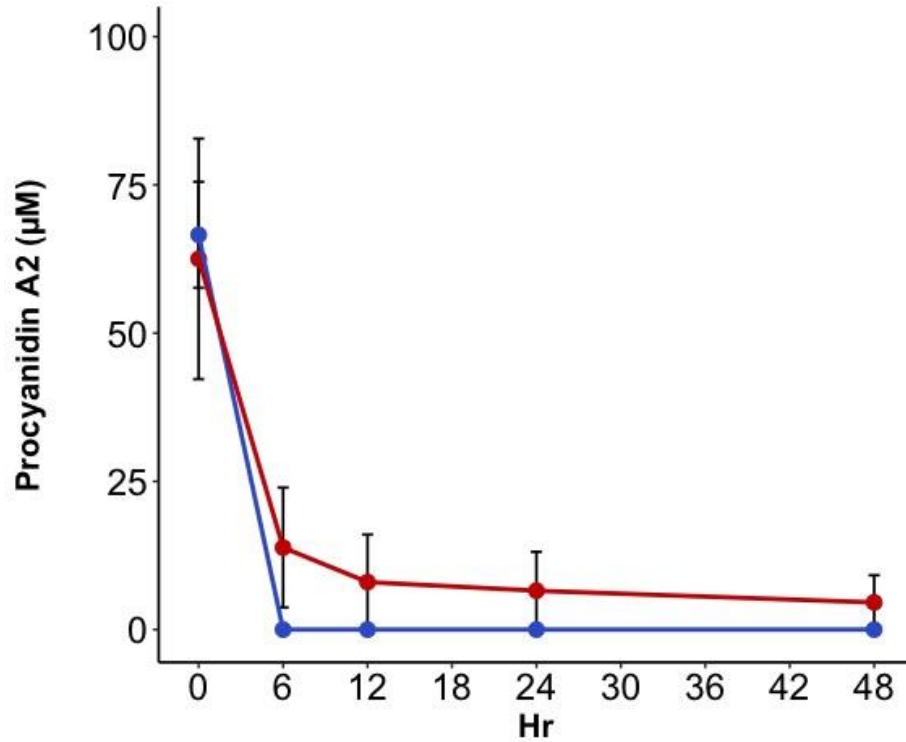
Figure 32 Comparison of the metabolism of procyanidin A2 between healthy (blue) and UC (red) fecal microbiomes over 48 hours of fermentation with a high-PAC cranberry extract. Data is presented as mean ± SEM μM. There were no significant differences (P<0.05) in the concentration of procyanidin A2 between groups at any hour.
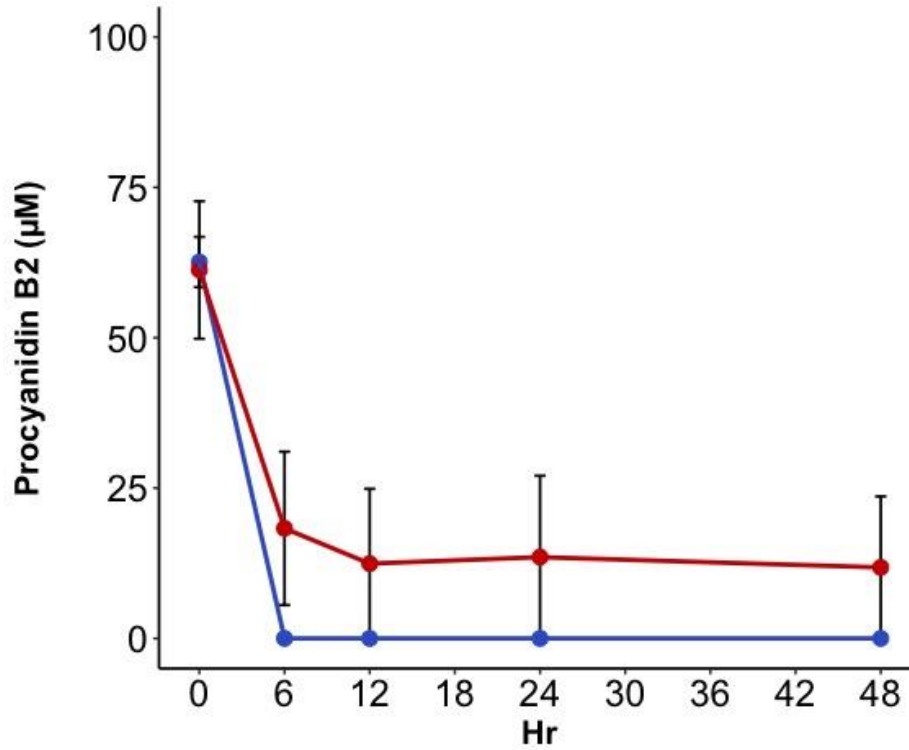
Figure 33 Comparison of the metabolism of procyanidin B2 between healthy (blue) and UC (red) fecal microbiomes over 48 hours of fermentation with a high-PAC cranberry extract. Data is presented as mean ± SEM μM. There were no significant differences (P<0.05) in the concentration of procyanidin B2 between groups at any hour.
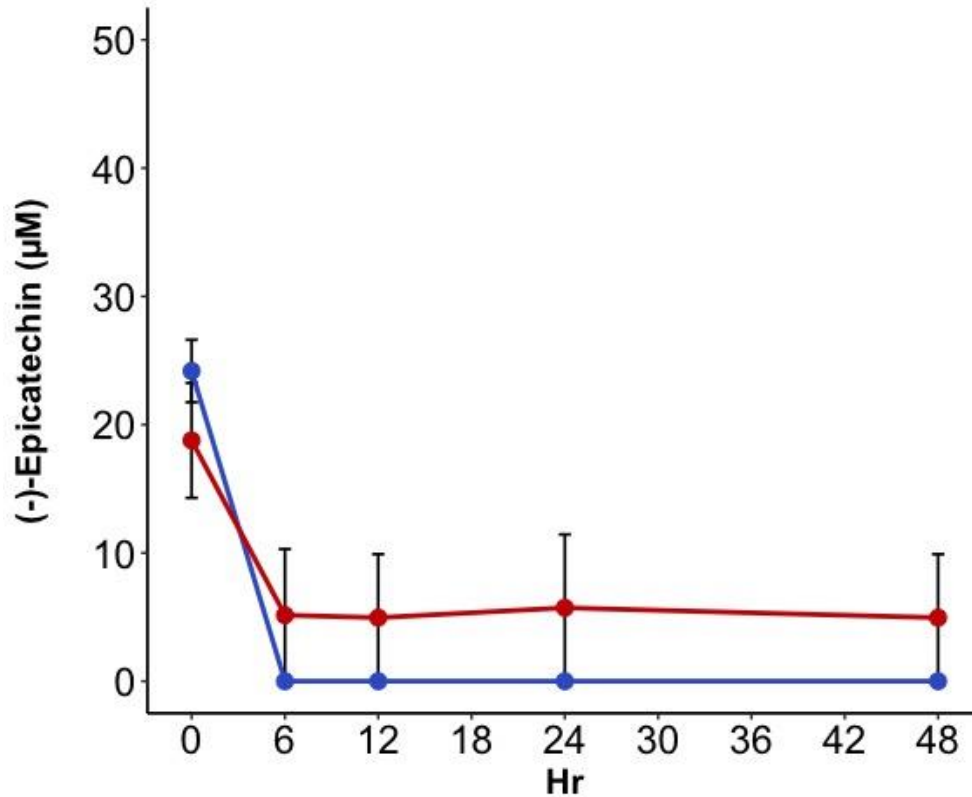
Figure 34 Comparison of the metabolism of (-)-epicatechin between healthy (blue) and UC (red) fecal microbiomes over 48 hours of fermentation with a high-PAC cranberry extract. Data is presented as mean ± SEM μM. There were no significant differences (P<0.05) in the concentration of (-)-epicatechin between groups at any hour.

The inefficient metabolism of procyanidin dimers by UC individuals corresponded to the production of dimeric microbial metabolites of procyanidin A2 and B2 (Figures 35 & 36). Healthy microbiomes produced higher concentrations of procyanidin A2 dimeric metabolite (*m/z* 577) from 6-48 hr. For both groups, this compound reached its maximum concentration after 12 hr with 238.11 ± 45.75 μM for the healthy group and 122.53 ± 46.44 μM for the UC group. The concentration of the procyanidin A2 dimeric metabolite began to decrease after 12 hr but was

still present after 48 hr in both healthy and UC fermentations with $102.32 \pm 27.08\ \mu M$ and $34.42 \pm 19.95\ \mu M$, respectively. By contrast, the procyanidin B2 dimeric metabolite reached its maximum concentration of $30.28 \pm 9.23\ \mu M$ in the healthy group after 6 hr and was not detected again after 12 hr (Figure 35 & 36). This demonstrates that procyanidin B2 is more easily metabolized than procyanidin A2 by healthy microbiomes. However, the dimeric procyanidin B2 metabolite reached its maximum concentration in the UC group of $33.73 \pm 22.63\ \mu M$ after 12 hr and was still detected after 48 hr with $9.67 \pm 6.33\ \mu M$. This indicated that metabolism of procyanidin A2 and B2 into dimeric intermediates was impacted by bacterial dysbiosis in UC. Differences in concentrations and rate of production of procyanidin A2 and B2 dimeric metabolites could explain the differences in concentrations of low molecular weight phenolic acid metabolites between healthy and UC microbiomes that were observed in chapter III.
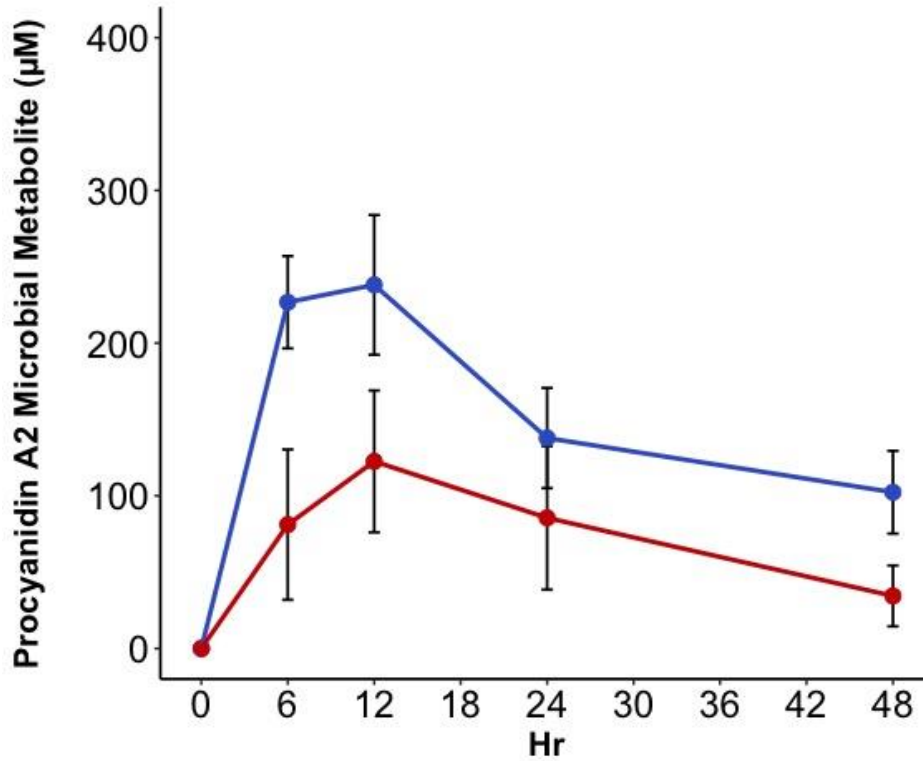
Figure 35 Comparison of the production of the procyanidin A2 dimeric metabolite (m/z 577) between healthy (blue) and UC (red) fecal microbiomes over 48 hours of fermentation with a high-PAC cranberry extract. Data is presented as mean ± SEM μM. There were no significant differences (P<0.05) in the concentration of the dimeric metabolite between groups at any hour.
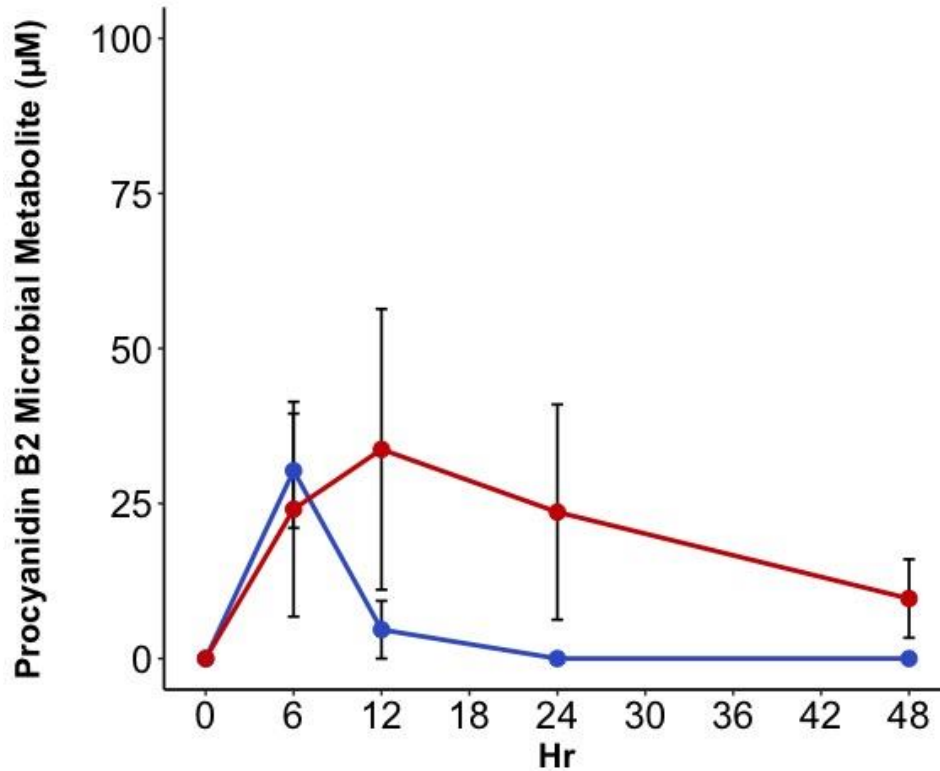
Figure 36 Comparison of the production of the procyanidin B2 dimeric metabolite (m/z 579) between healthy (blue) and UC (red) fecal microbiomes over 48 hours of fermentation with a high-PAC cranberry extract. Data is presented as mean ± SEM μM. There were no significant differences (P<0.05) in the concentration of dimeric metabolite between groups at any hour.

There were 2 a-type trimers identified in the cranberry extract (Table 6). The first eluting a-type trimer had an ion of *m/z* 863 and MS_2 fragments of *m/z* 711 and 573, indicating that the a-type bond is in the extension unit (Tarascou *et al*., 2011). The later eluting trimer had an ion of *m/z* 863 and MS_2 fragment ions of *m/z* 711 and 575 indicating that the a-type bond is in the terminal unit (Tarascou *et al*., 2011). There were no significant differences (P<0.05) in their rate of metabolism between healthy and UC microbiomes (Figure 37 & 38). 33.33% of the trimer

with the a-type bond in the extension unit was metabolized in the first 6 hr compared to 54.00% of the trimer with the a-type bond in the terminal unit, which could indicate that a-type bond position can impact rate of metabolism. More than 80.00% of both trimers were metabolized after 24 hr for both the healthy and UC group, in comparison to the dimers which were both completely metabolized by the healthy group within 6 hr. Evidence of depolymerization, as seen with procyanidin B2, was also not apparent with PAC trimers. Although a-type dimers not originally present in the cranberry extract were produced during fermentation with fecal microorganisms, it is more likely that these were epimers of procyanidin A2. This indicates that as degree of polymerization increases, PAC residence time in the large intestine could increase, as it is more difficult to metabolize compounds with a higher degree of polymerization. Considering the lack of difference in the rate and extent of metabolism of trimers between UC and healthy microbiomes, it is unlikely that differences in phenolic acid metabolites that occurred in chapter III were a result of PAC trimer metabolism.
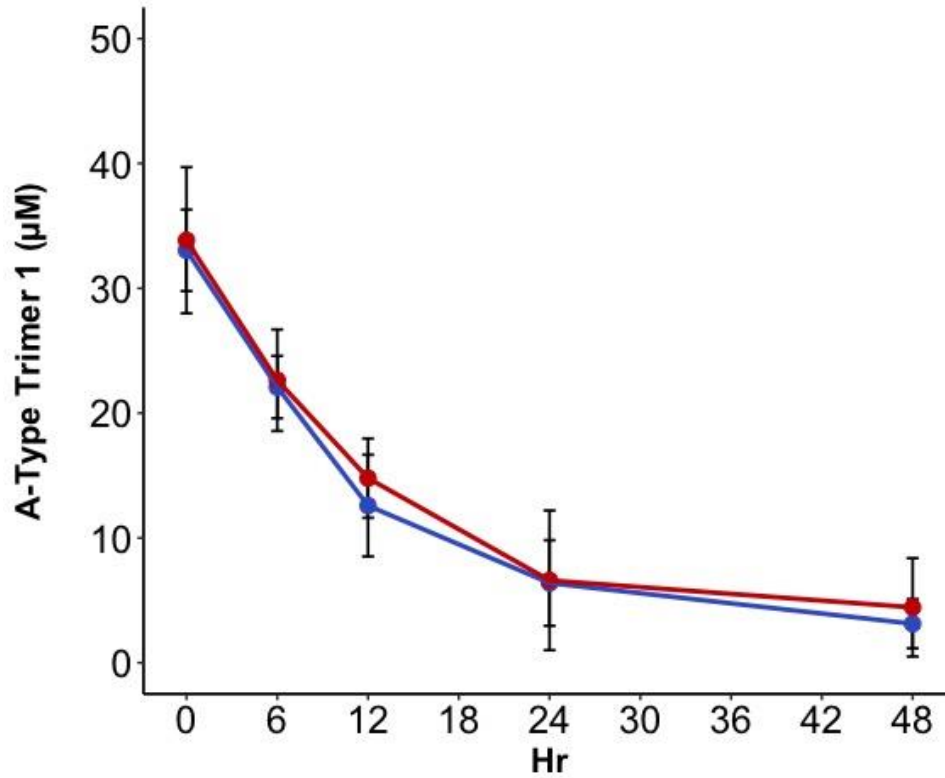
Figure 37 Comparison of the metabolism of an a-type trimer (a-type bond in extension unit) between healthy (blue) and UC (red) fecal microbiomes over 48 hours of fermentation with a high-PAC cranberry extract. Data is presented as mean ± SEM μM. There were no significant differences (P<0.05) in the concentration of the a-type trimer between groups at any hour.
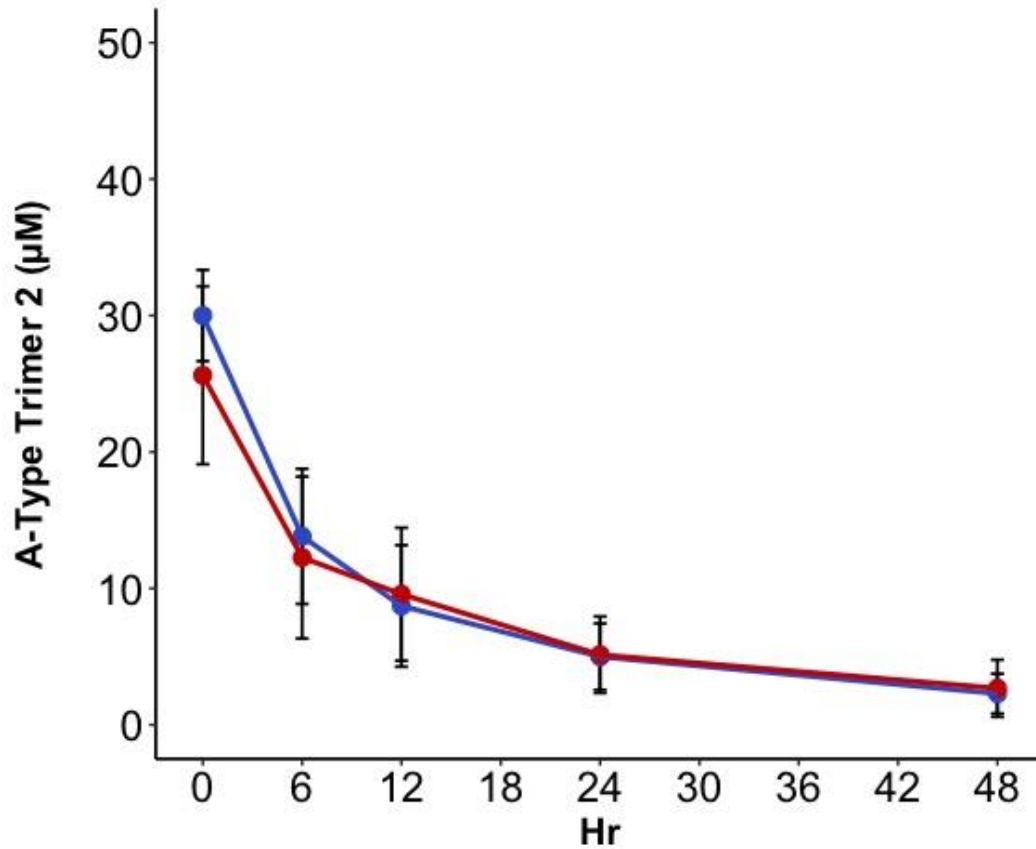
Figure 38 Comparison of the metabolism of an a-type trimer (a-type bond in terminal unit) between healthy (blue) and UC (red) fecal microbiomes over 48 hours of fermentation with a high-PAC cranberry extract. Data is presented as mean ± SEM μM. There were no significant differences (P<0.05) in the concentration of the a-type trimer between groups at any hour.

Conclusion

Cranberries contain both a and b-type PACs, which each have different routes of metabolism dependent on the degree of polymerization and interflavan bond type and position. Procyanidin B2 was more rapidly metabolized than procyanidin A2 by fecal microorganisms into a variety of phenolic acid metabolites. Procyanidin A2 was metabolized into a limited number of phenolic acid metabolites and instead its dimeric metabolite accumulated and resided in fermentations for a longer period of time than that of procyanidin B2. Differences in metabolism by UC and healthy microbiomes were more apparent comparing procyanidin dimers opposed to monomers and trimers, especially when comparing the rate of production of their dimeric metabolites. B-type PACs may then be more rapidly metabolized into bioavailable metabolites in comparison to a-type PACs, which more likely localize in the large intestine. Therefore a-type and b-type PACs may have differing distribution and locale where they exert their bioactivity.

CHAPTER V

FRACTIONATION OF CRANBERRY POLYPHENOLS REVEALS CLASS DEPENDENT

METABOLISM BY THE COLON MICROBIOME *EX VIVO*

Introduction

Cranberries are a "super fruit" native to North America that have presumed health-promoting benefits linked to their phytochemical composition. The use of cranberry as a medicinal food dates to folklore as early as the 1600's (Zhao, Liu & Gu, 2018). Presently, cranberries are known for their ability to mitigate urinary tract infections (UTI), whereas many over-the-counter medicines and remedies for UTI's contain cranberry as the active ingredient (Fu *et al*., 2017). Appreciable evidence also exists to suggest that cranberries may help prevent diabetes and cancer, down-regulate inflammation and promote cardiovascular and intestinal health (Zhao, Liu & Gu, 2018). Much of the reported bioactivity is attributed to the many classes of polyphenols contained in cranberry, but especially the a-type proanthocyanidins (PACs) that are less common and appear in foods such as peanuts, avocado and cinnamon (Smeriglio *et al*., 2017). The a-type proanthocyanidins in cranberry typically exist in combination with the more common b-type PACs and vary in size, or degree of polymerization (DP), ranging from 2-50 (Cires *et al*., 2017). Although PACs are the most predominant class of polyphenol found in cranberry, accounting for 41.5-52.2% of polyphenols, cranberries also contain high concentrations of several other classes of polyphenols, including phenolic acids (5-12.1%), flavonols (18.6-30.5%) and anthocyanins (8.0-24.4%) (Oszmianski *et al*., 2018). Commonly found phenolic acids in cranberry include the hydroxycinnamates chlorogenic acid and *p*-coumaryl glycoside isomers and the hydroxybenzoate, benzoic acid. The predominant flavonols

in cranberry are quercetin glycosides and the main anthocyanins are cyanidin and peonidin glycosides (Oszmianski *et al*., 2018).

Due to the low absorption of most polyphenols in the small intestine, it is estimated that as much as 90% of polyphenols consumed will arrive to the large intestine where they can be metabolized by the colon microbiome into bioavailable metabolites (Etxeberria *et al*., 2013). To elucidate polyphenol metabolism by the colon microbiome, static *ex vivo* fermentations of polyphenols with fecal microorganisms are commonly utilized. This model is advantageous because it eliminates absorption and metabolism by the host and represents only substrate metabolism by the microbiota. A tentative pathway of polyphenol metabolism by the microbiome has been proposed after fermentation of authentic standards and polyphenolic extracts of PACs (Sanchez-Patan *et. al*, 2012, Cueva *et al*., 2012, Stoupi *et al*., 2010a). For example, 34PV has been identified as a unique metabolite of (-)-epicatechin that in turn can be cleaved and dehydroxylated to form 34PP, which is metabolized through beta or alpha-oxidation into 34PA and 34BA (protocatechuic acid) (Roowi *et al*., 2010). The microbiome was also shown to express dehydroxylase activity which results in monohydroxylated derivatives of phenyl propionates, acetates and benzoates, which have been identified after fermentation of polyphenol extracts (Mena *et al*., 2015). In comparison to PACs, phenolic acids, flavonols and anthocyanins require less extensive metabolism to produce common end-products such as phenyl acetates, phenyl propionates and benzoates, which are either derived from the B-ring of the flavonoid aglycone or through reduction of the aliphatic chain of phenolic acids (Gonzalez-Barrio, Edwards & Crozier, 2011, Tomas-Barberan *et al*., 2014).

Despite the advantages of static *ex vivo* fermentations to model microbiome metabolism of polyphenols, it is still difficult to translate these findings to *in vivo* pharmacokinetics. The use

97

of crude polyphenolic extracts is ineffective to deduce what may occur *in vivo* because of the diversity of polyphenol classes present, their differing ratios in the extracts, and likely their differential kinetics of metabolism. Further, the fate of the parent or precursor compounds is rarely determined. Therefore, in these trials, cranberry polyphenols were fractionated and concentrated by polyphenolic class (phenolic acid, flavonoid, condensed tannins, anthocyanins, and polymers) and each fraction was subjected to metabolism by the colon microbiome *ex vivo* to deduce the timing of metabolite production and identification of the primary metabolites derived from each class.

Materials and Methods

Tannin, Flavonol and Phenolic Acid Polyphenol Extraction from Cranberry Powder

Extracts were obtained from a cranberry powder concentrate provided from Ocean Spray Cranberries, Inc. 100 mg of powder was dissolved in 200 mL of 0.1% formic acid and extracted 3-times with 400 mL of ethyl acetate. The organic phases were pooled and evaporated under reduced pressure until dry and re-dissolved in 50 mL of 0.1% formic acid. The residual aqueous phase was retained to isolate the anthocyanin fraction. A 10 g C18 Sep Pak column (Waters Corp, Milford, MA) conditioned with 3 column volumes (CV) of methanol and 6 CV of 0.1% formic acid was utilized to fractionate polyphenols within the ethyl acetate extract. Polyphenols were adsorbed to the column and highly polar compounds were washed with 2 CV of 0.1% formic acid. Phenolic acids were then desorbed by washing the column with 200 mL of pH 7.0 deionized water. Remaining polyphenolics were eluted using a gradient of 0.1% formic acid in methanol and 0.1% formic in water with fractions collected in 50 mL increments. Fractions 1-2 were collected after 100 mL of 10% methanol, fractions 3-10 after 400 mL of 20% methanol, and fractions 11-12 after 100 mL of 30% methanol. Fractions 8-12 were confirmed to be free of

flavonols by HPLC-ESI-MS and retained as the tannin fraction. Fraction 13 and 14 were eluted with 25 mL of 0.1% formic in methanol, and fraction 15 was finally eluted with 50 mL 0.1% formic in methanol. Fractions 13 and 15 were pooled and retained as part of the flavonol fraction. Fractionation was repeated 8-times and collected fractions of phenolic acids (200 mL water), tannins (fractions 8-12) and flavonols (fractions 13 and 15) were pooled and concentrated on a conditioned C18 column. Flavonols were additionally fortified with a flavonol extract prepared by loading a conditioned C18 column with 100 mg of cranberry powder. Other polyphenols were eluted with 75 mL of 50% methanol and flavonols were desorbed with 0.1% formic in methanol, evaporated under reduced pressure and pooled with fractions 13 and 14.

Anthocyanin and Polymer Extraction from Cranberry Powder

Anthocyanins from aqueous solutions after ethyl acetate extraction were concentrated on a 10 g Sep Pak C18 cartridge as previously described and evaporated under reduced pressure. The crude anthocyanin extract was dissolved in 50% aqueous ethanol and loaded onto a 5 g Sephadex LH-20 column that was pre-conditioned in 100% ethanol for 12 hr. Anthocyanins were eluted with 200 mL of 100% ethanol acidified with 0.1% formic acid and polymers were subsequently eluted with 70% aqueous acetone acidified with 0.1% formic acid. Anthocyanins were separated from residual polyphenols of other chemical classes with a mixed-mode, strong cationic exchange (MCX) SPE cartridge as described by He & Giusti (2011) with some modifications. The crude anthocyanin extract was loaded onto a MCX column that had been conditioned with 2 CV of 0.2% formic acid, washed with 1 CV of 0.2% formic and 10 CV of 0.2% formic in MeOH. The anthocyanin fraction was finally desorbed with 1 CV each of 1% NH_4OH in Methanol and 1% NH_4OH in 40:60 water:methanol and acidified with 500 uL formic acid.

Characterization of Polyphenolic Fractions

To assess purity of the extracts, the 4-dimethylaminocinnamaldehyde (DMAC) assay was performed according to Prior *et al*. (2010). Polymeric anthocyanins were assessed in the anthocyanin and polymer extracts utilizing the sulfite assay by Rodriguez-Saona, Giusti & Wrolstad (1999). All final extracts were also screened utilizing LC-ESI-MS/MS and PDA monitoring chromatograms at 280 nm, 360 nm and 520 nm. Purity of total polyphenols, flavonols, and anthocyanins was quantified as the percentage of integrated compounds of interest at 280, 360 and 520 nm, respectively, over the integration of all peaks at 280 nm (Giusti, Rodriguez & Wrolstad, 1999).

Ex Vivo *Fecal Fermentation of Polyphenol Extracts*

Total soluble polyphenols were quantified in each of the 5 extracts by the Folin-Ciocalteu assay (Singleton, Orthofer & Lamuela-Raventos, 1999). Each fraction was then normalized to the same concentration of total polyphenolics by diluting with aqueous 0.1% formic acid. To begin the anaerobic fermentation, 11.24 mL of fermentation media described by Tsounis et al. was mixed with 5.86 mL of normalized extract from each fraction in a fermentation vessel. To this solution, 1.9 mL of fecal slurry prepared in pH 7.4 PBS (10 % w/v). A polyphenolic-free control was prepared with 5.86 mL 0.1% formic acid, 11.24 mL media and 1.9 mL of fecal slurry to control for metabolites not produced from polyphenols and analytical matrix effects. The pH of the solutions were adjusted to 6.4-6.8 and monitored throughout the fermentation. Aliquots were taken from the fermentation tubes at 0, 6, 12, 24, and 48 hr and acidified with 5 µL of formic acid before metabolite and polyphenol analysis via LC-ESI-MS/MS. The entirety of the fermentation was carried out in an anaerobic chamber (Coy Labs, Grass Lake, MI, USA) using carbon (5%) dioxide and hydrogen (5%) and nitrogen as gases. Anaerobiosis was monitored

using an oxygen and hydrogen detector (Coy Labs, Grass Lake, MI, USA) and resazurin strips (Sigma Aldrich, St. Louis, MO, USA).

Preparation of Fermentation Aliquots for LC-ESI-MS/MS Analysis

Samples were prepared for chemical analysis of polyphenols and their metabolites by diluting 250 µL of an aliquot from each fermentation time point with 300 uL 0f 0.1% formic acid. 50 µL of 25 mg L-1 of ethyl gallate was spiked into the matrix to serve as the internal standard. The diluted aliquot was partitioned from a 200 mg Sep Pak C18 column (Waters, Milford, MA, USA) pre-conditioned with 1 mL of MeOH and 1.5 mL of 0.1% formic acid. After loading, the cartridge was washed with 1.5 mL of 0.1% formic acid and polyphenols and their metabolites were eluted with 750 µL of 0.1% formic acid in MeOH. Each fermentation aliquot was extracted in triplicate for each time point. The control fermentation samples absent of polyphenols were likewise extracted to account for matrix effects during LC-ESI-MS analysis.

Polyphenol Microbial Metabolite Analysis Via LC-ESI-MS/MS

The quantification of cranberry polyphenol metabolites produced during fecal fermentations was performed using an Ultimate3000 UPLC equipped to a Thermo Scientific TSQ Quantiva mass spectrometer with an ESI source according to the method outlined in chapter III.

Tannin and Phenolic Acid Analysis Via LC-ESI-MS/MS

Identification and quantification of cranberry tannins and phenolic acids were analyzed via a Thermofinnigan LCQ Deca LC-MS with an ESI as source and ion trap as mass analyzer. Compounds were separated on a Kinetic C18 Column (2.6 µm, 4.6 x150 mm) with 0.1% formic acid as mobile phase A and 0.1% formic acid in methanol as mobile phase B. Gradient elution began with 90% A and 10% B for 5 min, decreased to 70% A after 40 min, decreased to 10% A

after 45 min, increased to 90% after 50 min and equilibrated for an additional 5 min with isocratic conditions before injection of another sample. The MS was run in the negative ionization mode with a capillary temperature was 320°C, source temperature 350°C, sheath gas 30 and auxiliary gas 10. Source parameters and collision energy were optimized utilizing a standard of *p*-coumaric acid for analysis of phenolic acids and for tannin analysis an a-type PAC trimer in cranberry extract was utilized. The mass range for detecting tannins was set from *m/z* 150-1500 while for phenolic acids it was *m/z* 150-600. Phenolic acids were quantified with authentic standards, with the exception of *p*-coumaryl glycosides and caffeic acid glycosides which were quantified in *p*-courmaric acid and caffeic acid equivalents, respectively. Tannins were quantified in (-)-epicatechin equivalents.

Flavonol Analysis Via LC-ESI-MS/MS

Flavonols were separated utilizing a Sunfire C18 column (2.6 µm, 250 x 4.6 mm) and identified and quantified via a Thermofinnigan LCQ Deca LC-MS. Mobile phases were 0.1% formic acid (A) and 0.1% formic acid in methanol (B). Gradient elution began with 95% A, decreased to 75% A after 5 min, to 60% A after 25 min, maintained isocratic conditions for 5 min and then decreased to 5% A after 45 min. 5% A was maintained for 5 min before returning back to initial conditions for the next injection. Source parameters and collision energy were optimized utilizing an authentic standard of rutin. The mass range for detecting flavonols was set from *m/z* 150-1500. Quercetin was quantified with an authentic standard while quercetin glycosides were quantified in rutin equivalents.

Anthocyanin Analysis via HPLC-PDA

Anthocyanins were separated and quantified utilizing a Waters 2695 Alliance HPLC equipped with a Waters 996 photodiode array detector. Separation was carried out according to

the method of Cipriano *et al.*, (2015) with some modification to the gradient scheme. Gradient

elution began with 100% mobile phase A, decreased to 90% after 15 min, 70% after 25 min,

50% after 35 min, 20% after 33 min and increased to 100% A after 40 min. Isocratic conditions

were maintained for 5 min before the next injection. Anthocyanins were quantified in cyanidin-

3-glucoside equivalents at 520 nm.

Polymer Analysis via HPLC-FD

Polymers were assessed via HPLC and fluorescence detection according to the method by

Girard *et al.*, 2016. Separation was carried out using a Develosil Diol column (250 mm x 4.6

mm, 5 µm, Phenomenex, UK) at a flow rate of 1 mL min-1. Mobile phase A consisted of acetic

acid and acetonitrile (2:98) and mobile phase B consisted of acetic acid: methanol: water

(2:95:3). Gradient elution began with 7% B for 0-3 min, which increased to 37.6% B after 57

min, increased to 100% B at 60 min and decreased back to 7% after 73 min. Fluorescence was

measured at 230 nm (excitation) and 321 nm (emission). Polymers were quantified in (-)-

epicatechin equivalents with correction for polymer size (Robbins *et al.*, 2009).

Statistics

For the analysis of parent polyphenol compounds, a one-way AVOVA was utilized

comparing concentrations of parent compounds at each hour of fermentation. Polyphenol

metabolites were analyzed with a two-way ANOVA where factors were time of fermentation and

polyphenolic fraction. Significance was designated if P<0.05.

Results and Discussion

Fractionation and Characterization of Cranberry Polyphenolics

Table 7 Purity of each fraction assessed by the percentage of area under the curve (AUC) of polyphenols at 280, 360 and 520 nm over the AUC of all compounds at 280 nm. Data is reported as mean ± standard deviation.

Fraction	% 280/280 Total	% 360/280 Total	% 520/280 Total	DMAC (µM)	Sulfite Assay (% Derived-Anthocyanins)
Tannins	53.68 ± 12.03	10.19 ± 1.58	4.39 ± 1.02	633.78 ± 19.49	-
Flavonols	95.41 ± 1.10	122.65 ± 4.45	0.49 ± 0.15	ND	-
Anthocyanins	86.10 ± 4.56	18.83 ± 3.43	73.58 ± 10.56	168.89 ± 21.79	6.16 ± 0.14%
Phenolic Acids	61.80 ± 0.46	9.31 ± 0.21	0.16 ± 0.03	ND	-
Polymers	87.73 ± 0.47	12.36 ± 0.08	13.98 ± 0.03	3130.67 ± 59.17	34.68 ± 0.74

Table 8 Characterization and identification of polyphenols in each fraction.
*m/z in positive ionization mode.

Fraction	Compound	[M-H]-	MS$_2$	Concentration (μM)
Anthocyanins	Cyanidin-3-hexose	449*	287*	932.41 ± 5.29
	Cyanidin-3-pentose	419*	287*	1,155.09 ± 4.17
	Peonidin-3-hexose	463*	301*	1,806.39 ± 5.67
	Peonidin-3-pentose	433*	301*	606.43 ± 5.00
Tannins	A-type trimer	575	539, 449, 423, 289	433.59 ± 21.00
	Procyanidin A2	863	711, 575	1,098.45 ± 95.79
Phenolic Acids	Caffeic acid 1	179	135	28.01 ± 9.56
	Caffeic acid 2	179	135	213.98 ± 15.03
	Caffeic Acid hexose 1	341	179	64.88 ± 9.93
	Caffeic Acid hexose 2	341	179	100.68 ± 11.94
	Chlorogenic acid 1	353	191	949.80 ± 33.46
	Chlorogenic acid 2	353	191	924.42 ± 43.90
	p-Coumaryl hexose 1	325	163	3,203.36 ± 111.88
	p-Coumaryl hexose 2	325	163	2,370.10 ± 175.27
	Protocatechuic acid	153	109	1,957.95 ± 88.93

Table 8 Continued

Fraction	Compound	[M-H]-	MS2	Concentration (µM)
Flavonols	Quercetin pentose 1	433	301	20.55 ± 0.57
	Quercetin pentose 2	433	300	26.25 ± 0.63
	Quercetin pentose 3	433	301	38.96 ± 0.49
	Quercitrin	447	301	104.18 ± 1.45
	Methoxy-quercetin pentose	447	315	7.84 ± 0.55
	Quercetin hexose	463	300	71.46 ± 0.25
	Quercetin aglycone	301	179, 151	187.19 ± 4.59
Polymers	-	-	-	444.59 ± 27.91

Tannin Fraction

Separation of tannins from other polyphenol classes in complex fruit extracts is challenging due to their unpredictable chromatographic behavior on C18, which does not depend on degree of polymerization. Purity of the tannin extract was 53.68 ± 12.03 % and low purity was due to the non-polyphenol, absisic acid-hexoside (*m/z* 425, MS2 263), which accounted for 36.97 ± 1.06 % of the tannin fraction (Table 7). Absisic acid-hexoside is not known to result in microbial metabolites similar to tannins and without this contaminant tannins accounted for 85.34 ± 20.52 % of total polyphenols in the fraction (Chmielewski *et al*., 2018). Polyphenols

identified in the tannin fraction were procyanidin A2 and an a-type proanthocyanidin trimer.

Procyanidin A2 had a *m/z* of 575 and MS2 fragments of 449 and 289, indicating loss of

phloroglucinol and a monomeric unit, respectively (Table 8) (Gu *et al*., 2003). Additionally, the

identity of procyanidin A2 in the fraction was confirmed with an authentic standard. The a-type

proanthocyanidin trimer had a *m/z* of 863 and MS2 fragments of 711 (Retro-Diehl's-alder

Rearrangement) and 575, indicating that the a-type interflavan bond was located in the terminal

unit (Tarascou *et al*., 2011). Because thiolysis was not performed, identification of the monomers

as (+)-catechin or (-)-epicatechin could not be confirmed, but others have found that cranberry a-

type proanthocyanidin trimers contain only (-)-epicatechin units (Tarascou *et al*., 2011).

Expectantly, the tannin fraction was reactive with DMAC, and the fraction contained $633.78 \pm$

19.49 μM soluble tannins (Table 7). It should be noted that monomers have greater sensitivity in

the DMAC assay, so this quantity may be an underestimation of the actual soluble tannin content

(Feliciano *et al*., 2012, Krueger *et al*., 2016). In comparison to the PAC composition of cranberry

products, this fraction is not representative of the diversity of PAC DP and percentage of a and b-

type PACs found in cranberry products, as the diversity was compromised for in order to achieve

higher fraction purity (Feliciano *et al*., 2014).

Phenolic Acid Fraction

Phenolic acids, due to their carboxylic acid functional groups, are easily separated from

complex fruit extracts by manipulating pH during extraction, which proved to be successful

when separating phenolic acids from cranberry (Ghiselli *et al*., 1998). Cranberry phenolic acids

were isolated to a purity of 61.80 ± 0.46 % (Table 7). Low purity was due to a non-polyphenol

contaminant that was unidentifiable, but most resembled a peptide from its PDA spectra. Further,

no tannins could be detected in the phenolic acid fraction, as the fraction was not reactive with

DMAC. Hydroxycinnamates identified in the fraction included two caffeic acid isomers (m/z 175/135), two caffeic acid hexosides (m/z 341/155), two chlorogenic acid isomers (m/z 353/191) and two *p*-coumaric acid hexoses (m/z 371/163) (Table 8). Hydroxybenzoates identified in the fraction included protocatechuic acid (m/z 153/109) and benzoic acid (m/z 121/77). Of the hydroxycinnamates, the *p*-coumaryl hexosides were the most abundant and of the hydroxybenzoates, benzoic acid was the most abundant polyphenolic present. This agrees with what phenolic acids have been previously identified in cranberry, as coumaryl hexosides and benzoic acid have been found to be the most predominant phenolic acids in a variety of cranberry products (Oszmianski *et al.*, 2018).

Flavonol Fraction

Flavonols, especially aglycones, are more non-polar in comparison to other polyphenol classes and typically are the last compounds to elute from fractionation on C18 solid phases. The flavonol fraction had the highest purity of any other fraction with 95.41 ± 1.10 % (Table 7). The high purity was also represented at 360 nm with 122.65 ± 4.45 %, which reflects the higher absorbance flavonols exhibit at 360 nm and why many authors quantify flavonols at 360 nm and not 280 nm when utilizing PDA detectors (Vvedenskaya *et al.*, 2004). The flavonol fraction was also not reactive with DMAC, indicating that tannins were not present. Flavonols identified in the fraction included several derivatives of quercetin pentoses and hexoses (Table 8). Three quercetin glycosides were characterized by an ion peak at m/z 433 and MS_2 fragment of 301, indicative of a loss of a pentose sugar from a quercetin moiety. Two flavonols with an ion of m/z 447 were identified and had MS_2 fragments of either 301 or 315, which corresponds to quercitrin (quercetin-3-alpha-rhamnoside) and a methoxylated quercetin pentoside, respectively. Quercetin-hexose was also identified with a m/z of 463 and MS_2 fragment of 301. Quercetin aglycone (m/z

301) was also identified and was one of the most abundant flavonols present in the fraction. Quercetin aglycone is typically not found in fresh cranberry fruit but may accumulate in cranberry powders during processing (Vvedenskaya *et al*., 2004). The pentose and hexose moieties could not be specified, although others have identified these sugars as galactose, xylose, arabinose, glucose or rhamnose (Chen & Zuo, 2007, Vvedenskaya *et al*., 2004). Myricetin glycosides were also identified but at levels below the limit of quantification. The flavonol fraction was representative of what would be found in a processed cranberry product, as the fraction mainly comprised of quercetin derivatives and aglycone.

Anthocyanin Fraction

The collective usage of ethyl acetate and cation-exchange chromatography to separate anthocyanins from other cranberry polyphenols resulted in a high purity cranberry anthocyanin fraction. Ethyl acetate and cation exchange separates other polyphenols from anthocyanins by manipulating the cationic nature of anthocyanins at pH 2 (Flores da Silva *et al*., 2017). The purity of the anthocyanin fraction was 86.10 ± 4.56 %, which is comparable to attempts made to isolate anthocyanins from other complex fruit polyphenol extracts (Table 7) (He & Giusti, 2011). However, the anthocyanin fraction was DMAC positive, indicating that the contaminants contained flavan-3-ol derived tannins. These tannin compounds were identified as residual 6.16 ± 0.14% derived-anthocyanins in the fraction, as determined by the sulfite assay, indicating that derived-anthocyanins still have cationic behavior despite polymerization (Table 7). Anthocyanins in the fraction were identified as cyanidin hexose and pentose and peonidin hexose and pentose. This is in accordance with Prior *et. al*., (2001) who previously identified cyanidin-3-galactoside, cyanidin-3-arabinoside, peonidin-3-galactoside and peonidin-3-arabinoside as the predominant anthocyanins in cranberry.

Polymeric Fraction

Cranberries contain high concentrations of polymeric compounds, which are typically isolated utilizing the solid-phase material Sephadex LH-20 and, due to their large size, are not separable on HPLC. Accordingly, the polymer fraction was evident by a large, broad and late-eluting peak and the purity was 87.73 ± 0.47 %. Analysis of polymeric compounds from cranberry, isolated utilizing Sephadex LH-20, has typically focused on PACs. However, cranberries also contain high concentrations of derived-PACs, or polymers consisting of both anthocyanins and PACs (Tarascou *et al*, 2011). Derived PACs have also been identified in other foods where both PACs and anthocyanins are present, including açai and wine (Pancheco-Palencia, Hawken & Talcott, 2007, Singleton & Trousdale, 1992). Their presence is indicated by a brownish-red tint, which is apparent by their absorbance at 510-520 nm, and in the polymeric fraction accounted for 13.98 ± 0.03 % of the fraction (Table 7). The sulfite assay also indicated that 34.68 ± 0.74% of the fraction was derived PACs. The polymer fraction also expectantly had the highest DMAC or soluble PAC concentration of 3130.67 ± 59.17 µM, indicating the high concentrations of flavan-3-ol monomer units within the polymers.

Metabolism of Parent Compounds by Fecal Microorganisms

The microbiome plays a critical role in the metabolism of polyphenols and the pharmacokinetics of their resulting metabolites. However, there is limited information about the fate of polyphenol parent compounds during microbiome metabolism. Therefore, the rate of metabolism of parent polyphenols was investigated in order to better interpret *in vivo* pharmacokinetic data, especially in food matrices where there are mixed classes of polyphenols present, such as cranberry.

Phenolic acids and anthocyanins were rapidly metabolized into low molecular weight metabolites, as no compound from either isolate was detectable after 6 hr (Table 9). Polymers within the polymeric isolate were also no longer detectable after 6 hr, but this may be due to their precipitation with proteins over time, which may limit their detection (Tajchakavit *et al*., 2001). In comparison to other fractions, cranberry tannins were metabolized by the microbiome at a slower rate, as they were still detectable in the fermentation after 24-48 hr. However, the majority of the tannins were catabolized/degraded within the first 6 hr. Procyanidin A2 decreased by 66.72 ± 3.59 % and the a-type trimer decreased by 73.03 ± 3.78 % after 6 hr and their initial concentration was significantly higher ($P<0.05$) than what was found after 6-48 hr. This is in accordance with the observations of Ou *et al*. (2014) ,where it was reported that neither procyanidin A2 nor other PACs from cranberry were fully metabolized by human fecal microbiota after 24 hr. However, this can be dependent on concentration, as in chapter III it was shown that 66.58 ± 8.94 µM of procyanidin A2 was fully catabolized/degraded after 6 hr. The fermentation conditions of *ex vivo* models, in attempt to simulate the distal colon, are unfavorable to most polyphenolics due to the low acid environment. One such change that can occur with tannins at neutral to alkaline pH is the formation of epimers (Lu *et al*., 2011). An epimer of procyanidin A2 with a *m/z* of 575 was formed after 6 hr and had a max concentration of 96.68 ± 4.66 µM after 24 hr but was not detected after 48 hr (Table 10). Three additional a-type trimer epimers were also produced with a *m/z* 863 and MS$_2$ fragments of *m/z* 711 and 575. Trimer epimers reached maximum concentrations from 12 to 24 hr and, like dimer epimers, were

Table 9 Differing letters indicate significant differences (P<0.05) between concentrations of individual compounds at each hour of fermentation. Data is presented as mean ± SEM μM.

Fraction	Compound	Concentration (μM)				
		0 hr	6 hr	12 hr	24 hr	48 hr
Anthocyanins	Cyanidin-3-hexose	76.28 ± 14.43 a	ND	ND	ND	ND
	Cyanidin-3-pentose	95.82 ± 6.29 a	ND	ND	ND	ND
	Peonidin-3-hexose	164.56 ± 14.22 a	ND	ND	ND	ND
	Peonidin-3-pentose	67.69 ± 3.24 a	ND	ND	ND	ND
Tannins	A-type Trimer	39.58 ± 1.27 a	10.60 ± 1.23 b	6.00 ± 0.90 c	2.22 ± 0.24 cd	ND
	Procyanidin A2	370.87 ± 15.20 a	123.16 ± 12.62 b	87.29 ± 14.64 b	87.97 ± 5.16 b	6.69 ± 6.69 c
Phenolic Acids	Caffeic acid 1	0.22 ± 1.05 ns	ND	ND	ND	ND
	Caffeic acid 2	27.58 ± 0.75 a	ND	ND	ND	ND
	Caffeic acid hexose 1	8.77 ± 0.32 a	ND	ND	ND	ND

Table 9 Continued

Fraction	Compound	Concentration (µM)				
		0 hr	6 hr	12 hr	24 hr	48 hr
	Caffeic acid hexose 2	12.68 ± 1.04 a	ND	ND	ND	ND
	Chlorogenic acid 1	128.91 ± 1.96 a	ND	ND	ND	ND
	Chlorogenic acid 2	119.68 ± 1.68 a	ND	ND	ND	ND
	Coumaryl hexose 1	561.72 ± 6.45 a	ND	ND	ND	ND
	Coumaryl hexose 2	240.53 ± 4.45 a	ND	ND	ND	ND
Flavonols	Quercetin pentose 1	10.60 ± 0.58 a	ND	ND	ND	ND
	Quercetin pentose 2	10.31 ± 0.79 a	1.32 ± 0.16 b	ND	ND	ND
	Quercetin pentose 3	16.66 ± 1.36 a	5.23 ± 0.09 b	5.34 ± 0.19 b	3.47 ± 0.14 b	ND

Table 9 Continued

Fraction	Compound	Concentration (µM)				
		0 hr	6 hr	12 hr	24 hr	48 hr
	Quercitrin	50.37 ± 2.85 a	7.22 ± 0.15 b	ND	ND	ND
	Methoxy-quercetin pentose	4.16 ± 0.19 ns	2.45 ± 2.45	ND	ND	ND
	Quercetin hexose	31.61 ± 1.38 a	10.47 ± 0.47 b	5.73 ± 0.15 c	0.87 ± 0.02 d	ND
	Quercetin aglycone	72.86 ± 5.80 c	90.84 ± 4.66 bc	111.72 ± 3.81 ab	115.58 ± 11.30 ab	128.27 ± 7.95 a
Polymers	-	5.54 ± 0.12 a	ND	ND	ND	ND

Table 10 Concentrations of epimers and dimeric microbial metabolites produced from fermentation of the cranberry tannin fraction with fecal microorganisms over 48 hours. Data is presented as mean ± SEM µM and differing letters indicate a significant difference (P<0.05) between different hours of fermentation for each individual compound.

Compound	[M-H]-	MS$_2$	Concentration (µM)				
			0 hr	6 hr	12 hr	24 hr	48 hr
Microbial Derived Dimer	577	451, 291	ND	35.30 ± 7.54 cd	71.08 ± 5.08 c	144.73 ± 8.53 b	301.22 ± 20.81a
Microbial Derived Trimer	865	577	ND	ND	ND	ND	9.78 ± 3.33 a
Dimer Epimer 1	575	449, 289, 285	ND	<LOQ	<LOQ	2.72 ± 0.22 b	24.37 ± 10.62 a
Dimer Epimer 2	575	-	ND	54.58 ± 6.99 b	91.66 ± 17.11 ab	98.68 ± 4.66 a	ND
Trimer Epimer 1	863	711, 575	ND	7.32 ± 1.17 b	11.69 ± 0.76 a	6.33 ± 0.26 b	ND
Trimer Epimer 2	863	711. 575	ND	4.76 ± 1.04 c	16.81 ± 1.89 b	24.83 ± 1.20 a	ND
Trimer Epimer 3	863	711, 575	ND	ND	3.47 ± 0.36 b	6.43 ± 0.10 a	ND

also not detectable after 48 hr. The dimer and trimer epimers had identical mass spectral characteristics to their parent compounds from cranberry which indicates that the a-type bond is in the same location, further providing evidence that these compounds are epimers of the parent cranberry dimer and trimer in the isolate (Tarascou *et al*. 2011).

The tannin fraction also produced a unique large molecular weight microbial metabolite. This microbial metabolite had a *m/z* of 577 and accumulated over time to reach a maximum concentration of 301.22 ± 20.81 µM after 48 hrs. This microbial metabolite had similar mass spectral characteristics to an (-)-epicatechin dimer that had undergone c-ring fission in the terminal unit, as indicated by the MS_2 fragment ion at *m/z* 291, and was reported as a derivative from procyanidin A2 isolated from lychee fruit after fermentation with porcine fecal bacteria (Engemann *et al*., 2011). This study is the first to report the production of this metabolite by fecal microorganisms from cranberry polyphenols.

Flavonols were more slowly metabolized in comparison to other fractions, as quercetin was still detected after 48 hr. Quercetin aglycone increased from 72.86 ± 5.08 µM at 0 hr to a maximum concentration of 128.27 ± 7.95 µM after 48 hr. By contrast, quercetin-glycosides were not detectable after 24 hr of fermentation, which could indicate their contribution to the increasing concentrations of quercetin. The deglycosylation of flavonoids is a known step in flavonol metabolism and is likely from ß-glucosidase expression by the microbiota (Serra *et al*., 2012, Jaganath *et. al*., 2009, Rechner *et. al*., 2004). Overall**,** parent compound metabolism/degradation was highly variable between fractions and may differ due to their differing chemical structures and/or anti-bacterial activity.

3,4-Dihydroxyphenyl-gamma-valerolactone (34PV)

34PV is a unique metabolite of the flavonoid class, flavan-3-ols. Specifically, 34PV has been identified as a metabolite of (-)-epicatechin, (+)-catechin, procyanidin B2 and grape b-type PACs, and is one of the first metabolites produced through reduction of the C-ring of the flavonoid aglycone (Cueva *et al.*, 2012, Appeldoorn *et al.*, 2009, Stoupi *et al.*, 2010a). 34PV was a minor microbial metabolite identified in 2 fractions – the anthocyanin and polymer fractions (Figure 39). The maximum concentration of 34PV produced in the polymeric fraction was after 6 hr with $0.45 \pm 0.06 \, \mu M$ and was not detected in the fraction thereafter. Possible routes for the production of 34PV from the polymer fraction are depolymerization into free (-)-epicatechin which could be subsequently metabolized into 34PV, or production from residual procyanidin dimer identified in the fraction. Both mechanisms could explain the low concentrations of this metabolite found, especially in comparison to concentrations of 34PV produced from fermentation of grape seed extract and (-)-epicatechin (Sanchez-Patan et al., 2012, Roowi et al., 2010). Unexpectedly, 34PV was detected after 6 hr of fermentation of the anthocyanin fraction and reached a maximum concentration after 12 hr of $3.35 \pm 0.38 \, \mu M$. As a unique metabolite of flavan-3-ols, the detection of 34PV in the anthocyanin fraction was likely due to the presence of residual anthocyanin-proanthocyanidin polymers, as indicated by the DMAC concentration of $168.89 \pm 21.79 \, \mu M$, which in the fermentation was ~13 µM. This supports the idea that anthocyanin-derived polymers can depolymerize into free (-)-epicatechin. Additionally, 34PV was not detected after fermentation of the tannin fraction. Considering that the tannin fraction only contained a-type PACs, this may indicate that the a-type linkage prevents the formation of

34PV, which has also been speculated by others (Ou & Gu, 2014). Although not detected in the

tannin fraction, 34PV is still considered a unique metabolite of flavan-3-ols, and can possibly be

produced from larger PAC polymers, but its formation is dependent on the type of interflavan

bond.

Figure 39 Concentration of 3,4-dihydroxyphenyl-gamma-valerolactone (34PV) produced by fermentation of anthocyanins (circle), flavonols (triangle), polymers (cross), phenolic acids (squares) and tannins (square with x) with fecal microorganisms over 48 hours. Data is presented as mean ± SEM μM.

3,4-Dihydroxyphenyl Propionic Acid (34PP)

34PP is hypothesized to be produced from delactonization, dehydroxylation and beta-oxidation of 34PV when produced from flavan-3-ols, directly derived from the B-ring of flavonols and anthocyanins or from reduction of the aliphatic chain of phenolic acids. 34PP was unique to the phenolic acid fraction and was not detected in any other cranberry polyphenol fraction. After 6 hr, the phenolic acid fraction resulted in 63.20 ± 2.86 µM of 34PP and after 12 hr, 34PP was never again detected (Figure 40). 34PP has been previously identified as a microbial metabolite derived from the phenolic acid chlorogenic acid following its hydrolysis into free caffeic acid and reduction of the aliphatic chain. (Tomas-Barberan *et al*., 2014, Gonthier *et al*., 2006) In cranberry, not only chlorogenic acid, but also caffeic acid glycosides and aglycone are likely sources of 34PP, as caffeic acid can be deglycosylated by ß-glucosidase produced by the microbiota (Duda-Chodak et al., 2015).

Figure 40 Concentration of 3,4-dihydroxyphenyl propionic acid (34PP) produced by fermentation of anthocyanins (circle), flavonols (triangle), polymers (cross), phenolic acids (square) and tannins (square with x) with fecal microorganisms over 48 hours. Data is expressed as mean ± SEM μM.

Monohydroxyphenyl Propionic Acid (MPP)

MPP is hypothesized to be formed from dehydroxylation of 34PP and has been identified as a metabolite of several classes of flavonoids and phenolic acids (Mena *et al.*, 2015). Despite only detecting 34PP in the phenolic acid fraction, MPP was a metabolite produced from all cranberry polyphenol fractions (Figure 41). Phenolic acids produced the highest ($P<0.05$) concentration of MPP among the cranberry fractions with a peak concentration of 223.67 ± 1.44 μM after 12 hr. MPP has been reported as a metabolite of chlorogenic acid and caffeic acid after dehydroxylation of 34PP (Gonthier *et al.*, 2006). MPP may also be produced from the reduction

Figure 41 Concentration of hydroxyphenyl propionic acid (MPP) produced by fermentation of anthocyanins (circle), flavonols (triangle), polymers (cross), phenolic acids (square) and tannins (square with x) with fecal microorganisms over 48 hours. Data is presented as mean ± SEM μM.

of the aliphatic chain of *p*-coumaric acid and hydrolyzed *p*-coumaric acid hexoses, as *p*-coumaric

acid has the same hydroxylation pattern on the phenyl substituent as MPP. This could explain the

3.54-fold higher concentration of MPP in comparison to 34PP produced by phenolic acids, or the

lack of 1:1 molar conversion rate of 34PP into MPP. Although not higher than the phenolic acid

fraction, cranberry anthocyanins were metabolized predominantly into MPP. After 48 hr, MPP

reached a maximum concentration of 99.89 ± 4.11 μM in the anthocyanin fraction, which was

higher ($P<0.05$) than that of the tannin, flavonol and polymer fractions. MPP also reached a

maximum concentration of 18.67 ± 0.17 and 4.22 ± 0.06 μM in the tannin and flavonol fractions,

respectively, after 48 hr. This could indicate that flavonoid metabolism into MPP occurs at a slower rate than phenolic acid metabolism. By contrast, and similar to phenolic acids, the concentration of MPP produced by the polymer fraction reached a maximum concentration of $26.12 \pm 1.50\ \mu M$ after 12 hr. MPP has not always been identified as a metabolite of all flavonoids. Serra *et al*., (2012) found that 34PP, and not MPP, was produced from fermenting flavonol standards with rat fecal microorganisms. A reason for this discrepancy could be due to tyrosine metabolism, which results in the same compound (Windey, De Preter & Verbeke, 2012).

3,4-Dihydroxyphenyl Acetic Acid (34PA)

34PA is a microbial metabolite produced from alpha-oxidation of 34PP or directly from the flavonoid B-ring. The cranberry flavonol fraction resulted in the highest ($P<0.05$) concentration of 34PA (Figure 42). This agrees with Rechner *et al*. 2004, who found that rutin was first metabolized into quercetin before producing 34PA. Further, due to the lack of production of 34PP from the flavonol extract, this also suggests that 34PA is produced directly from the flavonoid B-ring opposed to alpha-oxidation of 34PP. 34PA has also been identified as a major metabolite of condensed tannins, specifically (-)-epicatechin, procyanidin B2 and grape seed PACs (Appeldorn *et al*., 2009, Stoupi *et al*., 2010a). However, low concentrations of 34PA was produced from the cranberry tannin fraction and it was not the major metabolite produced from the tannin fraction. This could suggest that the a-type interflavan bond could prevent C-ring fission and production of 34PA from the B-ring. This contradicts what was found by Ou *et al*., (2014), whereas cranberry PACs resulted in higher concentrations of phenyl acetates.

122
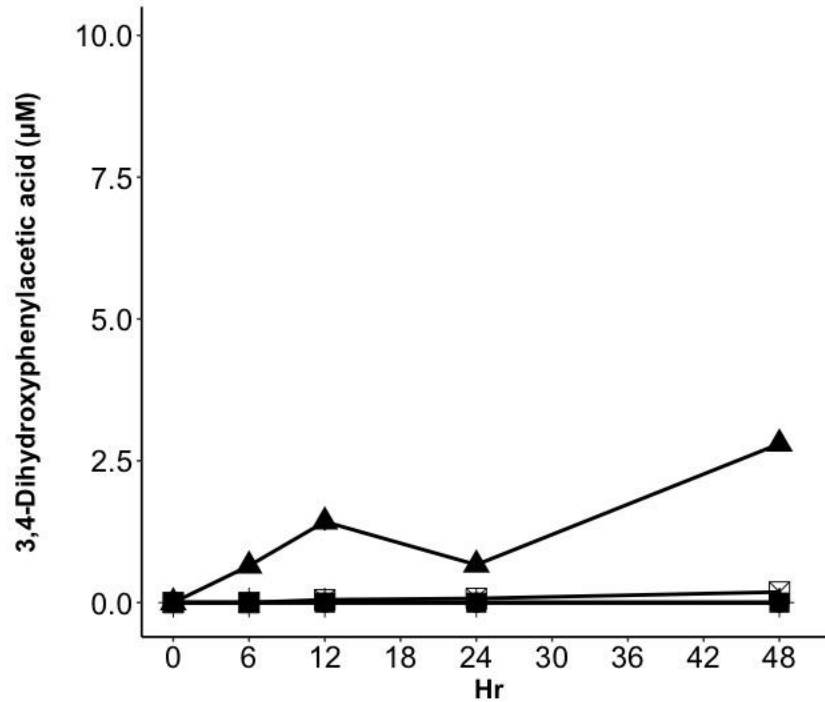
Figure 42 Concentrations of 3,4-diydroxyphenyl acetic acid (34PA) produced by fermentation of anthocyanins (circle), flavonols (triangle), polymers (cross), phenolic acids (square) and tannins (square with x) with fecal microorganisms over 48 hours. Data is presented as mean ± SEM μM.

Monohydroxyphenyl Acetic Acid (MPA)

MPA is hypothesized to be derived from alpha-oxidation of MPP. However, MPA was not found in any fraction (Figure 43). This was unexpected as there were significant concentrations of its alpha-oxidation precursor, MPP, produced in the phenolic acid, tannin and anthocyanin fractions (Appeldoorn *et al*., 2009, Stoupi *et al*., 2010a). This is in accordance with Tomas-Barberan *et al*., (2014) who found that the end products of chlorogenic acid metabolism by human fecal microorganisms resulted in phenyl propionates and not phenyl acetates.
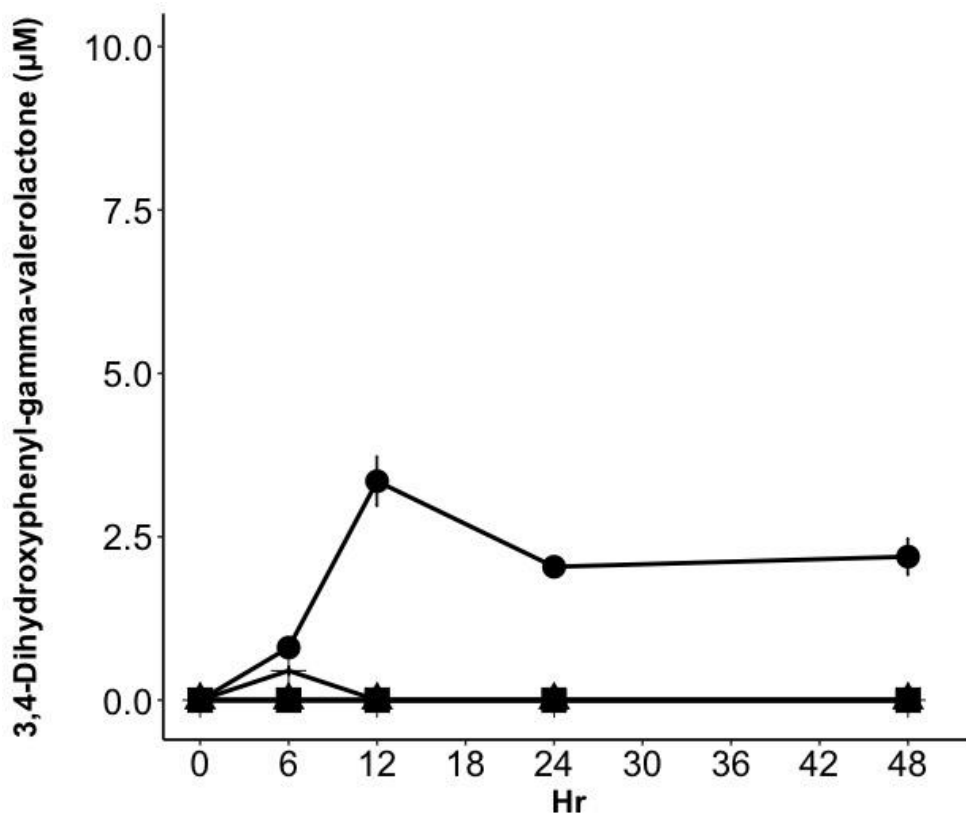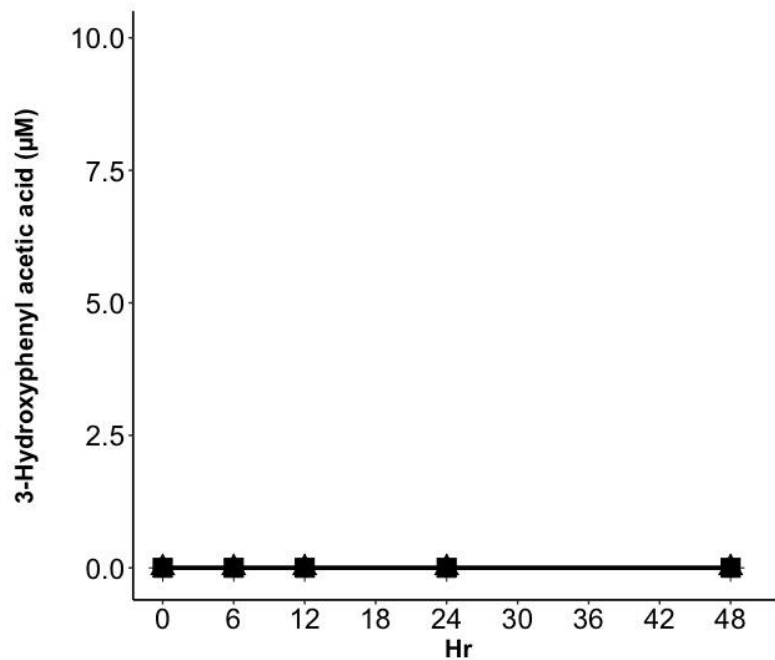
Figure 43 Concentrations of hydroxyphenyl acetic acid (MPA) produced by fermentation of anthocyanins (circle), flavonols (triangle), polymers (cross), phenolic acids (square) and tannins (square with x) with fecal microorganisms over 48 hours. Data is presented as mean ± SEM µM.

Protocatechuic Acid (34BA)

Protocatechuic acid was produced from the cranberry tannin, flavonol and anthocyanin fractions (Figure 44). The tannin fraction resulted in the highest ($P<0.05$) amount of produced protocatechuic acid after 48 hr with 24.22 ± 0.30 µM, with the exception of the phenolic acid fraction, which contained naturally present 34BA from cranberry. Levels of 34BA did not increase in the phenolic acid fraction at any time during the fermentation indicating that these

124

compounds were likely not metabolites of other phenolic acids. 34BA was also produced as a metabolite of the flavonol fraction and reached a maximum concentration of $4.19 \pm 0.12\,\mu M$ after 12 hr. Serra *et al.* (2012) and Jaganath *et al.* (2009) also reported 34BA as a metabolite of a quercetin glycoside standard and hypothesized that 34BA was derived from flavonols directly from the flavonoid B-ring or through alpha-oxidation of 34PA. 34BA was also produced in the anthocyanin fraction and reached a maximum concentration of $2.69 \pm 0.28\,\mu M$ after 6 hr. 34BA has been reported as the predominant metabolite of cyanidin-3-glucoside standard and also from high-anthocyanin blackberry, raspberry and blueberry extracts. However, fermentation of cranberry anthocyanins into 34BA in comparison to other cranberry polyphenol fractions was significantly lower ($P<0.05$) and suggests that the production of MPP from anthocyanins may be favored (Dall'Asta *et al.*, 2012, Fleschhut *et al.*, 2006).
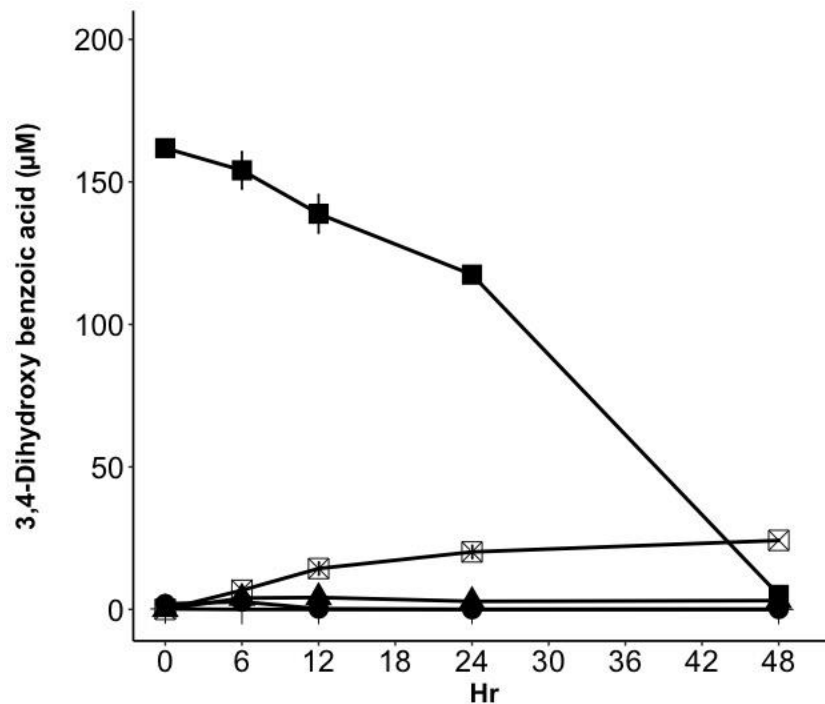
Figure 44 Concentration of 3,4-dihydroxy benzoic acid (34BA), or protocatechuic acid, produced by fermentation of anthocyanins (circle), flavonols (triangle), polymers (cross), phenolic acids (square) and tannins (square with x) with fecal microorganisms over 48 hours. Data is presented as mean ± SEM μM.

Monohydroxy Benzoic Acid (MBA)

MBA is hypothesized to be a metabolite produced from alpha or beta-oxidation of MPA or MPP, respectively. However, MBA was only produced in the phenolic acid fraction after fermentation with fecal microorganisms and reached its maximum concentration after 48 hr at 7.29 ± 0.35 μM (Figure 45). Gonthier *et al.* (2006) previously identified MBA as a metabolite of phenolic acids from the dehydroxylation of benzoic acid, which was abundant in the phenolic acid fraction. Although MBA was identified as a metabolite after fermentation of procyanidin A2 and cinnamtannin B1 isolated from lychee fruit with porcine fecal microorganisms, MBA was

not identified as a cranberry tannin fraction metabolite (Engemann *et al*., 2011). Evidence suggests that the production of MBA through the dehydroxylation of 34BA is favored over production from alpha or beta oxidation from phenyl acetates and propionates, respectively.
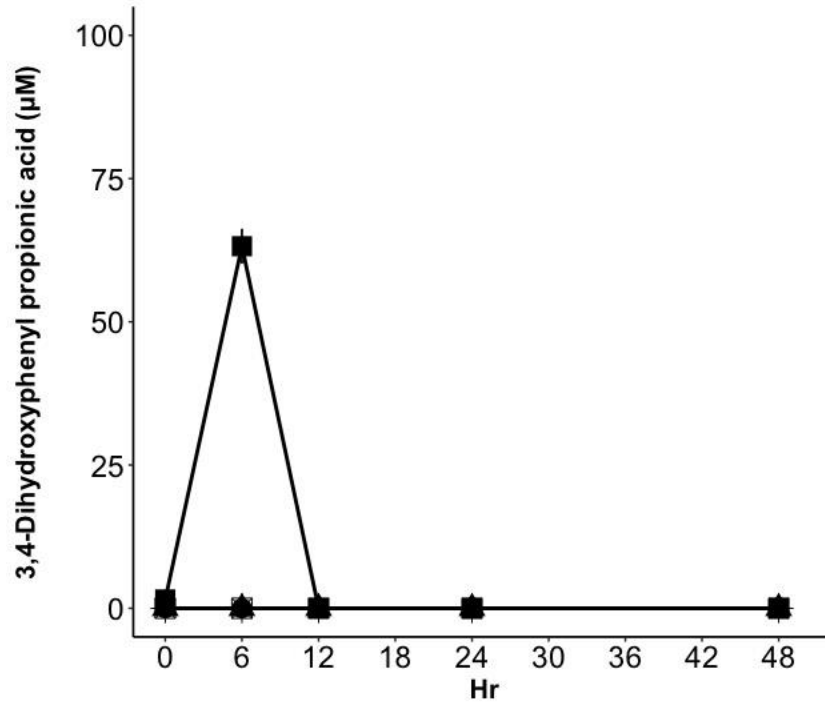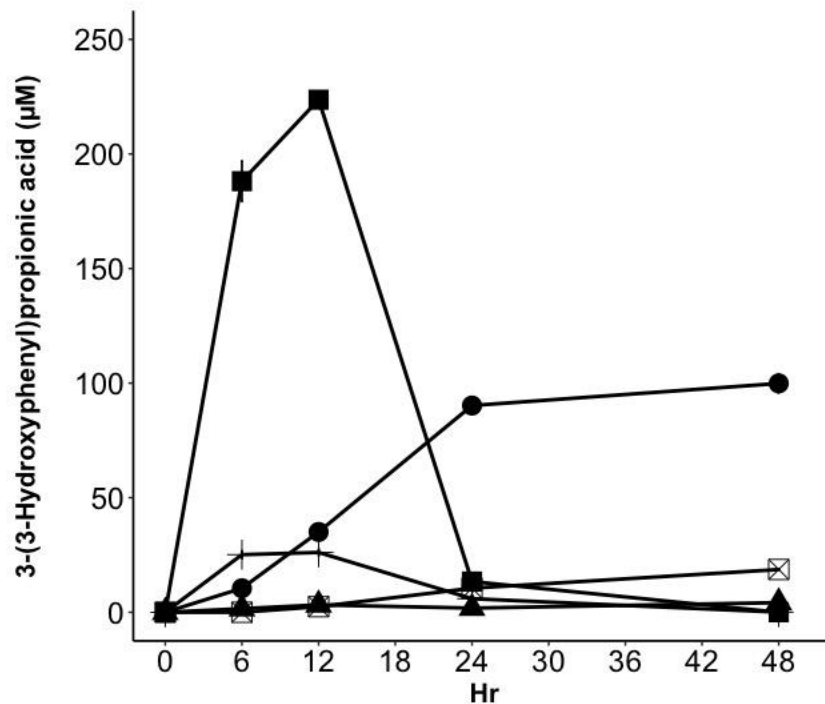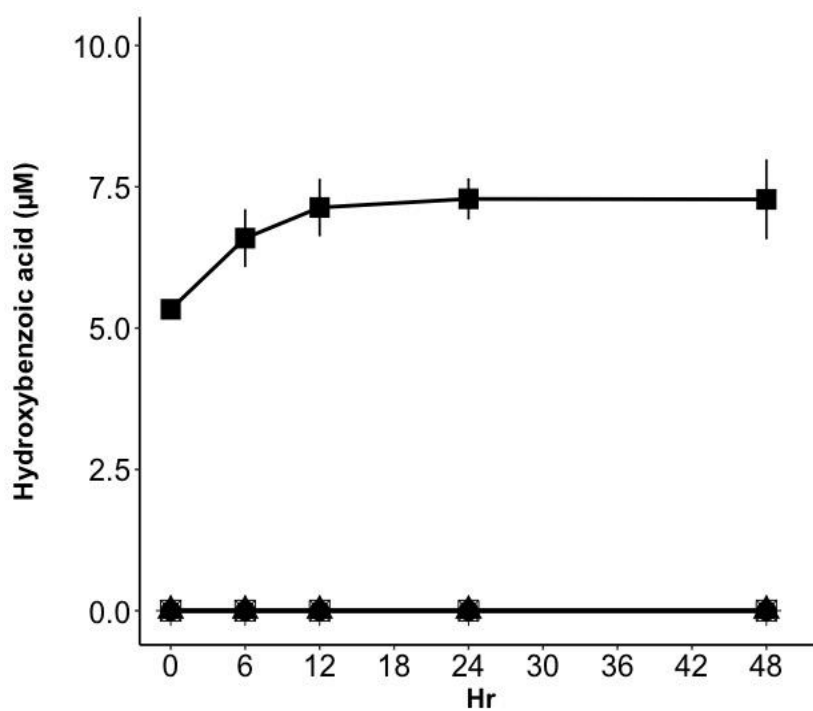

Figure 45 Concentrations of hydroxy benzoic acid (MBA) produced by fermentation of anthocyanins (circle), flavonols (triangle), polymers (cross), phenolic acids (square) and tannins (square with x) with fecal microorganisms over 48 hours. Data is presented as mean ± SEM μM.

Benzoic Acid (BA)

      Benzoic acid (BA) was identified as a metabolite of the cranberry tannin and anthocyanin fractions (Figure 46). BA is a phenolic acid naturally present in cranberry that is also used as a preservative in processed foods (Ermis *et al*., 2015). Accordingly, BA was present in the phenolic acid fraction with 2,665.40 ± 160.12 µM at 0 hr and decreased slowly over the 48 hr period. Levels of BA did not increase significantly at any time during the fermentation indicating that these compounds were likely not metabolites of other phenolic acids. By contrast, BA was not detected in the tannin fraction at 0 hr and reached a maximum concentration after 48 hr of 15.19 ± 0.50 µM, which was significantly higher ($P<0.05$) than the other fractions. Benzoic acid was also produced in the anthocyanin fraction and reached a maximum concentration after 24 hr with 3.54 ± 0.25 µM.
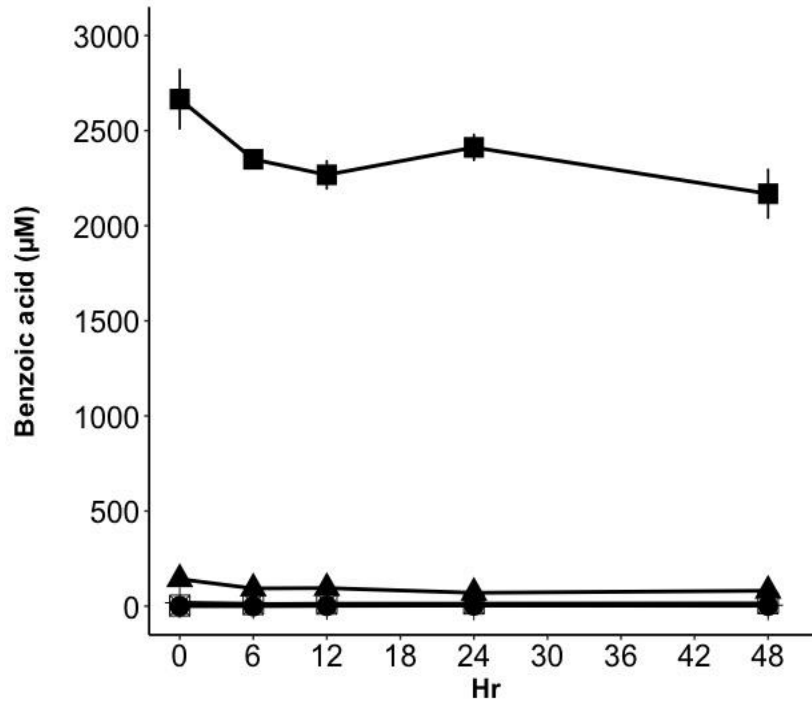
Figure 46 Concentrations of benzoic acid (BA) produced by fermentation of anthocyanins (circle), flavonols (triangle), polymers (cross), phenolic acids (square) and tannins (square with x) with fecal microorganisms over 48 hours. Data is presented as mean ± SEM μM.

Table 11 Identification (+) or no detection (-) of microbial metabolites in each fraction after fermentation with fecal microorganisms for 48 hr.

Metabolite	Anthocyanins	Flavonols	Tannins	Phenolic Acids	Polymers
34PV	+	-	-	-	+
34PP	-	-	-	+	-
MPP	+	+	+	+	+
34PA	-	+	+	-	-
MPA	-	-	-	-	-
34BA	+	+	+	-	-
MBA	-	-	-	+	-
BA	+	-	+	-	-

Conclusion

Cranberry polyphenol fractionation revealed differential metabolism of polyphenols by the fecal microbiome into phenolic acid metabolites which was dependent on polyphenol class (Table 11). Parent compound metabolism differed by fraction whereas phenolic acids and anthocyanins were rapidly metabolized and tannins and flavonols persisted in fecal fermentations throughout the 48 hr. Metabolite production was also dependent on polyphenol class and evidence suggests that polymerization and C-ring substitution and aromaticity impacts phenolic acid metabolite production, as indicated by differential metabolism between classes of flavonoids. Further, direct metabolism of phenolic acid metabolites from the flavonoid B-ring

appeared to be favored over alpha and beta-oxidation reactions. These results will help translate *in vivo* pharmacokinetic studies and also predict pharmacokinetics of polyphenol metabolites of cranberry products that may have differing compositions of polyphenols.

CHAPTER VI

PROCESSING TANNIN-RICH FRUITS FOR HIGHER QUALITY AND INCREASED

BIOAVAILABILITY*

Introduction

Mangos are commercially produced in over 80 countries and are among the most

commonly consumed fruits worldwide (Sivakumar *et al*., 2011). Mangos are consumed in a

variety of ways, either as a whole fruit or processed into juice, puree, nectar, jam and dried slices

(Delgado & Fleuri, 2016). Beyond their sweet and tropical fruit flavor, mangos are attractive to

consumers because of their potential health benefits from their high fiber content, pro-vitamin A

carotenoids, and an abundance of polyphenolics (Kalia & Rajinder, 2017).

Polyphenolics have been investigated for their potential to prevent diseases and there is

evidence that suggests they help prevent cardiovascular and neurodegenerative diseases, cancer,

and diabetes (Guasch-Ferre *et al*., 2017, Kotecha *et al*., 2016, Rienks *et al*., 2017, Pasinetti *et al*.,

2015). Among the different polyphenolic classes mangos predominantly contain gallotannins, a

high molecular weight polymer of gallic acid (GA) esterified to a polyol, most commonly

glucose (Smeriglio *et al*., 2017). Although gallotannins have demonstrated anti-inflammatory,

anti-carcinogenic, and anti-mutagenic properties they have also shown hepatotoxic activity and

may cause reduced protein digestibility or iron bioavailability (Chunga *et al*., 1998). For these

reasons, attempts to limit the presence of tannins in commercial foods and animal feed are common.

In vitro digestion models are a useful tool to assess the chemical changes that polyphenols undergo during digestion related to their chemical stability as opposed to metabolomic transformations. Digestion models can vary in the biological processes they represent such as the inclusion and/or exclusion of oral, gastric, and intestinal phases, time of digestion, and physicochemical conditions such as pH and the use of digestive enzymes (Minekus *et al.*, 2014). Many polyphenolics are considered to have low absorption from foods and *in vitro* modeling can serve to identify the extent to which absorption is influenced by the physiochemical conditions of the intestines, enzymatic activity, and interactions with macro- and micronutrients during digestion, or the bioaccessibility of polyphenols from food matrices (Ozdal *et al.*, 2016).

Hydrolysable gallotannins can be depolymerized by tannase (tannin acyl hydrolase E.C. 3.1.1.20), which acts on both ester and depside bonds to release GA (Raghuwanshi et al., 2013, Zhang et al., 2015). Tannase has been used in the food industry as a processing aid to resolve food and beverage quality issues caused by hydrolysable tannins including clarification of alcoholic and coffee beverages (Belmares et al., 2004), prevention of tea creaming along with color and flavor enhancement (Lu et al., 2009) and as a de-bittering agent in fruit juice preparations (Sharma et al., 2014). The use of tannase is proposed to be a viable option to enhance the bioaccessibility of GA in mango products as long as the resultant chemical composition does not adversely impact quality during storage. It is hypothesized that the hydrolysis of gallotannins into GA will result in an increase of bioaccessible GA, or GA solubilized in digesta (Dufour et al., 2018). Therefore, it was investigated if tannase impacts

133

mango juice auto-oxidative browning as well as GA bioaccessibility during oral, gastric, and intestinal phases of *in vitro* digestion.

Materials and Methods

Chemicals

Chromatography standards of GA, 1,2,3,4,6-Pentagalloyl glucose (5-GG) and L-ascorbic acid (L-AA), porcine pancreatin (4 x USP), and porcine bile were acquired from Sigma Aldrich (St. Louis, MO, USA). Gastric juice, formic acid, and HPLC grade methanol and water both containing 0.1% formic acid were purchased from Fisher Scientific (Hampton, NH, USA). Ethanol was purchased from Koptec (King of Prussia, PA, USA). Tannase isolated from *Aspergillus oryzae* (EC 3.1.1.20) was obtained from the Kikkoman Corporation (Chiba Prefecture, Japan).

Mango Juice Preparation

Fresh Mexican mangos cv. Ataulfo were shipped refrigerated to the Department of Nutrition and Food Science at Texas A&M University as mature, green fruit. Fruit were ripened under ambient conditions and edible pulp was cut from the fruit, vacuum-sealed in plastic bags, and stored at -20°C until processed into juice. 0.02% (v/w) cellulase (Validase TRL, DSM, USA) and 0.01% (v/w) pectinase (Crystallzyme 200 XL, DSM, USA) were added to homogenized pulp and incubated for 3 h at 40°C to liberate juice. The pulp was then centrifuged at 3,500 *g* for 20 min and the supernatant was collected as juice and clarified under a mild vacuum through a 0.5 cm bed of diatomaceous earth previously washed with water acidified with 0.2% citric acid. The clarified juice was fortified with an additional 200 mg L^{-1} GA equivalents (GAE) of gallotannins that was extracted from mango pulp with ethanol, evaporated, partitioned from a reversed phase C_{18} cartridge (Waters Corporation, Milford, MA, USA), and dissolved in

134

water. The fortified juice was adjusted to pH 3.8 with citric acid, immersed in boiling water for 10 min to pasteurize and inactivate residual enzymes, and cooled in an ice-water bath.

Simulated In Vitro *Oral, Gastric and Intestinal Digestion of Mango Juice*

Clarified, pasteurized mango juice was subjected to *in vitro* digestion using an adapted model by Green *et al*. (2007). A mixture of 2 mL of mango juice, with or without 1 mg L-AA, 2 mL of gastric juice, and 1 mL 0.9% saline solution was adjusted to pH 2.0 ± 0.1 with 1 N HCl in a 15 mL falcon tube. An aliquot was then taken as the baseline (0 hr). After the 0 hr time point was collected, 0.5 U ml $_{-1}$ of tannase was added, the headspace was flushed with nitrogen, and the digesta was incubated in the dark at 37°C in a shaking water bath. Additional aliquots were taken after 0.5, 1, and 2 hr. After 2 hr, to begin the intestinal phase of digestion, 2 mL of an intestinal enzyme solution (2 mg mL$_{-1}$ pancreatin and 12 mg mL$_{-1}$ bile), 1 mL 0.2 M NaHCO$_3$ added to raise the pH to 7.0 ± 0.1, and 0.9% saline solution was then added until the final volume was 10 mL. Following a nitrogen flush the incubations were held for an additional 8 hr and aliquots were systematically removed for polyphenolic evaluation. Digestions were stopped by adding methanol acidified with 1% formic acid and held frozen at -20°C until analysis. To simulate digestion with the addition of an oral phase, 2 mL of mango juice was mixed with 1 mL of human saliva before mixing with 2 mL of gastric juice. 2 mL of mango juice mixed with 1 mL 0.9% saline solution was used to serve as the control. Gastric and intestinal digestion were then further carried out as previously described by the adapted method of Green *et al*. (2007). Saliva was collected from 2 non-smoking volunteers of the opposite sex and was centrifuged at 10,000 *g* for 10 min to remove insoluble material. To simulate how tannase effects protein-tannin interactions in the oral phase of digestion, 400 µL of saliva free of insoluble solids was mixed with 400 µL of 100 mg L $_{-1}$ GAE of gallotannins that were isolated from mango prepared as

described by Strumeyer & Malin (1975). The saliva and mango gallotannin isolate were centrifuged for 10 min at 10,000 g to form a pellet. The supernatant was subsequently removed and the pellet was washed with DI water, centrifuged, and weighed once the supernatant was discarded. 1 U ml $^{-1}$ of tannase was added to the pellet and incubated for 2 hr and a control that did not contain tannase was incubated in parallel. After 2 hr, the pellets were weighed again and re-solubilized to analyze via LC-MS.

Mango Juice Color and Polyphenol Storage Stability

The storage stability of polyphenols in gallotannin-fortified mango juice was evaluated in the presence and absence of tannase and/or fortification with L-AA. Clarified mango juice was divided into two and one half of the juice was treated with 167 U 100 mL^{-1} tannase compared to an untreated control both held for 3 hr at 35°C in a static water bath. Following tannase hydrolysis, juices were again divided and half was fortified with 200% of the recommended daily allowance of L-AA compared to a non-fortified control. Sodium azide (50 mg L^{-1}) was then added to prevent microbial growth. Aliquots of the four juices were added in triplicate to 10 mL screw cap borosilicate tubes, flushed with nitrogen, pasteurized at 100°C for 3 min, cooled in an ice-water bath, and stored in the dark at 20°C. Juices were assessed for concentration of GA after 0, 1, 2, 4, and 6 weeks of storage by LC-MS and absorbance at 420 nm to indicate browning.

Identification of Tannase Activity on Surface of Mangos

The presence of naturally-occurring tannase on the surface of fresh, unblemished fully ripe mangos (cv. Ataulfo) was evaluated to see if tannase is naturally present during mango processing. The surface of 3 mangos were repeatedly washed in 100 mL of 0.1 M citric acid buffer at pH 5.5 to solubilize tannase to which 50 mg L^{-1} of 1,2,3,4,6-pentagalloyl glucoside (PGG) was added and allowed to react at 35°C for 0, 10, 30, 60, and 120 min ran in parallel to a

non-enzyme containing buffered control. The reaction was stopped by acidification with 0.1% formic acid in methanol and analyzed by HPLC for GA and other GD and loss of PGG.

HPLC-ESI-MS Analysis

Polyphenolics were analyzed using a Thermo-Finnigan Surveyor HPLC-PDA in tandem with a LCQ Deca XP Max ion trap spectrometer with an ESI source. Compounds were separated on a Kinetex C_{18} 2.6 μm column (150 x 4.6 mm) and eluted by a gradient of 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in methanol (mobile phase B) at a flow rate of 0.45 mL min$_{-1}$. The gradient began with 100% mobile phase A for 2 minutes then decreased to 90% A over 10 minutes, 60% at 25 minutes, 35% at 35 minutes, and 15% at 41 minutes and held for an additional 15 minutes. The capillary temperature was 325°C, sheath gas flow was 8.00, and the source voltage, source current, and capillary voltage were 4.50 kV, 80 μA, and 7.00 V, respectively. GA was quantified using a 6-point external calibration curve of known concentrations of GA ranging from 3-100 mg L$_{-1}$ with an average R_2 of 0.994 ± 0.002.

Statistics

All treatments were performed in triplicate and compared using Tukey's HSD statistical test unless there were only two treatments being compared in which a two-tailed t-test was utilized. Statistical analysis was conducted via JMP statistics software package (NC, USA). Values are reported as mean ± standard deviation.

Results and Discussion

In Vitro *Oral Digestion of Mango Juice and Effect of Tannase on Mango Gallotannins Subjected to Salivary Proteins*

Modeling the oral phase of digestion that includes saliva is often omitted in *in vitro* digestion models, which could lead to the overestimation of bioaccessibility of polyphenols due

to polyphenol-protein interactions (Obreque-Slier *et al.*, 2011). It was investigated whether the addition of saliva during oral digestion would decrease the bioaccessibility of GA. At 0, 1, and 2 hr GA was significantly lower ($P<0.05$) in mango juice when in the presence of human saliva in comparison to mango juice that had no added saliva (Table 12**).** This shows that the presence of saliva can potentially reduce GA bioaccessibility due to protein-polyphenol interactions as evidenced by the lack of solvation of GA in the gastric digesta. During the intestinal phase of digestion (3-10 hr), there were no significant differences between the amount of free GA in orally digested mango juice and mango juice that was not exposed to saliva, with the exception of 8 hr where GA in orally digested mango juice was significantly higher ($P<0.05$) at 152.5 ± 16.1 mg L_{-1} than mango juice that was not exposed to saliva with 118.3 ± 12.9 mg L_{-1} (Table 12). However, GA in mango juice with added saliva sharply decreased after 10 hr which could be due to oxidative degradation of GA which occurs more rapidly in low acid conditions (Friedman & Jurgens, 2000). Polyphenol-protein complexes are favored in the high acid conditions of gastric digestion (Delimont, *et al.*, 2017) and these complexes are more resistant to digestive proteases (Papadopoulou & Frazier, 2004). In accordance with this, it was shown here that saliva impacted GA bioaccessibility during gastric digestion but did not impact the bioaccessibility of GA during the intestinal phase of digestion.

Table 12 Concentration of gallic acid (mg L-1) in mango juice during each hour of digestion. *In vitro* **digestion for each treatment was performed in triplicate and is expressed as mean ± standard deviation.**

hr	0	0.5	1	2	3	4	6	8	10
Control	69.2 ± 7.1	66.3 ± 5.9	71.4 ± 7.8	73.4 ± 5.4	100.48 ± 7.83	107.44 ± 13.69	125.04 ± 4.84	118.26 ± 12.91	139.74 ± 3.51
Saliva	46.1 ± 8.8*	47.0 ± 15.1	49.3 ± 8.74*	54.1 ± 6.8*	80.58 ± 22.66	77.01 ± 43.19	123.27 ± 16.77	152.54 ± 16.14	125.34 ± 9.88

It was investigated whether tannase can improve GA bioaccessibility after gallotannins are subjected to salivary proteins as there is evidence that tannase produced by the colonic microbiota can disrupt polyphenol-protein complexes (Osawa, 1992). Isolated mango gallotannins were combined with saliva and centrifuged to form a pellet and when treated with tannase the pellet decreased in mass by 21.6 ± 9.4 mg (data not shown) in comparison to the non-tannase treated control which did not change. Further, the re-suspended pellet contained a 44.8 ± 23.6 % decrease in gallotannins and also contained GA compared to the control which had no GA detected post-incubation (Figure 47). This demonstrates that tannase can potentially increase bioaccessibility of GA by disrupting polyphenol-protein complexes allowing for the solubilization of GA in gastric and intestinal digesta.
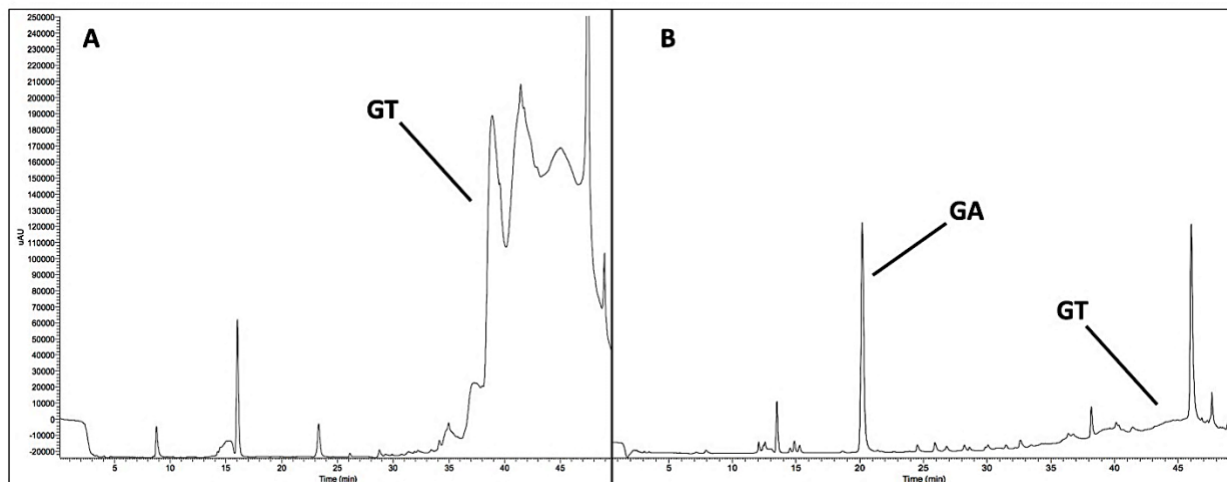
Figure 47 Composition of re-suspended pellet of human saliva and mango gallotannin extract. (A) Pellet that was not treated with tannase prior to re-suspension contains only gallotannins (GT). (B) Pellet treated with tannase prior to re-suspension contains remaining gallotannins and gallic acid (GA).

Effect of Tannase on Gallic Acid Bioaccessibility During In Vitro *Gastric and Intestinal*

Digestion

The ability of tannase to increase the bioaccessibility of GA in mango juice during gastric and intestinal *in vitro* digestion was evaluated. The gastric phase was modeled from 0-2 hr. In the first 0.5 hr, the presence of tannase in mango juice significantly increased (P<0.05) concentrations of GA by 64.8 ± 9.6% from baseline while the concentration of GA in tannase-free juice was unchanged (Figure 48). The increase in GA in tannase treated juice indicated that the enzyme remained active under gastric conditions despite tannase from *Aspergillus oryzae* having an optimal activity near pH 5.0 (Mizuno *et al*., 2014)**.** After 2 hr of gastric digestion, GA increased by 94.3 ± 7.0% from baseline in tannase treated juice and only 6.30 ± 3.4% for the

non-treated juice. The small increase in GA in the control was likely the result of acid hydrolysis of gallotannins. By contrast, Krook & Hagerman (2012) found that 5-GG did not hydrolyze under gastric conditions at pH 1.8 after 1 hr. However, Mosele *et al.* (2016) found that GA content increased by 772% during gastric digestion of *Arbutus unedo* and this was also hypothesized to be due to extensive gallotannin acid hydrolysis. Here it was found that acid hydrolysis does occur to an extent to produce more bioaccessible GA, but tannase lead to a more pronounced increase in bioaccessibility of GA.

After 2 hr, the pH was raised from 2 to 7 along with the addition of pancreatin to simulate intestinal digestion conditions. The estimated time to traverse the small intestine is 2-4 hr post-prandial and the small intestine is the site of absorption for many low-molecular weight polyphenolics, including GA (Hur *et al.*, 2011). The concentration of GA in tannase treated juice continued to increase from hour 2-4 with 56.11 ± 16.46 % and 47.70 ± 27.80% increases of GA for tannase and non-tannase treated juice, respectively (Figure 48). However, the rate at which GA increased for tannase treated juice during upper intestinal digestion was slower in comparison to the gastric phase, despite there being a higher ($P<0.05$) concentration of bioaccessible GA from tannase treated juice in comparison to non-tannase treated juice. Simulated digestion in the colon began after 4 hr without modification of the digestion conditions and mango juice treated with tannase from 4-10 hr continued to experience slower rates of GA production (4.90 ± 4.86%). Tannase treated juice did not exhibit the same rate of hydrolysis as observed in the gastric phase despite a more favorable pH for enzyme function, and this indicated that the activity of tannase was sub-optimal due to inhibition by the excess of GA that had been produced or that smaller galloyl glucose oligomers produced after initial hydrolysis would hydrolyze at a slower rate than larger gallotannins (Bradoo *et al.*, 1997). It has been

141

shown that tannase has a higher affinity and preference for larger gallotannin polymers than lower molecular weight polymers and has slower activity at pH 7 which explains the slower rate of GA production during large intestinal digestion (Rodriguez *et al*., 2008, Mizuno *et al*., 2014). Although there was not as high of a concentration of GA in non-tannase treated juice, the largest increase in GA for the non-tannase treated juice was observed in the large intestinal phase that resulted in an increase of 31.4 ± 16.5% from 4-10 hr. Gallotannins are known to auto-hydrolyze and release free GA in neutral to alkaline pH conditions (Krook & Hagerman, 2012, Mosele *et al*., 2016, Osawa & Walsh, 1993) which could explain the increase in GA in non-tannase treated juice during intestinal digestion.

L-AA fortification did not significantly ($P<0.05$) effect the concentration of GA in the presence or absence of tannase (Figure 48). The reducing capacity of L-AA during gastric digestion was hypothesized to aid in stabilizing GA as it was being liberated from tannins as was previously reported by Green *et al*. (2007). However, the high acid conditions of gastric digestion were sufficient to stabilize polyphenolics and the low acid conditions of the simulated intestinal conditions likely rendered L-AA ineffective as previously observed (Rodriguez-Roque *et al*., 2013). Vallejo *et al*. (2004) reported that L-AA from broccoli inflorescence degraded 91% during *in vitro* digestion and likewise Mosele *et al*. (2016) found that the majority of L-AA degraded during the intestinal digestion phase. Therefore, L-AA did not have any significant impact on GA bioaccessibility during *in vitro* digestion of mango juice.

It is hypothesized that the hydrolytic release of GA by tannase compared to its auto-hydrolytic production in the intestines has the potential to deliver higher concentrations of GA and its metabolites systemically upon consumption. The use of tannase was considerably more effective at increasing the bioaccessibility of GA in all phases of digestion and likely increases

its bioavailability *in vivo*. Tomas-Barberan *et al*. (2007) found that by increasing the concentration of bioaccessible (-)-epicatechin from condensed tannins its bioavailability increased in a porcine model. Further, more than 60% of consumed 1 g kg $^{-1}$ wt. tannic acid has been shown to be excreted via feces of rats *in vivo*, indicating a lack of GA bioaccessibility from gallotannin auto-hydrolysis (Nakamura *et al*., 2003). Tannase helps increase GA bioaccessibility by hydrolyzing GA from gallotannins that would otherwise be excreted/underutilized.
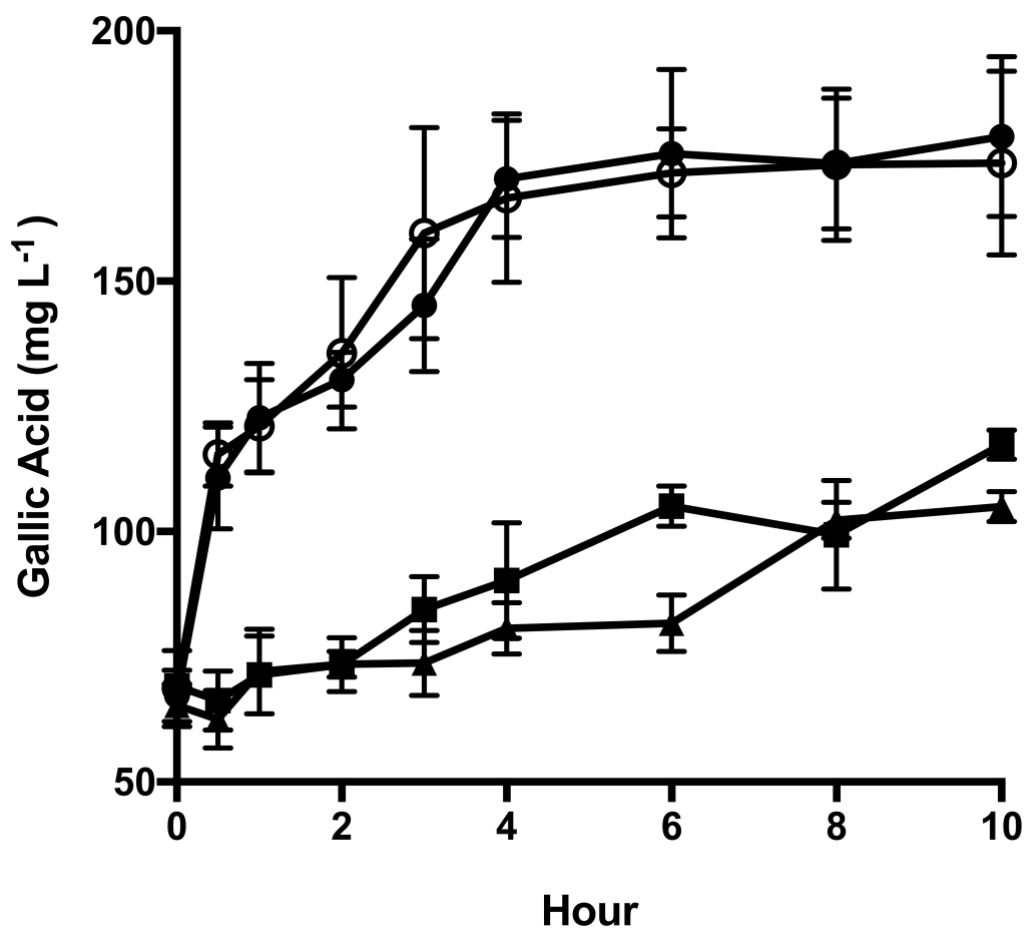
Figure 48 Change in the concentration of gallic acid in mango juice during in vitro digestion. (■) Control, or mango juice without any added L-ascorbic acid or tannase. (▲) Mango juice fortified with L-ascorbic acid. (●) Mango juice treated with tannase during digestion. (○) Mango juice fortified with L-ascorbic acid and tannase during digestion.

Effect of Tannase on Mango Juice Color and Polyphenol Storage Stability

The extent of browning was measured to assess if tannase helped prevent auto-oxidative browning in mango juice and as a result improve its quality. The presence of L-AA initially delayed the onset of browning. After pasteurization and during the first 2 weeks of storage, juice

144

without tannase had significantly (P<0.05) more browning than tannase treated juice. However, by week 4 there were no differences in browning among tannase and non-tannase treated juice (Figure 49) indicating that the action of tannase or addition of L-AA had no adverse effects on the browning of mango juice.
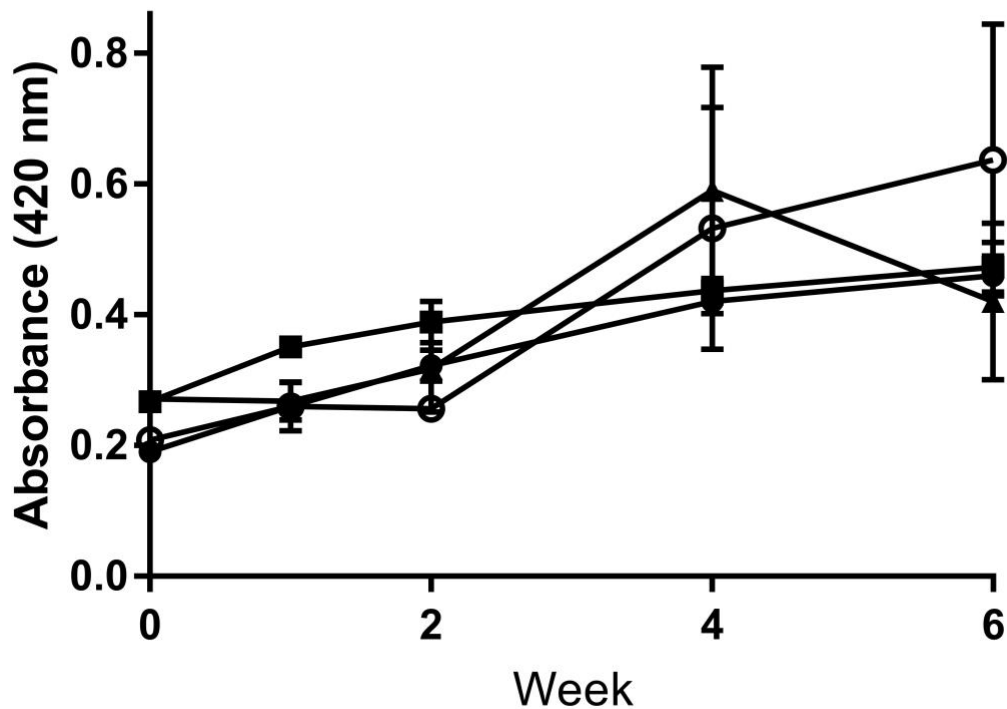
Figure 49 Change in absorbance at 420 nm of mango juice over a 6-week storage period. Juice was stored in the dark at ambient temperature. An increase in absorbance corresponds to a change in color from yellow to brown. (■) Control, or mango juice without added L-ascorbic acid or tannins. (▲) Mango juice fortified with L-ascorbic acid. (●) Mango juice treated with tannase during digestion. (○) Mango juice fortified with L-ascorbic acid and tannase during digestion. Color was analyzed for each treatment and absorbance is expressed as mean ± standard deviation.

Loss of GA was also monitored to ascertain if this was related to browning or the reduction of juice quality. At week 0, tannase treated juice had significantly higher ($P<0.05$) concentrations of GA than non-tannase treated juice with 684.1 ± 20.8 and 581.5 ± 123.4 mg L^{-1} for tannase and tannase treated, ascorbic acid fortified juice, respectively. At week 0 non-tannase treated mango juice and L-AA fortified juice contained 209.4 ± 33.2 and 190.7 ± 36.4 mg L^{-1}, respectively. The concentration of free GA decreased during storage for all four treatments (Figure 50). However, at the end of week 6, tannase treated juice still had significantly ($P<0.05$) more GA present at 376.2 ± 13.0 mg L^{-1}. Mango juice not treated with tannase but fortified with L-AA had the lowest concentration of GA after week 6 with 97.5 ± 25.9 mg L^{-1}, followed by untreated mango juice which had 138.1 ± 16.2 mg L^{-1}. The loss of GA may be due to auto-oxidation of the compound, which is also indicated by the increase of absorbance at 420 nm over time for all of the juices (Figure 50). Auto-oxidation reactions with GA have shown to promote polymeric brown pigments, even in acidic conditions (Singleton, 1987, Tulyathan *et al.*, 1989).
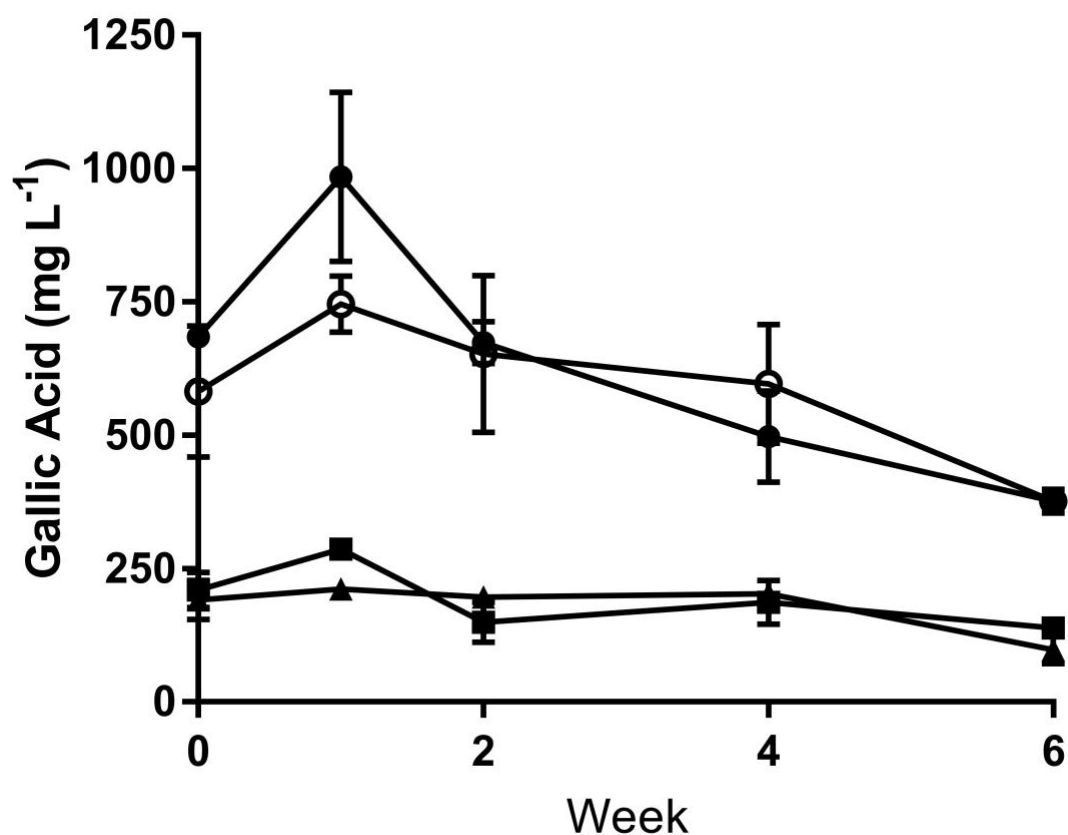
Figure 50 Change in the concentration of gallic acid in mango juice over a 6-week storage period. (■) Control, or mango juice without any added L-ascorbic acid or tannase. (▲) Mango juice fortified with L-ascorbic acid. (●) Mango juice treated with tannase during digestion. (○) Mango juice fortified with L-ascorbic acid and tannase during digestion. Concentration of gallic acid over time for each treatment was assessed in triplicate and is expressed as mean ± standard deviation.

Identification of Tannase Activity on the Surface of Fresh Mango Fruits

It was investigated whether naturally occurring tannase could be found on the surface of

imported mangoes that had been previously subjected to a commercial hot-water thermal

quarantine treatment. Exposure of PGG to mango surface wash water significantly decreased

147

(P<0.05) its concentration in comparison to a buffered control. After 2 hr, there was $99.76 \pm 5.86\%$ recovery of PGG in the control compared to $22.74 \pm 1.69\%$ after treatment with mango wash waters (Figure 51). Hydrolytic products produced from PGG after 2 hr of incubation with mango surface wash water included two tetragalloyl glucosides (*m/z* 787), six trigalloyl glucosides (*m/z* 635), three digalloyl glucosides (*m/z* 483), and free GA (*m/z* 169) (Figure 52) and none of these products were present in the buffer control. It is likely that tannase was expressed by naturally-occurring microbiota on the surface of the mangos, since previous studies have found bacterial families on the surface of mangos that contain tannase-expressing genes (Kalia, 2017). Further, tannase can be found naturally within diverse environments and tannase-producing organisms have been isolated from effluent of tanneries and tea-processing facilities (Wilson *et al*., 2009, Murugan *et al*., 2007, Kumar *et al*., 2016, Vidyalakshimi *et al*., 2016, Kachouri *et al*., 2005). Tannase producing microbes have also been isolated from soil and rotting wood which are other materials with high tannin concentrations (Chowdhury *et al*., 2004). It is probable that tannase can be transferred to edible pulp during handling by consumers or during commercial fruit processing and therefore alter the polyphenolic composition. However, it is unknown how post-harvest quarantine treatments may impact the growth of tannase-producing microbiota and likely depends on growing location and processing conditions (Kalia, 2017).
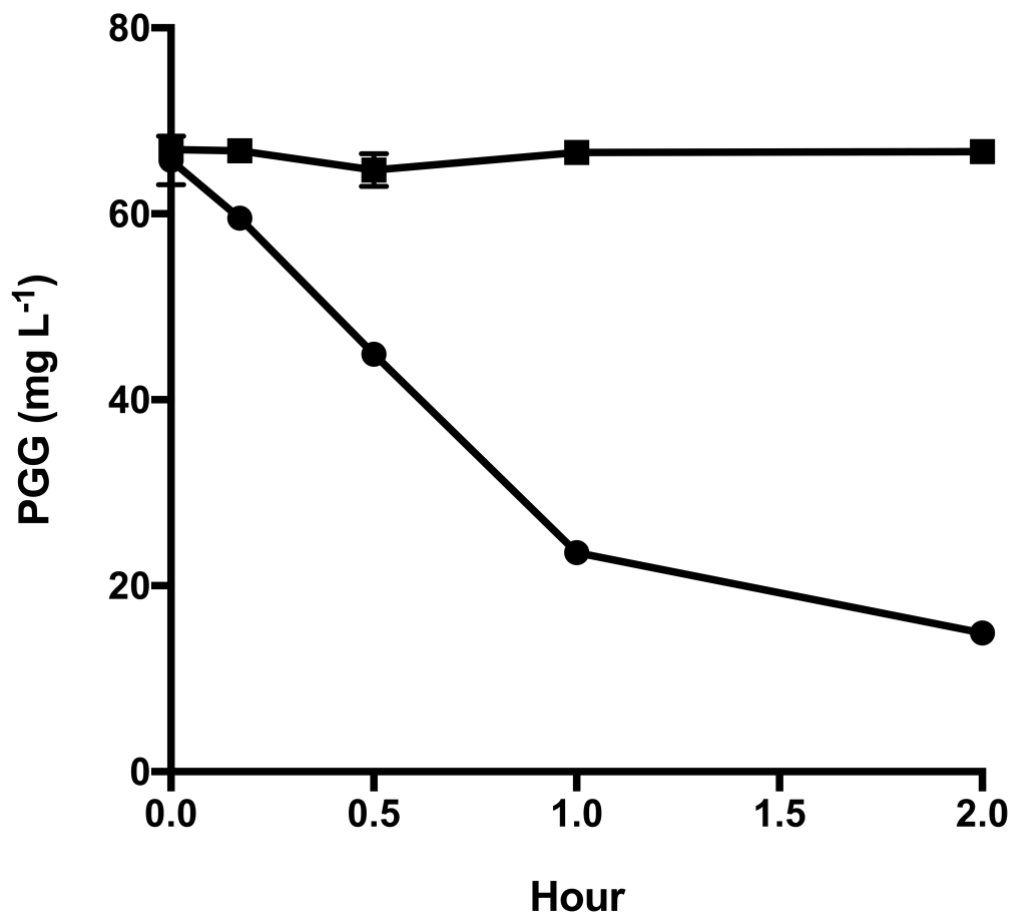
Figure 51 Percent change in the concentration of 1,2,3,4,6-pentagalloyl glucoside (PGG) after 2 hours of exposure to tannase crudely extracted from the surface of mangos.
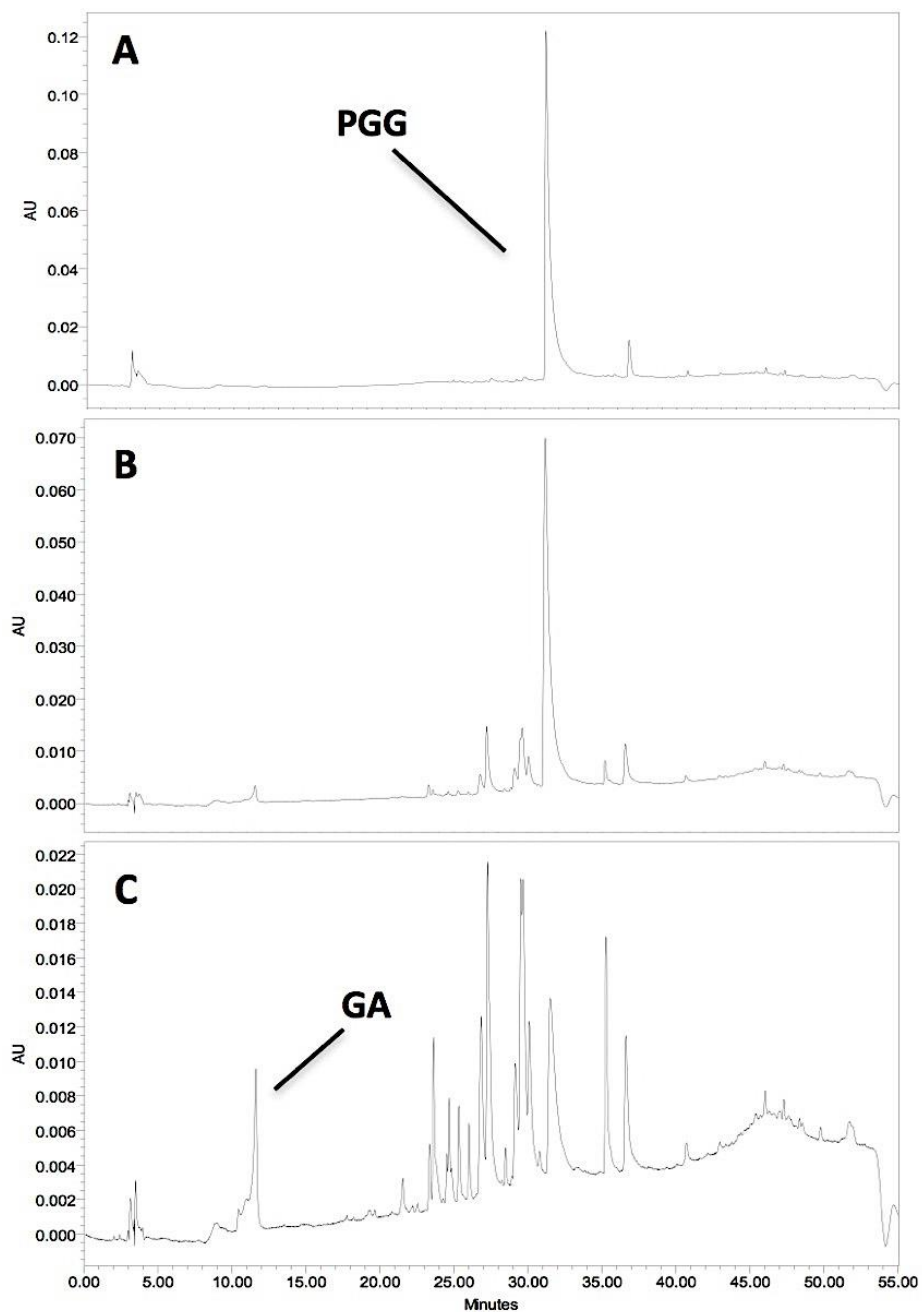
Figure 52 Hydrolysis of pentagalloyl-glucoside (PGG) by tannase from the surface of mangos. (A) PGG after 0 hours of hydrolysis. (B) PGG after 0.5 hours of hydrolysis. (C) PGG after 2 hours of hydrolysis. Gallic acid (GA) and galloyl glucoses of various sizes were formed after 2 hours, or characteristic products of tannase hydrolysis of PGG.

Conclusion

Tannase increased GA bioaccessibility in mango juice without compromising juice color. During the oral phase of *in vitro* digestion, tannase hydrolyzed GA from gallotannins despite their association with salivary proteins, thereby increasing GA that can be absorbed during gastric and intestinal phases. At all of the time points of gastric and intestinal phases of *in vitro* digestion bioaccessible GA was significantly higher ($P<0.05$) in tannase treated juice than mango juice that was not treated with tannase, with or without the addition of L-AA. There were also no differences in color between tannase treated and non-tannase treated mango juice after 6 weeks of storage, yet the tannase treated mango juice had significantly higher GA. Tannase can be a viable processing and/or digestive aid for the production of quality mango juice with more bioaccessible GA.

CHAPTER VII

SUMMARY AND CONCLUSIONS

Tannins are polymers of both phenolic acids and flavonoids that, due to their large size, localize in the large intestine where they are metabolized by the colon microbiome. The metabolism of cranberry proanthocyanidins by the microbiome, simulated with fecal microorganisms, resulted in the production of 3,4-dihydroxyphenyl-gamma-valerolactone, 3,4-dihydroxyphenyl propionic acid, hydroxyphenyl propionic acid, 3,4-dihydroxyphenyl acetic acid, hydroxyphenyl acetic acid and 3,4-dihydroxy benzoic acid as metabolites. The effect of bacterial dysbiosis on cranberry proanthocyanidin metabolism was also evaluated utilizing fecal donors who had been diagnosed with ulcerative colitis (UC), a form of inflammatory bowel disease. Bacterial dysbiosis was confirmed in the UC group and was characterized by decreased abundance of *Ruminococcaceae* and increased abundance of *Enterobacteriaceae*. The UC group produced significantly lower concentrations of cranberry proanthocyanidin metabolites in comparison to healthy fecal donors and at differing rates. In particular, the UC group had lower dehydroxylase activity in comparison to healthy microbiomes. Observing the disappearance of the parent proanthocyanidins, differences in the production of polyphenol metabolites between UC and healthy microbiomes resulted from differential metabolism of procyanidin dimers and their dimeric microbial metabolites. Cranberry proanthocyanidins modulated the microbiome of both UC and healthy microbiomes by increasing the abundance of *Coriobacteriaceae*, which contains genera that can metabolize proanthocyanidins. Proanthocyanidins may then modulate the microbiome by selecting for the growth of proanthocyanidin-resistant bacteria that can metabolize proanthocyanidins.

152

Other classes of polyphenols are metabolized into the same microbial metabolites as proanthocyanidins, therefore cranberry polyphenols were fractionated by polyphenol class in order to identify which metabolites are genuinely derived from proanthocyanidins. Hydroxyphenyl propionic acid, 3,4-dihyroxyphenyl acetic acid, 3,4-dihydroxy benzoic acid and benzoic acid were identified as metabolites from procyanidin A2 and an a-type trimer isolated from cranberry. Considering that 3,4-dihydroxyphenyl-gamma-valerolactone was not identified as a metabolite of isolated cranberry a-type proanthocyanidins, this demonstrated that type of interflavan bond influences how proanthocyanidins are metabolized.

Understanding the metabolism of tannins by the microbiome allows for the development of improved processing strategies for functional foods. As tannin degree of polymerization increases, bioavailability, bioaccessibility and metabolism by the microbiome decreases. Therefore, tannase was investigated for its ability to increase the bioaccessibility of gallic acid from gallotannins in mango juice while maintaining juice quality. Tannase was able to increase gallic acid bioaccessibility ~2-fold, and promote the absorption of gallic acid in the small intestine. Tannase also did not cause any detriment to the quality of mango juice. The hydrolysis of tannins into monomers and oligomers can then be a processing strategy utilized to increase the bioaccessibility of tannins and their metabolites.

Overall, tannin metabolism by the human intestinal microbiome is dependent on class, degree of polymerization, type of bond between monomers and the microbiota composition of the individual.

153

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