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Potential influence of blueberry and black raspberry pomace phenolics on inflammatory cytokines in coronary cells

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POTENTIAL INFLUENCE OF BLUEBERRY AND BLACK RASPBERRY POMACE PHENOLICS ON INFLAMMATORY CYTOKINES IN CORONARY CELLS

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This dissertation is dedicated to two great men that have made a difference in my life. To my father Carlos, who instilled in me the value of education and shares my success from Heaven; and to my husband Franklin, who believed in my dreams and supported me with unconditional love through the road that has brought us here today.

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To you, the reader, just some words of wisdom in the language of my predecessors: "*In papalotl, in huitzitzilin patlanih: xochitemoah. In momachtique noyuhqui patlanih: tlamatiliztemoah."* (The butterfly and the hummingbird fly: they search for flowers. Those who study also fly: they are in pursuit of knowledge). *Miguel León Portilla*

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ABSTRACT

Elevated free fatty acids (FFAs) in obesity are known risk factors associated with chronic inflammatory conditions including cardiovascular inflammation and thus cardiovascular disease (CVD), the leading cause of death in the US. Anthocyanins from berries have the potential to provide protection against inflammation in various tissues; however they are poorly absorbed and undergo major transformations by the microbiome. The work presented in this dissertation involves the development and utilization of an *in vitro* model to characterize the products of colonic fermentation and absorption of blueberry and black raspberry pomace phenolics, and evaluate the potential efficacy of these products compared to anthocyanin-rich extracts as anti-inflammatory compounds in fatty acid-induced inflammation. A previous study identified myristic and palmitic acids as the most inflammatory, and linoleic acid as least inflammatory. In the first study, a model of colonic fermentation and transport through Caco-2 cells was used to evaluate the changes in phenolic profile of blueberry and black raspberry pomaces. Phenolic acids deriving from anthocyanin degradation were identified as the major products of berry pomace digestion and absorption. The second study involved the use of palmitic acid-stimulated HCAE and HCASM cells to evaluate the anti-inflammatory properties of anthocyanin-rich extracts and phenolic acids produced by microbiota fermentation and transport across Caco-2 monolayers using the prevention and intervention approaches. Protein and gene expression of inflammation markers demonstrate that phenolic acids derived from fermentation and absorption of both blueberry and black raspberry pomaces were equally or more effective than anthocyanin-rich extracts in modulating the inflammatory response through a proposed effect on NF-κB transcription, especially when used in the prevention

approach. This suggests that phenolic acids may be responsible for some of the beneficial effects of blueberries and black raspberries. We have also demonstrated that berry pomaces, could be utilized in foods, providing anthocyanins and phenolic acids that may prevent inflammation in cardiovascular cells.

CHAPTER 1
LITERATURE REVIEW

1.1 INTRODUCTION

Blueberries and caneberries are some of the oldest fruits known to humans and have been used not only for survival but medicinal purposes. Caneberries is a term encompassing many common berries that grow on leafy canes at temperate climates around the world, including raspberries, marionberries, blackberries, and boysenberries.[2] These berries are widely consumed in the US, with per capita consumptions of 0.09 lb blackberries, 1.5 lb blueberries, and 0.75 lb raspberries according to the data from 2009.[3] Blueberries and caneberries are rich in phenolics, especially anthocyanins, which give them their characteristic red, blue, or purple pigments and represent major sources of antioxidant capacity.[4] Daily intakes of anthocyanins in the United States had been estimated to range according to season from 180-215 mg/d,[5] however the latest estimations indicate that anthocyanin consumption in the US is 12.5 mg/day/person.[6] Studies of dietary anthocyanins have suggested various cardiovascular benefits, including vascular anti-inflammatory effects and influence on blood vessels and platelets.[7-9] However, neither the mechanisms of action for the parent anthocyanins nor the metabolites responsible for these anti-inflammatory effects are well understood.

As public awareness of dietary effects on health and wellness increases, consumers are seeking foods to help preserve their health. Consumers are now considering “functional foods” as alternatives or complements to Western medicine. Functional foods focus more on prevention rather than intervention. For example, in the United States 6 out of 10 consumers take some type of food supplement with the main objectives of feeling better, preventing

illness, and living longer.[10] It has been estimated that consumption of functional foods that contain effective bioactives in the US could result in annual health cost savings of $2 to $3 billion from anticipated prevention and reduction of the incidence of chronic disease.[11] However, more detailed fundamental research is necessary to understand how bioactive compounds in foods are digested, absorbed, metabolized and excreted in the body in order to establish science-based claims that ethically and factually inform the consumers of the benefits of the wide variety of potentially bioactive compounds found in foods.

1.2 REVIEW OF LITERATURE RELEVANT TO THIS APPLICATION

1.2.1 Cardiovascular Disease (CVD)

Cardiovascular disease (CVD) is a group of disorders of the heart and blood vessels which include coronary heart disease, cerebrovascular disease, peripheral arterial disease, rheumatic heart disease, congenital heart disease, deep vein thrombosis, and pulmonary embolism.[12] CVD is the number one cause of death in the United States and the world, accounting for close to 25% of all US deaths in 2009 (down 3.7% from the previous year) and 30% of the World deaths in 2008.[13,14] Additionally, 8.1% of Americans have self-reported having some type of CVD.[15]

According to the American Heart Association, atherosclerosis, a form of CVD, is a process in which fat, cholesterol, cellular waste, calcium and other substances build up in the inner lining of an artery; rupture of this plaque causes blood clots that can block blood flow to the heart (heart attack), brain (stroke) or limbs (gangrene).[16] It was previously believed that atherosclerosis was primarily a “plumbing” problem, where plaque buildup would eventually

block the flow of blood thru the vascular system. However, research has revealed that atherosclerosis derives from an inflammatory process, where proinflammatory stimuli trigger the expression of adhesion molecules and chemoattractant factors (such as MCP-1) that allow the infiltration of leukocytes to the intima and the maturation of monocytes into macrophages.[17,18] Macrophages then engulf modified lipoproteins and are converted into foam cells, while other macrophages and T cells continue to amplify the inflammatory response by the secretion of various cytokines and growth factors that also promote the migration and proliferation of smooth muscle cells (SMCs) forming a fibrous cap over the foam cell cluster. Eventually, inflammatory mediators weaken the fibrous cap and make it prone to rupture while procoagulant tissue factor is produced. When the fibrous cap is ruptured, the tissue factor is released forming a thrombus that can then create blockage in blood vessels as described before.[17]

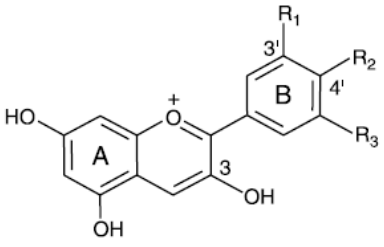
Research has shown that circulating free fatty acids (FFAs) induce inflammation, thus making them a risk factor for CVD. An increase in plasma FFA concentrations induces proinflammatory changes and oxidative stress including an increase in nuclear factor-κB (NF-κB) binding activity and p65 expression without changes in inhibitor κB (IκB) in circulation mono-nuclear cells (MNCs). Reactive oxygen species (ROS) are generated by MNCs and polymorphonuclear leukocytes (PMNs) in subjects with high levels of FFAs [19]. An increase in macrophage migration inhibitory factor (MIF), a proinflammatory cytokine, and an impairment of the endothelium-dependent flow-mediated vasodilation was also observed as a result of plasma FFA increase. Another study based on the Atherosclerosis Risk in Communities study, showed that FFA levels were positively associated with an inflammation score quantifying levels

of six systemic inflammation markers (interleukin-6, C-reactive protein, orosomucoid, sialic acid, white cell count, and fibrinogen) [20].

1.2.2 Bioactive Compounds in Blueberries and Caneberries

Several studies have identified different bioactive compounds in blueberries and caneberries, mainly phenolics, however it is still not clear which compounds are the most effective in protecting cardiovascular health or the mechanisms by which these compounds produce an anti-inflammatory effect.

Anthocyanins represent 57-93% of the total phenolics found in blue and caneberries.[4] Anthocyanins are a group of flavonoids commonly found in nature and present in many fruits and vegetables as glycosides or acylglycosides of their respective aglycone anthocyanidins, which are not usually found in fresh plant material.[6,21] Six major anthocyanidins are widely found in plants, namely cyanidin (Cy), delphinidin (Dp), malvidin (Mv), peonidin (Pn), pelargonidin (Pg), and petunidin (Pt).[6] All anthocyanins share the same basic anthocyanidin skeleton (Figure 1.1), however they vary in the number and position of hydroxyl/methoxyl groups on the skeleton, number and positions of the attached sugars, and the extent and identity of sugar acylation.[22]



Anthocyanidin	R_1	R_2	R_3
Pelargonidin	H	OH	H
Cyanidin	OH	OH	H
Delphinidin	OH	OH	OH
Peonidin	OMe	OH	H
Petunidin	OMe	OH	OH
Malvidin	OMe	OH	OMe

Figure 1.1. Basic structure of six major anthocyanins

Major anthocyanins for blueberries and caneberries have been identified (Table 1.1), namely cyanidin-3-glucoside in blackberries, malvidin 3-galactoside and malvidin 3-glucoside in blueberries, cyanidin 3-sophoroside in red raspberries, and cyanidin 3-rutinoside in black raspberries.[21,23-25] Procyanidins, ellagitannins, flavonols, and ellagic acid derivatives have also been identified as flavonols present in these fruits.[23] Identified phenolics include flavonols kaempferol, quercetin, and myricetin, and phenolic acids chlorogenic acid, p-coumaric acid, caffeic acid, ferulic acid, *p*-hydoxybenzoic acid, gallic acid, and ellagic acid.[24,26]

Table 1.1. Average concentration of anthocyanins grouped by aglycones in US blueberries and caneberries. Adapted from Wu et al., 2006.[6]

Fruit	**mg anthocyanin/100 g fresh fruit**							**Total anthocyanins per serving (mg)**
	Delphinidin	Cyanidin	Petunidin	Pelargonidin	Peonidin	Malvidin	Total	
Blackberry		244		0.7			245	353
Marionberry		297.7		1.7	1.1		300.5	433
Blueberry, cultivated	120.7	28.6	71.9		34.2	131.3	386.6	529
Blueberry, wild	141.1	66.3	87.6		36.9	154.6	486.5	705
Chokeberry		1478		2.3			1480	2147
Raspberry, black		669		16.7	1.1		687	845
Raspberry, red		90.2		1.9			92.1	116

1.2.2.1 Anthocyanin Absorption and Excretion

In vitro studies have focused on the differential absorption of anthocyanins based on their differences in aglycones, attached sugars, and chemical structure. Transport efficiency using Caco-2 cell monolayers was 3-4%, with delphinidin 3-glucoside having the lowest absorption/transport efficiency (0.72-1.18%) and malvidin 3-glucoside the highest (2.01-6.03%). Additionally, this study showed that anthocyanin bioavailability is reduced by more free hydroxyl groups and less OCH_3 groups, and increased by attached glucoses rather than galactoses.[27]

Anthocyanin absorption and excretion studies carried out in humans agree that absorption of ingested anthocyanins is very low (ppm), and may only raise human plasma concentrations to the ng/ml order of magnitude. A study carried out in humans and rabbits fed with black currant juice showed that both species absorbed a larger proportion of the anthocyanin rutinosides than glucosides, and absorption in humans was proportional to dose. Maximum anthocyanin plasma levels were observed at 30-45 minutes, for a maximum level of 50 ng/ml.[28]

In another study, individuals fed freeze-dried blueberry powder also absorbed most of the individual anthocyanins, with serum concentrations of total anthocyanins ranging 5.43-16.9 ng/mL and peak concentrations at 4 h after consumption (average 13.1 ng/mL), however this represented only 0.002-0.003% of the ingested amount. The most abundant anthocyanins found in plasma after blueberry consumption were malvidin 3-galactoside, malvidin 3-glucoside, and delphinidin 3-glucoside (27, 20, and 10% of the total anthocyanins, respectively).[29]

Studies carried out in Japan had also shown low absorption after intake of pure anthocyanin preparations. In the first study average plasma concentration peaks were 11 μg/l cyanidin 3-glucoside and trace amounts of cyanidin 3,5-diglucoside, but no free aglycones. In the same study, anthocyanins were found in rat livers with peaks of 150 pmol/g at 15 min after consumption of a dose of 320 mg/kg.[30] In the second study, black currant anthocyanin peaks ranged 5-73.4 nmol/l between 1.25-1.75 h after intake, with variations due to the individual anthocyanin, however the anthocyanins were found in their intact forms.[31]

Human urinary excretion of anthocyanins up to 8 h after consumption of a pure anthocyanin preparation have been reported as 0.05% of ingestion.[31] Urinary excretion of anthocyanins from juice has also been reported to represent only 0.035% during the first 4 h after ingestion.[28] Thus, by calculation we can determine that there are more than 90% of consumed anthocyanins not accounted for that may reach colon.

1.2.2.2 Microbial Anthocyanin Metabolism in the Gut

Metabolism of phenolics, including anthocyanins, by gut microflora has been previously investigated. A study carried out in France supplemented rats with red wine powder, and identified microbial metabolites in the rats' urine, including benzoic acids (hydroxybenzoic, hippuric, vanillic), phenylacetic acids, phenylpropionic acids, *p*-coumaric acid, cinnamic acids (caffeic, ferulic), and catechins. Most of these microbial metabolites were not initially present in the supplemented powder.[32]

In vitro studies have also demonstrated microbial metabolism of anthocyanins. A Finnish study treated pure anthocyanins (Cy-3-glu and cy-3-rut) with human fecal inoculum, and identified cy-3-gluc and cyanidin aglycone as intermediary metabolites, followed by conversion to protocatechuic acid as the major metabolite. Most anthocyanins disappeared after 1 h of treatment with 5% fecal inoculum, indicating that microbial degradation involves deglycosylation and breakdown of the anthocyanidin heterocycle.[33] These results were confirmed by later studies using porcine and human fecal inoculums, where anthocyanins were also deglycosylated and hydrolized into metabolites incluiding protocatechuic acid (from cyanidins), syringic acid (from malvidins), vanillic acid (from peonidins), 4-hydroxybenzoic acid (from pelargonidis) and phloroglucinol aldehyde, for a total disappearance of anthocyanins

after 0.5-2 h of treatment.[34,35] Phenolic acids derived from the B ring of the correspondent anthocyanidin, while aldehydes derive from the A ring.[35]

All authors suggested that these metabolites, rather than their intact precursors, may be responsible for the beneficial effects of anthocyanins, since these metabolites are more readily absorbed and resistant to further metabolism.[32-35] However, models utilizing anaerobic fermentation for the production of these metabolites from berry pomaces have not been explored.

1.2.2.3 Anthocyanin Toxicology

Limited studies have been performed on the toxicity of anthocyanins. An initial *in vitro* study tested a cyanidin 3-glycoside rich extract from black carrot on HT29 human colon cells, and observed toxicity after 24 h in the form of reduced cell viability (88-175 μM) and cell growth (45 μM), and induction of DNA damage (88-175 μM).[36] In another study, an anthocyanin-rich extract from strawberries (mainly pelargonidins) showed to low toxicity in human prostate cells. Only a 10% decrease in cell survival was observed at 25 μg extract/ml media for both tumorigenic and normal prostate cells. In contrast, 4-6 μg/ml of the total strawberry extract, containing other phenolics, displayed a 50% reduction in survival for both prostate and breast cells.[37]

Hormesis, a non-linear toxicity effect, has been proposed as an alternative regulation mechanism by which plant phenolics such as chalcone, ferulic acid, epicatechin, and luteolin exert their health benefits.[38] Hormesis is defined as a "dose-response relationship phenomenon characterized by low-dose stimulation and high-dose inhibition" (Figure 1.2), in other words it is a U-shaped dose-response curve in which low doses of a compound cause a beneficial effect,

while high doses induce toxicity.[1,39,40] This is not a new concept, as Paracelsus (1492-1591), had first described that the toxicity of any substance was dependent on dose, and noted that various toxic substances could be beneficial in small quantities with his famous quote "All substances are poisons; there is none which is not a poison. The right dose differentiates a poison from a remedy".[41] Further research is required to determine the hormetic effects of anthocyanins.

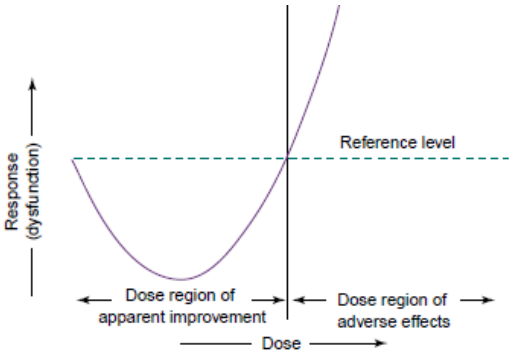


Figure 1.2. The classical hormetic U-shaped dose response illustrating a reduction of dysfunction at low doses.[1]

1.2.3 Anthocyanins and Heart Health

Numerous health benefits of anthocyanins have been reported including improved brain function, modulation of obesity, antioxidant effects, improvement of cardiovascular function, cancer prevention, and anti-inflammatory effects.[22] We will focus this review on anti-inflammatory effects and the improvement of cardiovascular function.

The anti-inflammatory effects of anthocyanins have mainly been studied *in vitro*. The aglycones delphinidin and cyanidin, but not malvidin, peonidin, or petunidin, have shown to inhibit LPS-induced COX-2 expression in murine macrophages, possibly due to the *o*-dihydroxyphenyl on the B ring of the anthocyanidin structure.[42] Delphinidin dose-dependently inhibited COX-2 expression, IκB-α degradation, p65 nuclear translocation, c-Jun phosphorylation, and the activators of MAPK (JNK, ERK, and p38 kinase); thus inhibition of COX-2 expression by delphinidin occurred through the blockage of pathways mediated by MAPK,

including the activation of nuclear factor-κB (NF-κB), activator protein-1 (AP-1) and CCAAT/enhancer-binding protein (C/EBPd).[42]

Cyanidin, but not its correspondent anthocyanins (cyanidin 3-glucosylrutinoside, cyanidin 3-rhamnoside, cyanidin 3-glucoside) from tart cherries, have demonstrated anti-inflammatory effects by inhibiting the enzymes prostaglandin endoperoxide H synthase-1 and -2 (PGHS-1, and -2), which are responsible for the conversion of arachidonic acid to prostaglandins. Cyanidin IC_{50} against PGHS-1 and PGHS-2 was 90 and 60 mM respectively (compare to naproxen and ibuprofen, with IC_{50} 11 and 25 mM).[43] Similarly, cyanidin (5 µM) and anthocyanins isolated from tart and sweet cherries, cranberry, bilberry, elderberry, strawberry, blackberry, and raspberry have shown to significantly inhibit COX-1 and COX-2 enzyme activity at 125 µg/ml.[44] Cyanidin 3-glucoside has also been observed to dose-dependently reduce LPS-induced NO and PGE_2 in mice eye aqueous humor, with effects observed after anthocyanin intravenous injection of 10mg. These results suggest a decrease in NO-induced COX-2 expression, putatively through suppression of NF-kB activity. Additionally, this effect was stronger when a Russian berry extract, containing cy 3-glu, was administered, indicating synergistic effects between anthocyanins and other phenolics in the anti-inflammatory properties of berries.[45] Similar effects were observed in LPS-stimulated THP-1 monocytes pretreated with cy 3-glu. Pretreatment with as low as 1 µM cy 3-glu significantly and dose-dependently reduced gene and protein expression of iNOS and COX-2, as well as release of NO and PGE_2, by up-regulation of liver X receptor α (LXRα) transcriptional activation. Furthermore, the latter led to suppression of NF-kB activity, and an effect on functional transcription but not nuclear translocation was suggested.[46]

Polyphenol-rich extracts from blueberries at 50 μg/ml have effectively reduced LPS-induced inflammatory mediators inducible nitric oxide synthase (iNOS) and COX-2 in microglial cells (macrophages in brain and spinal cord). The modulation mechanism was through the suppression of the iNOS and COX-2 promoter activity and NF-kB nuclear translocation.[47] Treatment of polymorphonuclear cells (PMN) with 0.01-1 g/l of an anthocyanin-rich juice blend containing mainly cyanidin-based anthocyanins from acai reduced migration towards inflammatory stimuli bacterial peptide f-Met-Leu-Phe (fmlp), leukotriene B4 (LTB4), and IL-8.[48] Treatment of LPS/IFN-γ-activated RAW 264.7 macrophages with cyanidin or blueberry concentrate also reduced NO (31 μM, 125 μg/ml) but increased TNF-α (125 μM, 250 μg/ml) production, a mechanism that has been proposed to be responsible for chemopreventive and anti-inflammatory potential.[49]

Anthocyanin and hydroxycinnamic acid from blueberries and cranberries have been shown to effectively reduce TNFα-induced inflammatory mediators IL-8, MCP-1, and intercellular adhesion molecule 1 (ICAM-1). Pretreatment of human microvascular endothelial cells with these compounds at 0.1 mg/ml significantly reduced IL-8 and ICAM-1, while only anthocyanins reduced MCP-1.[7]

Cyanidin 3-glucoside in the form of blackberry juice or pure anthocyanin has effectively reduced peroxynitrite-induced vascular hyporeactivity in thoracic aortic rings and impairment of the endothelium-dependent relaxations at concentrations as low as 14.5 ppm from juice or 0.0085 μM pure anthocyanins *in vitro.*[50] In contrast, a similar study demonstrated that fractions from red raspberries rich in phenolic acids lambertianin C and sanguiin H-6, but not anthocyanins, promoted vasorelaxation of rabbit thoracic aorta.[51]

In vivo, blueberry supplementation in the diet has resulted in significant cardioprotective and anti-inflammatory effects. Rats fed a 2% blueberry diet for 3 months showed reduced cardiomyocyte mitochondrial permeability transition (MPT) susceptibility to reactive oxygen species (ROS), which was proposed as the cellular mechanism of cardioprotection.[52] Additionally, blueberry fed rats had smaller myocardial infarction (MI) areas and 40% less inflammatory cells (monocytes and lymphocytes) in cardiac tissue following induced ischemia by arterial ligation. Furthermore, blueberry diet, even when started after MI, attenuated cardiac remodeling and MI expansion.[52] Tart cherry anthocyanins have also shown to effectively reduce carrageenan or complete Freund's adjuvant induced paw inflammation in mice gavaged with 85-400 mg anthocyanins/kg bw. The anti-inflammatory effect, measured by paw withdrawal and paw thickness, was comparable to indomethacin at 5 mg/kg, and the mechanism of action was suggested to be the inhibition of COX-1 and -2 mediated synthesis of prostaglandins.[53] In another study, mice fed with 10% or 50% tart cherry juice dilutions for 14 days showed significant improvements in lipid oxidation in liver and superoxide dismutase in liver and blood. Furthermore, COX-2 activity of peritoneal macrophages obtained from these mice was dose-dependently inhibited in 33-41%.[54] In contrast, a previous study did not report significant differences in various markers of coronary health when healthy adults were supplemented with 750 ml/day of cranberry juice. Markers included homocysteine, HDL, LDL, and total cholesterol, triglycerides, plasma antioxidant potential, glutathione peroxidase, catalase, and superoxide dismutase activities.[55] These results, however, may be considered positive, as the subjects preserved their health.

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CHAPTER 2
IN VITRO MODEL FOR ASSESSING THE COLONIC FERMENTATION AND ABSORPTION OF BLUEBERRY AND BLACK RASPBERRY POMACE PHENOLICS

2.1 INTRODUCTION

Obesity is a major health problem in the United States, where according to the latest information from the Center for Disease Control and Prevention (CDC), 35.7% of adults and 16.9% of children and adolescents are obese.[1] It is now widely accepted that chronic inflammation is a major consequence of obesity, and that this type of inflammation may be the underlying cause of many other chronic conditions, to the extent that it has been called "the secret killer".[2] Obesity-induced inflammation has been closely linked with chronic conditions including insulin resistance, type 2 diabetes, and cardiovascular disease.[3,4] The goal of prevention or delay of these conditions has led the scientific community to explore dietary interventions including bioactives derived from fruits.

Fruit juice production, including berry juices, is a growing industry around the world. For example, 17% of the 2008 US blueberry production (around 225 million lb) was allocated in the non-alcoholic beverage industry, while 95% of the Oregon Valley black raspberry production (which accounts for most of the black raspberries produced in the US) is destined to processed products including individually quick frozen (IQF) berries, purees, and juice concentrates.[5,6] Berry juice yields range 50-85%, resulting in at least 15% byproducts, mainly pomace consisting of seeds, hulls, and pulp.[7] These byproducts used to be considered of low value, and were mainly destined for composting or animal feed. However, studies have shown that berry and

grape pomaces are rich in anthocyanins, polyphenols, and dietary fiber, and are potential ingredients for functional foods.[8-10]

Anthocyanins and phenolics derived from berries are recognized as potent antioxidants.[11-13] While antioxidant activity is readily measured chemically the *in vivo* effects of antioxidants remain in doubt. Emerging literature suggests that the anti-inflammatory effects of these compounds may be of greater significance *in vivo*.[14-16] Studies have identified anti-inflammatory effects of polyphenols including anthocyanins in several tissues including lungs, joints, and macrophages by inhibiting the production of nitric oxide and TNF-α.[17-20] Fruits, like berry fruits and fruit pomace contain substantial levels of fiber.[21,22] Fermentable fiber derived from plant tissues can produce short-chain fatty acids (SCFAs) as a result of fermentation by the microbiome. It has been demonstrated that the butyrate produced during the fermentation of dietary fiber is the preferred substrate for colon cells.[23] Furthermore, SCFAs derived from fiber fermentation have been linked to the control of healthy body weight by stimulating the expression of gut hormones peptide YY (PYY) and glucagon-like peptide (GLP)-1 in *in vivo* studies.[24] However, the anti-inflammatory and SCFA-producing effects of berries, and especially their byproducts, have not been explored in depth.

This study evaluated of blueberry and black raspberry pomaces as sources of bioactive compounds including anthocyanins and other phenolics, and fermentable fiber. Models for the fermentation of berry pomaces and absorption of phenolics deriving from the aforementioned fermentation have been established. These models offer low cost and time efficient alternatives to larger-scale *in vivo* testing. Demonstrating the anti-inflammatory and overall health-promoting effect of these byproducts will offer insight for their further use as food

ingredients, thus minimizing waste in juice production and offering a new economic opportunity for juice producers.

2.2 MATERIALS AND METHODS

2.2.1 Materials

Blueberry (*Vaccinium spp.*) and black raspberry (*Rubus occidentalis* 'Munger') pomaces were provided by Milne Fruit Products (Prosser, WA) and were freeze dried in a VirTis Genesis pilot lyophylizer (Warminster, PA). Digestive enzymes, phenolic acids (gallic, protocatechuic, cinnamic, chlorogenic, caffeic, *p*-coumaric, and ferulic), and phloroglucinol aldehyde were purchased from Sigma Aldrich (St. Louis, MO). Human epithelial colorectal adenocarcinoma (Caco-2) cells were purchased from ATCC (Manassas, VA). Cell culture media, buffers, and materials were obtained from Life Technologies (Carlsbad, CA). All other reagents were of analytical or HPLC grade accordingly and obtained from Fisher Scientific (Waltham, MA). Water was Milli Q (Millipore Corporation, MA) for all the studies.

2.2.2 Digestion and Fermentation of Berry Pomaces

Digestion of berry pomaces was based on the method previously employed by Fazzari and others for cherries.[25] In this method, pepsin-HCl simulates gastric digestion, while pancreatin and bile salts simulate the small intestine conditions. Briefly, 100 g of homogenized pomace acidified to pH 2 with HCl (60 g dry pomace + 100 ml water) were mixed with 31500 units of pepsin and incubated at 37 °C while shaking at 100 rpm for 2 h. After incubation, the pH was adjusted to 7.5 with NaOH or $NAHCO_3$, and 20 g of the digest were mixed with 5 ml enzyme solution (pancreatin 4 mg/ml and porcine bile extract 25mg/ml) in a 90 ml

polypropylene screw cap container and incubated as before. The digest was microwaved 3-5 minutes to denature enzymes, filtered through Fisher P8 filter paper, washed with ethanol to remove hydrolyzed sugars and aminoacids, filtered, and dried overnight. The final dry digest was frozen at -20 °C until fermentation could be conducted.

A modified *in vitro* anaerobic fermentation system established by our research group was used to mimic colonic fermentation of berry pomace. Briefly, the system consists in two vessels, the fermentation mixture preparation vessel, and the anaerobic incubation system. Each preparation vessel contains 350 ml of anaerobic solution, consisting in a 9:1 ratio of autoclaved solution A ($NaHCO_3$ 0.14 M, hemin 8.7 µM, menadione 0.39 µM, resazurin 4.4 µM as redox indicator) and solution B (NaCl 0.48 M, K_2HPO_4 0.02 mM, cysteine-HCl 0.63 mM), adjusted to pH 7.5 with NaOH.[26] Fecal inoculum was prepared from freshly collected human feces from healthy donors kept at 0°C within 4 hours of collection then transferred to -80°C. Fifty grams of feces were homogenized with 200 ml of anaerobic solution, filtered through cheese cloth or a filter whirlpak bag, transferred into a sterile bag, and incubated at 37 °C for 1 hour under anaerobic conditions using a BD GasPak™ EZ Pouch System. Following incubation, 50 ml of the anaerobic inoculum was added to each fermenter vessel containing 10 g of digested pomace and the vessels were flushed with 80% N_2, 10% H_2, and 10% CO_2 for 10 min while incubating at 37 °C. An anaerobic strip was added to each vessel to verify the anaerobic conditions. To simulate colonic fermentation conditions, the contents from the incubated vessels were aseptically transferred to 50 ml centrifuge tubes, sealed leaving little or no head space, and placed in a hermetically sealed container using an anaerobic environment

generating pouch system. The container was incubated at 37 °C while shaking for up to 24 h, at which time the vessels were removed for analysis.

The contents of the fermentation vessels were centrifuged, and the supernatants were analyzed by HPLC for anthocyanin and phenolic acid content. Supernatants were also sterile-filtered for transport studies.

2.2.3 Anthocyanin Extraction and Characterization

Anthocyanin rich fractions for cell culture studies were extracted from pomace using the acidified-methanol method.[27] Briefly, 50 g of ground material were homogenized with 100 ml of acidified methanol (0.01% v/v HCl in methanol) and macerated for 1 h. The slurry was filtered by vacuum suction through Whatman 1 filter paper and re-extracted two times until a faint-colored extract was obtained. Methanol was evaporated from the filtrate using a rotary evaporator, freeze-dried, and re-dissolved to a final known volume with sterile water, and sterile filtered. Anthocyanin quantification in this extract was performed by HPLC analysis.[28]

Phenolic and anthocyanin profiles were determined by HPLC using the method previously developed in our laboratory by Jang and Xu.[29] Briefly, the HPLC system consisted in a Discovery C18 column (3 mm i.d. x 25 cm) by Supelco (Bellefonte, PA), Waters 2690 separation module, 996 photodiode array detector, and Millenium32 chromatography manager. The mobile phase consisted in a mixture of 0.4% TFA in water (A) and acetonitrile (B), with the percentage of A=0.4% TFA in water ramped from 100-90% in 5 minutes, and then from 90-45% at 45 min with 0.8 ml/min constant flow rate. Anthocyanin extracts from pomace were used for quantification, and chromatograms were obtained at 264 nm (gallic, protocatechuic, and cinnamic acid), 232 nm (phloroglucinol aldehyde), 314 nm (chlorogenic, caffeic, *p*-coumaric, and

ferulic acid), and 520 nm (anthocyanins). Quantification was achieved through comparison with previously performed phenolic or anthocyanin standard curves.

2.2.4 Cell Culture

Cell lines of heterogeneous human epithelial colorectal adenocarcinoma (Caco-2) were grown using supplemented Cascade Medium DMEM (Gibco, Portland, OR). Cells were fed every other day until 80% confluence and split at 1 to 3 or 1 to 2. Cascade trypsin solution was applied to lift the cells; and it was subsequently neutralized with CMF-PBS + 10% FBS. Cells were spun down and resuspended in media. For all the treatments, cells were incubated at 37 °C with 5% CO_2 in a humidified incubator (Sanyo Biomedical, Wood Dale, IL).

2.2.5 Transport Study

Apical to basolateral transport of anthocyanin rich fractions and pomace digests was assessed using a Caco-2 monolayer model previously established for drug transport studies.[30-32] Briefly, Caco-2 cells were seeded on non-coated Costar Transwell permeable supports with 0.4 mm polycarbonate membrane from Corning (Lowell, MA) at 2.6 x 10^5 cells per cm^2. The plating media (DMEM supplemented with 10% FBS, 1% L-glutamate, 1% NEAA and 5% antibiotic-antimycotic solution) was replaced 6-16 h after plating and then every 48 h up to 21-29 days to allow differentiation. Transepithelial electrical resistance (TEER) of the monolayers was measured routinely before and after the transport experiment using a Millicell®-ERS system (Millipore, Bedford, MA).

The transport experiment was conducted between days 21-29. The compounds of interest (anthocyanin rich extracts or digests) were diluted to 0.1mM in transport medium

consisting in Hank's balanced salts solution (HBSS) supplemented with 25mM glucose adjusted to pH 6.0 (apical) or 7.4 (basolateral) with HEPES or MES accordingly. Monolayers were incubated with "drug-free" transport medium for 20 minutes, after which the medium with the diluted anthocyanins was introduced to the apical side. Aliquots were obtained from the basolateral side every 15-30 minutes for 2 h and the basolateral volume was replaced with fresh medium. The experiment was carried out at 37 °C with 5% CO_2 in a humidified incubator.

Basolateral anthocyanin/phenolic concentrations were determined by HPLC as before to calculate the apical to basolateral permeability. The apparent permeability coefficient (P_{app}), in cm/s, was determined based on HPLC data for the amount of compound of interest transported per time according to the formula $P_{app} = (dQ/dt)(1/(AC_0))$ where dQ/dt is the steady-state flux (mmol/s) based on the slope of the linear area of the absorption curve, A is the surface area of the filter (cm^2) and C_0 is the initial concentration in the donor chamber (mM).[32]

After the completion of the transport experiment, phenolics from the cell layers were extracted based on the method reported by Yi et al.[33] Briefly, the cell layers were rinsed with HBSS 3 times and scraped off the membrane using 0.5 ml of acidified methanol (0.01% v/v HCl). The cells and methanol were collected and sonicated 15 min followed by centrifugation at 2000 xg for 5 minutes. The supernatant was collected and the cells were mixed with 0.5 ml of acidified methanol, sonicated, and centrifuged 2 more times as before. The combined supernatants were evaporated under N_2 and then reconstituted in 0.6 ml of acidified methanol for HPLC analysis.

2.2.6 Statistical Analysis

Results are presented as mean ± SD. Values obtained by HPLC for non-fermented extract characterization are presented as the mean of three repetitions, and had SE ≤ 1% (not shown). Statistical analysis was performed using one-way analysis of variance (ANOVA). Separation of means was performed by the Tukey's Studentized Test (SAS®, version 9.2). Significant differences were considered at an alpha level of 0.5.

2.3 RESULTS AND DISCUSSION

Characterization of phenolics in non-fermented anthocyanin-rich extracts deriving from blueberry and black raspberry pomaces (Figure 2.1) revealed three major phenolics, namely anthocyanins, gallic acid, and anthocyanidins (only found in black raspberry pomace extract), and two minor phenolics, protocatechuic acid and p-coumaric acid. Further characterization of anthocyanins (Table 2.1) revealed that blueberry anthocyanins were 57.6% malvidins and 21.5% cyanidins, while close to 95% of black raspberry anthocyanins were cyanidins. The major anthocyanins found were malvidin-3-galactoside and cyanidin-3-rutinoside in the blueberry and black raspberry extract, respectively. These results are consistent with previous studies evaluating the anthocyanin profile of whole berries.[34-36] Additionally, black raspberry pomace contained significantly more anthocyanin than blueberry pomace, which is consistent with a previous study comparing blueberries (230 mg anthocyanin/100 g fruit) to black raspberries (566 mg anthocyanin/100 g fruit).[37] Previous studies evaluating fruit pomaces have focused on grapes given its wide availability deriving from juice and wine processing. Authors have reported blueberry pomace anthocyanins at levels of 1757 to 11900 mg/kg, however a

breakdown of other phenolics or studies on anthocyanin profile of black raspberry pomace were not found in the literature.[38-40] Our results show anthocyanin contents of 1200 and 3800 mg/kg blueberry and black raspberry pomace, respectively. Differences with previous studies may be due to the anthocyanin quantification method employed.

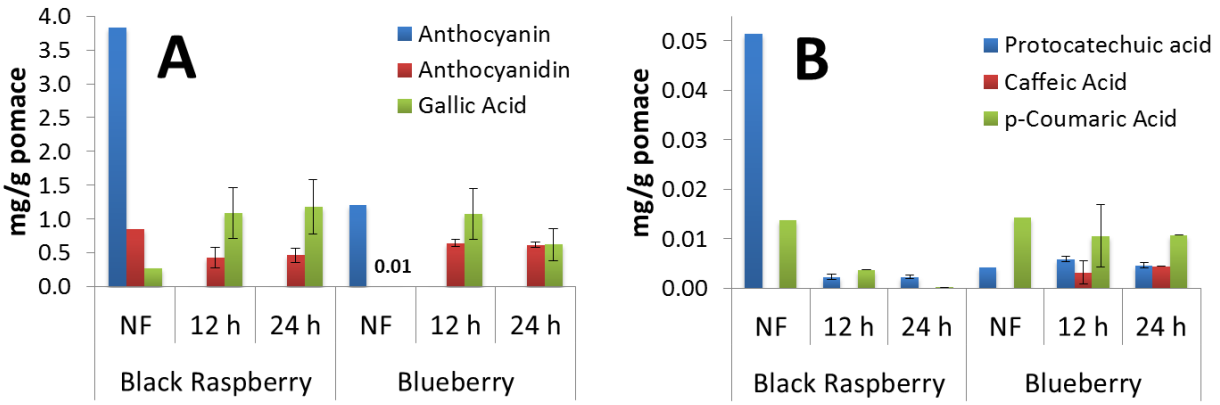

Figure 2.1. Effect of fermentation time on major (A) and minor (B) polyphenols in black raspberry and blueberry pomaces. NF – Non fermented. Different superscripts in the same row indicate significant differences between treatments.

Table 2.1. Anthocyanin characterization of berry pomace extracts.

Compound	% Total Anthocyanins	
	Blueberry	Black Raspberry
cyanidin-3-arabinoside	7.1%	
cyanidin-3-galactoside	5.5%	
cyanidin-3-glucoside	8.9%	7.1%
cyanidin-3-rutinoside		68.8%
cyanidin-3-sambubioside-5-rhamnoside		18.2%
delphinidin-3-galactoside	5.4%	
malvidin-3-arabinopyranoside	19.3%	
malvidin-3-galactoside	23.8%	
malvidin-3-glucoside	14.5%	
pelargonidin-3-glucoside		6.0%
peonidin-3-galactoside	4.9%	
petunidin-3-glucoside	10.7%	

Fermentation of berry pomaces resulted in significant changes in phenolic profiles (Figure 2.1). For both berries, anthocyanins, which were the major phenolic identified, were completely degraded after 12 h of fermentation. Anthocyanidins, initially only found in non-fermented black raspberry significantly decreased after 12 h of fermentation. In contrast, gallic acid from both berries significantly increased with fermentation. Minor phenolics protocatechuic and p-coumaric acid significantly decreased with fermentation in black raspberry, however no significant changes were observed for the same compounds in blueberry. Caffeic acid was only identified in the fermented blueberry samples. For all identified phenolics, no significant differences were observed between the 12 and 24 h fermentation samples, thus 12 h fermentation samples were used for the transport experiment. Complete degradation of anthocyanins after fecal inoculum incubation has previously been observed by Fleschhut et al. and Keppler et al.[41,42] For black raspberry, we hypothesize that bacterial fermentation by the fecal microflora initially resulted in production of protocatechuic acid from cyanidin as previously reported,[41-43] however protocatechuic acid was further converted by the microflora to gallic acid by hydroxylation as proposed by Kambourakis et al.[44] For blueberry, a clear deglycosylation of anthocyanins into anthocyanidins was observed, however this was also accompanied by an increase in gallic acid which may have derived from the O-demethylation of syringic acid, the main microbial metabolite of malvidins.[41,42]

In the transport experiment, further transformation of major and minor phenolics from both non-fermented and fermented berry pomaces was observed. Apparent permeability (Papp) could only be calculated for protocatechuic acid in blueberry (BB), black raspberry (BRB), and fermented black raspberry (FBRB), *p*-coumaric acid in BB and BRB, and anthocyanin in BRB

(Table 2.2). Papp could not be calculated for any phenolics in fermented blueberry (FBB) due to the apical to basolateral transport of compounds that were not initially in the apical side, but derived from the degradation of anthocyanidins and gallic acid. Calculated Papp for anthocyanin, protocatechuic and *p*-coumaric acid had values of $0.5x10^{-9}$ to $1.1x10^{-7}$ cm/s, which according to previous authors correlates to a poor *in vivo* absorption of less than 1%.[45,46] A previous study reported anthocyanin transport efficiency in Caco-2 monolayers of 0.9-4.7%, however a main difference with our study is that we did not utilize purified anthocyanins but rather an anthocyanin rich extract.[33] In comparison, a study evaluating a black currant extract, resulted in no anthocyanins being detected in the basolateral side after 80 minutes, similar to our results.[47] A study evaluating the permeability of *p*-coumaric acid through Caco-2 cell monolayers[48] reported a basolateral recovery of 11%, however our recovery was more than 35%; this may be due to a synergistic effect of other phenolics in the matrix evidenced by significant differences in the Papp of the same compound in different matrixes. Effect of phenolic acids and polyphenols on the Caco-2 transport efficiency of various compounds has previously been reported.[49-51] Gallic acid (GA) transport through Caco-2 monolayers has previously been reported at less than 1%,[48] however our higher values in selected treatments further confirm the synergistic/antagonistic effect of phenolics in the matrix.

Table 2.2. Apparent permeability coefficient (Papp) of phenolics in different berry matrixes.

Phenolic	**Papp (10^{-9} cm/s)**		
	Blueberry	**Black Raspberry**	
			Fermented
Protocatechuic Acid	6.3 ± 2.28 [a]	27.1 ± 7.31 [b]	110.8 ± 52.25 [c]
p -Coumaric Acid	15.0 ± 6.79 [a]	86.6 ± 21.82 [b]	-
Anthocyanin	-	0.5 ± 0.12	-

A detail of the changes and absorption of phenolic compounds deriving from non-fermented and fermented berries can be observed in Figure 2.2. For blueberry, the phenolics initially present were anthocyanin (ACY), p-coumaric acid (pCA), gallic acid (GA), and protocatechuic acid (PCA). After the 2 h transport experiment period, all of the initial phenolics were found on the apical side, in addition to anthocyanidin (ACD), caffeic acid (CA), phloglucinol aldehyde (PG), syringic acid (SA), chlorogenic acid (CGA), ferulic acid (FA), *p*-hydroxybenzoic acid (HBA) and traces of ellagic acid (EA), while ACY greatly decreased to 3% of the initial amount. Phenolics transported into the basolateral side were CA, SA, PG, GA, PCA, pCA, and FA. Traces of vanillic acid (VA) were also found exclusively in the basolateral side. Similar results were observed for black raspberry, where the initial phenolics ACY, ACD, GA, PCA, and pCA were also found in the apical side after 2 h with increases in PCA but significant decreases in ACY and ACD, however the ACY was better preserved, as 62% of the initial ACY but only 10% of the initial ACD were recovered. In the apical side after 2 h SA, VA, CGA, HBA, pCA, EA, CA, and traces of cinnamic acid (CNA) were also identified. The major phenolic transported into the basolateral side, although not found in the apical side or the cell monolayer, was PG; other phenolics transported into the basolateral side were PCA, VA, pCA, and CNA.

Results of the transport experiment of the fermented berry pomaces also displayed changes in phenolic profiles (Figure 2.2). Although the initial profiles were very similar, consisting in GA, ACD, pCA and PCA, the differences in individual anthocyanidins may have contributed to the difference in final profiles. For fermented blueberry, all the initial compounds except ACD were found in the apical side after 2 h in addition to PG, HBA, SA, VA,

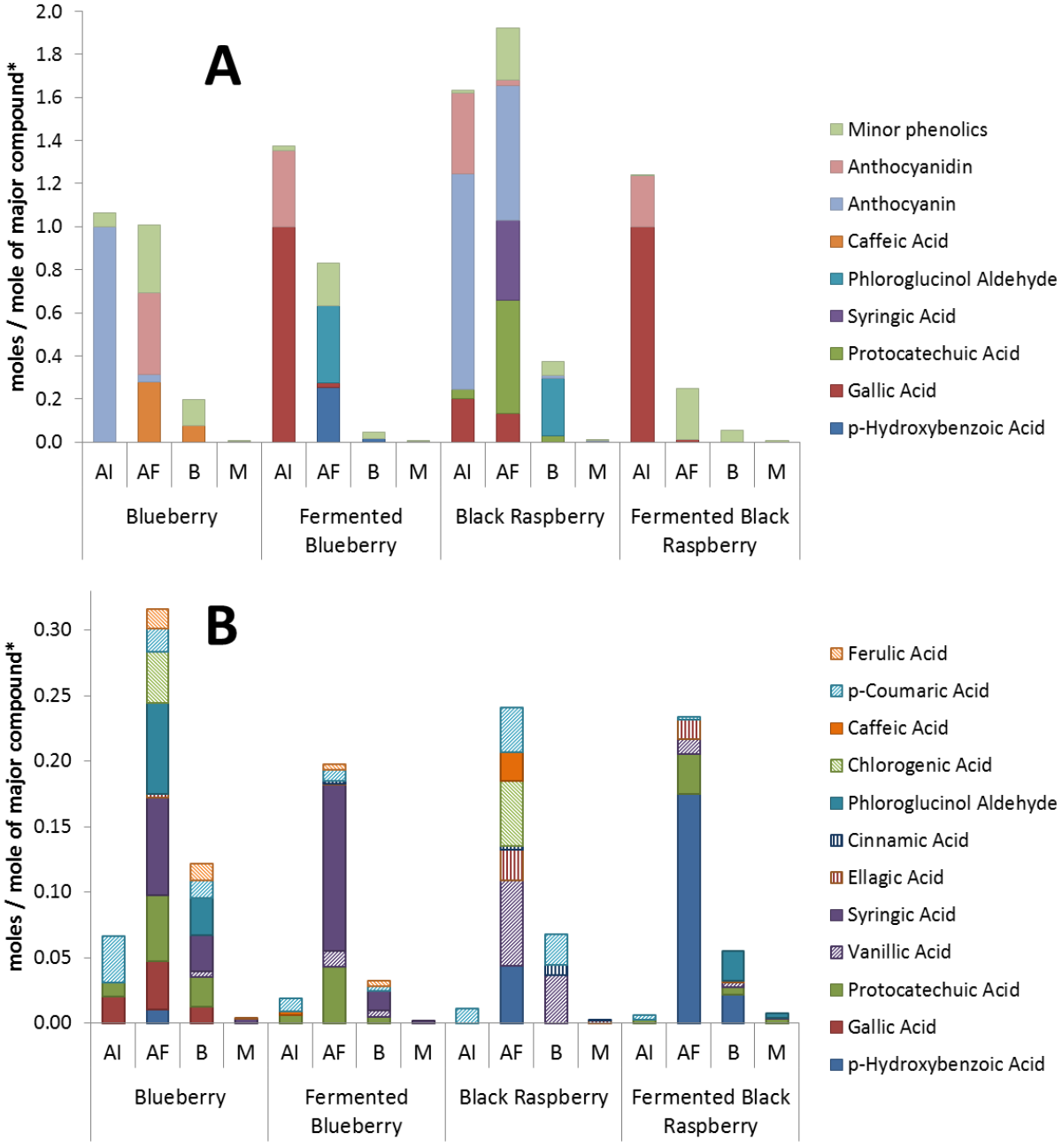

Figure 2.2. A Phenolic profiles of recovered Caco-2 permeability experiment fractions: apical initial (AI), apical final (AF), basolateral (B), and cell monolayer (M). B Breakdown of minor compounds. *Molar amounts have been normalized to the major phenolic identified, anthocyanin for non-fermented extracts or gallic acid for fermented extracts, where the major phenolic =1.

FA, and traces of CNA. Transport to the basolateral side only occurred for HBA, SA, PCA, VA,pCA, and FA. Similarly, for fermented black raspberry all the initial compounds except ACD were found in the apical side after 2 h, but the additional compounds found were HBA, EA, and VA. Phenolics transported to the basolateral side for fermented black raspberry were HBA, PG, PCA and VA. For all the treatments, phenolics found in the cell monolayers generally corresponded to those transported to the basolateral side, however the recovered amounts generally represented less than 10% of the transported fraction.

Major reductions in anthocyanins after exposure to Caco-2 cells in culture media or HBSS have been previously reported to levels up to less than 10% and 20%, respectively, depending on the individual anthocyanin due to oxidation and physiological actions of the cells.[33,47,52] In the study by Yi et al.[33] individual anthocyanins were recovered in the basolateral side up to 6%, however this study also demonstrated that the transport efficiency of total anthocyanins was affected by the individual anthocyanins present in the matrix, sugar moieties, and anthocyanidin base, which may explain why anthocyanins were identified in the basolateral side only for BRB. Although no anthocyanidins were identified in the basolateral side for any of the treatments, deglycosilation of anthocyanins from BB and BRB on the apical side after 2 h was evident. Several authors have observed deglycosilation of polyphenols by intestinal cells.[42,53-55] Enzymes involved in deglycosilation of anthocyanins may include lactase-phlorizin hydrolase and β-glucosidase from the intestinal mucosa.[53,55]

After deglycosylation, further degradation of anthocyanidins typically occurs with the fission of the C-ring of the anthocyanin resulting in phloroglucinol aldehyde and phenolic acids.[42,43] Theoretically, the main phenolic acids deriving from BB would be SA (from malvidins),

PCA (from cyanidins), 3-O-methylgallic acid (from petunidin) GA (from delphinidin), and VA (from peonidin); while from BRB we would expect to find PCA and HBA (from pelargonidin).[41,52] The aforementioned compounds corresponded to those found in our experiment both in the apical and basolateral sides; however other phenolics were identified such as EA, CNA, CGA, CA, and FA. Structures of anthocyanidins and the phenolic acids identified in this study are shown in Figure 2.3. EA may have derived from metabolism of ellagitannins,[56] which were not a target for quantification in the initial extracts given our interest in the anthocyanins and phenolic acids. EA absorption into the basolateral layer was extremely low or non-detectable, perhaps due to its large molecule size as compared to other phenolic acids. CNA, CGA, CA, and FA, all hydroxycinnamic acid derivatives may have resulted from anthocyanin/anthocyanidin degradation by the cells in a manner different from the classical aldehyde-phenolic acid C-ring fission or from EA degradation. CGA, the largest molecule of these four, was not detected in the basolateral side for any treatments, probably due to its size in a similar manner to EA. Previous studies have reported basolateral transport of pure CGA at less than 0.1% and CA at 1.5%,[49] however for BB the basolateral transport of CA in the matrix represented 25% of that recovered in the apical layer after 2 h.

Previous studies have reported unchanged anthocyanin absorption into the bloodstream by humans and other species, however while these studies typically involve the consumption of highly concentrated extracts and not the berries themselves, recoveries are quite low (less than 0.5%) and do not represent a normal berry consumption setting.[57-60] Authors have even named anthocyanins and anthocyanidins as the “least well-absorbed

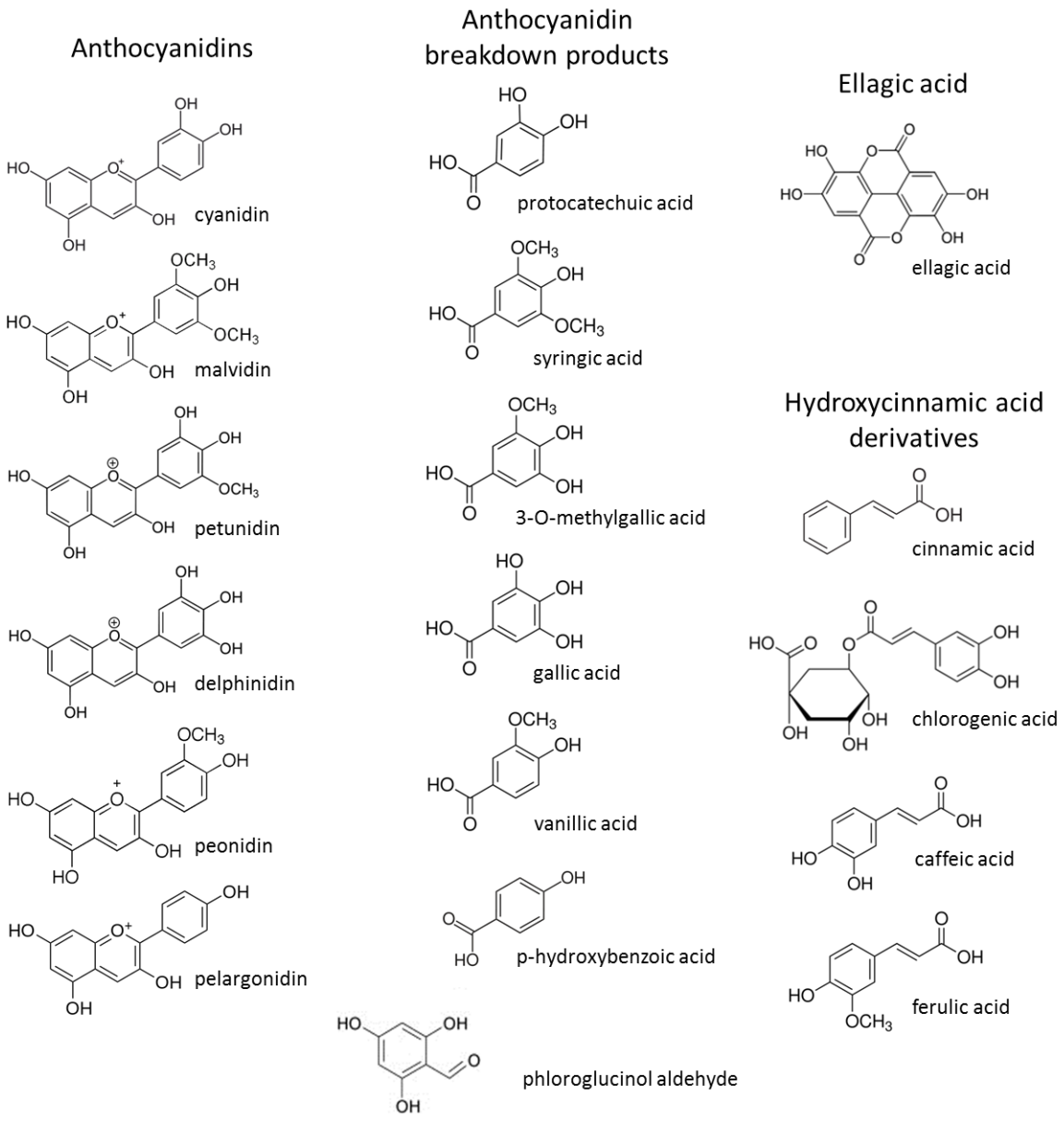

Figure 2.3. Structures of anthocyanidins and their corresponding breakdown products, ellagic acid, and hydroxycinnamic acid derivatives relevant to this study.

polyphenols".[61] Studies also show that the site for intact anthocyanin absorption may be the stomach, a mechanism which we did not simulate in our model.[62] In addition, authors have noted that anthocyanin metabolites may have undergone further transformation when excreted in the urine, a common method to quantify absorption, and thus may impede individual phenolic quantification.[63]

In this study, we have demonstrated major changes in phenolic profiles of BB and BRB pomaces due to colonic fermentation by human fecal microflora and further absorption by intestinal cells. Our results suggest that BB and BRB pomaces represent a source of bioavailable polyphenols and could be utilized in food formulations as a functional ingredient due to their inherent fiber content and phenolic availability after fermentation. In addition, we have proposed a model to screen bioavailability of food polyphenols *in vitro*, allowing for a detailed study of metabolites in a cost and time efficient manner as compared to *in vivo* studies.

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CHAPTER 3
IN VITRO SCREENING OF BLUEBERRY AND BLACK RASPBERRY POMACE PHENOLICS AND THEIR ANTI-INFLAMMATORY POTENTIAL IN CORONARY CELLS

3.1 INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death in the United States accounting for 33.6% of the deaths in the country. More than 1 out of 3 American adults have at least one type of CVD.[1] Low grade chronic inflammation, a major consequence of obesity, is a widely recognized risk factor for CVD and other diseases such as diabetes and some cancers.[2-8] Previous studies, as well as research carried out in our laboratory, have shown a positive correlation between free fatty acids (FFA) in plasma and inflammation scores.[9-12] It is now widely accepted that atherosclerosis, a form of CVD, is the result of a chronic inflammatory process, leading to atherosclerotic plaque rupture and formation of thrombus which results in events such as heart attack and stroke. The increased understanding of this type of chronic diseases has created interest in new opportunities for health promotion. Foods and some food production byproducts, such as berry fruits and pomace offer an attractive means to deliver anti-inflammatory compounds in a convenient and well established form.

Anthocyanins are a group of flavonoids commonly found in many fruits and vegetables.[13,14] Anthocyanins exhibit characteristic red, blue, or purple colors in fruits such as blueberries (*Vaccinium spp.*) and black raspberries (*Rubus occidentalis*). In addition to color anthocyanins have been shown to exhibit antioxidant capacity.[15] Intakes in the United States have been estimated at 12.5 mg/day/person.[13] In recent studies it has been shown that anthocyanins exhibit a range of biological activities, including vascular anti-inflammatory effects

and influence in blood vessels and platelets, leading to the suggestion that they reduce the risk of cardiovascular disease (CVD).[16-18] It has been shown that *in vivo* absorption of anthocyanins is very low, raising blood plasma levels to levels of the ng/ml order.[19-21] Anthocyanins undergo major changes during fermentation by the microbiome in the colon. Fermentation in the microbiome results in the production of phenolic acids that exhibit excellent absorption into the bloodstream and as a result may be responsible for some of the beneficial health effects attributed to anthocyanins.[22-25] It is postulated that the smaller size of the phenolic acids from fermentation accounts for the enhanced absorption.[26,27] We have also demonstrated these effects by utilizing an *in vitro* model for digestion, colonic fermentation and transport through Caco-2 cell monolayers (see Chapter 2). We have further been able to propose a profile of the intestinal transport products of the colonic fermentation of berry pomace.

Several biomarkers of inflammation have been associated with CVD, including interleukin-6 (IL-6), interleukin-8 (IL-8), monocyte chemoattractant protein 1 (MCP-1), C-reactive protein (CRP), and others.[28-33] Additionally, other circulating biomarkers have been proposed as anti-inflammatory such as interleukin-10 (IL-10) and interleukin-1 receptor antagonist (IL-1ra).[34-36] It has been proposed that berry anthocyanins are cardioprotective; however, the mechanism by which CVD risk is reduced is not well understood. Furthermore, the potential anti-inflammatory effects of a combination of phenolic acids resulting from the fermentation and absorption of said berries has not been explored.

The approach of this research was to test the impact of blueberry and black raspberry anthocyanin-rich extracts and phenolic acid mixtures representative of berry pomace colonic fermentation and absorption on the expression of various inflammation markers in human

coronary arterial smooth muscle (HCASM) and endothelial (HCAE) cells in response to stimulation with palmitic acid (PA). In this study, a model for screening food bioactives and induction of inflammation with free fatty acids in human coronary cells has been proposed as a time saving and cost efficient alternative to *in vivo* studies.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Blueberry (*Vaccinium spp.*) and black raspberry (*Rubus occidentalis* 'Munger') pomaces were provided by Milne Fruit Products (Prosser, WA) and freeze dried in a VirTis Genesis pilot lyophylizer (Warminster, PA). Palmitic acid (PA), p-hydroxybenzoic acid (HBA), protocatechuic acid (PCA), vanillic acid (VA), syringic acid (SA), ellagic acid (EA), p-coumaric acid (pCA), ferulic acid (FA), and fatty acid free bovine serum albumin (BSA) were purchased from Sigma Aldrich (St. Louis, MO). Human Primary Coronary Artery Endothelial (HCAE) and Smooth Muscle (HCASM) Cell lines were purchased from ATCC (Manassas, VA) and proceeded from healthy adult volunteers. All other reagents were of analytical or cell culture grade and obtained from Sigma Aldrich (St. Louis, MO). Water was Milli Q (Millipore Corporation, MA) and autoclaved as required for all the studies.

3.2.2 Anthocyanin Extraction and Characterization

Anthocyanin rich extracts were obtained from blueberry (BB) or black raspberry (BRB) pomace using the acidified-methanol method.[37] Briefly, pomace was homogenized with 2 volumes of acidified methanol (0.01% v/v HCl) and macerated for 1 h, filtered through Whatman 1 filter paper and re-extracted twice until a faint-colored extract was obtained. The

extract concentrated in a rotary evaporator to remove the methanol, freeze-dried, re-dissolved with sterile water, and sterile filtered. Anthocyanins and phenolics were quantified by HPLC using the method developed in our laboratory by Jang and Xu that has been previously described.[38,39]

3.2.3 Preparation of Palmitic Acid and Phenolic Solutions

PA was conjugated to BSA in a 12.5 mM solution based on the methods developed by McIntosh, Toborek and Henning, and van Greevenbroek el al.[40-43] Briefly, cell culture grade PA was aseptically weighed and dissolved in hexane and mixed with an equimolar amount of KOH, dried under nitrogen and immediately diluted in warm sterile water. The solution was then combined with a 30% BSA solution (30% BSA in DPBS+HEPES, pH 7.2, sterile filtered) and the pH was readjusted to 7.2. The solution was aliquoted into sterile vials, flushed with argon, and frozen at -80 °C. Aliquots of the BSA solution were used as control. Volumes of these solutions did not exceed 2% in the media.

Dilutions of berry extracts and phenolic acids were prepared immediately before the cell culture experiments. Berry extract stocks were prepared to achieve a 1 mM concentration, based on initial anthocyanin (ACY) + anthocyanidin (ACD) concentrations (BB=3.79 mM ACY; BRB=7.92 ACY + 2.98 ACD=10.9 mM) by diluting the extract with DPBS+HEPES at pH 7.4. Subsequent dilutions were prepared to achieve concentrations of 20, 10, and 5 µM to be added directly to the cell culture media at volumes not exceeding 0.5%. Phenolic solutions were prepared based on previous experiments dealing with the absorption of phenolics from fermented berry pomaces through Caco-2 monolayers. Table 3.1 illustrates the average composition of fermented BB and BRB permeates. Phenolic stocks were prepared to a

concentration of 10 mM with sterile water, based only on the phenolic acids deriving from the direct degradation of ACYs, namely HBA, PCA, VA, and SA (FBB=3.58mM HBA + 1.45mM PCA + 1.28mM VA + 3.69mM SA = 10mM; FBRB=6.43mM HBA + 2.31 mM PCA + 1.26 VA = 10mM); however other phenolic acids found in the permeate (EA in FBRB, pCA and FA in FBB) were also included in the stocks at the appropriate concentrations as shown in Table 3.1. Subsequent dilutions were prepared at concentrations of 20, 10, and 5 µM with DPBS+HEPES at pH 7.4 for direct addition to the media at volumes not exceeding 0.5%.

Table 3.1. Average molar phenolic acid profile of fermented blueberry and black raspberry pomace Caco-2 permeates. Values have been normalized for the concentrations of HBA, PCA, VA, and SA to add to 10.

Phenolic Acid	Fermented Blueberry	Fermented Black Raspberry
p-hydroxybenzoic acid	3.58	6.43
Protocatechuic acid	1.45	2.31
Vanillic acid	1.28	1.26
Syringic acid	3.69	
Ellagic Acid		0.34
p-coumaric acid	0.94	
Ferulic Acid	1.18	

3.2.4 Cell Culture and Inflammation Model

Cell lines of HCAE and HCASM cells were grown using Cascade Medium 200 or 231 (Gibco, Portland, OR), respectively. Cells were fed every other day until 80% confluence and the contents of each flask split into 2 or 3 subsequent flasks. The cells were lifted with Cascade Trypsin solution which was then neutralized with CMF-PBS, followed by centrifugation at 180 *x*g for 3 minutes and suspension at a density of 10,500 cells/cm^2 in their corresponding media (see above) using T-25 flasks, 24-well plates or 96-well plates as appropriate. Cells were incubated at

37 °C and 5% CO_2 in a humidified incubator (Sanyo Biomedical, Wood Dale, IL) for all treatments.

Anti-inflammatory assessments were performed using two approaches: prevention and intervention. For the prevention approach, cells were treated with anthocyanins or phenolics at 100, 50 or 25 nM in the media (based on human plasma anthocyanin levels in the literature ranging 26-103 nM[19-21,44]) followed by 2.5 (HCAEC) or 8 h (HCASM) of incubation; PA was then added for a final concentration of 250 μM, and the cells were incubated for a total of 8 or 24 h. For the intervention approach the same PA and phenolic doses were used, except cells were first incubated with PA followed by treatment with phenolics. A control to which neither extracts nor PA was added was included in each group.

After incubation at specified times (HCAEC 8 h, HCASM 24 h) were completed, cell culture supernatants were harvested and levels of inflammation biomarkers IL-6, IL-8, MCP-1, TNFα, IL-1β, IL-10, and IL-1ra were measured by ELISA kits from PeproTech (Rocky Hill, NJ) or R&D Systems (Minneapolis, MN). Cells were washed twice with DPBS and removed from the culture flasks or plates using Cascade trypsin solution that was then neutralized with 5% FBS in DPBS; the trypsinized cells were centrifuged at 180 *x*g for 7 minutes, the supernatant removed, and the pellet frozen at -80 °C for further analysis. Cell viability was assessed using the Promega CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (MTS) (Madison, WI). Biomarker levels were calculated and adjusted to be expressed as a percentage of inflammation based on the PA control (100% stimulation) and corrected for cell viability.

RNA was extracted from cell pellets using the RNeasy® Mini kit from Qiagen (Valencia, CA). Following the manufacturer's protocol, 1 x 10^6 cells were disrupted and homogenized

using the QIAshredder homogenizer. Ethanol was added to the lysate to bind the RNA to the membrane in the spin column, residual DNA was digested on the column using the RNase-Free DNase Set, and the RNA was eluted with RNase-free water. RNA in the elute was measured by UV spectrophotometry using the NanoDrop 2000 from Thermo Scientific (Wilmington, DE) blanked with RNAse-free water. Before PCR analysis, cDNA was synthesized from 500 ng of RNA from each sample using the RT^2 First Stand Kit from Qiagen which consists in the elimination of genomic DNA followed by reverse transcription. Real time PCR was performed on selected samples using the custom RT^2 Profiler PCR array CAPH10908 with HotStart DNA *Taq* Polymerase and SYBR Green fluorophore from Qiagen that included 16 target genes in a 96-well plate format (16x6), namely: IL6, IL8, IL10, IL18, CCL2, IL1B, PTGS2, NFKB1, NFKB2, TLR2, TLR4, TNF, ICAM1, VCAM1, MAPK14, and GAPDH (housekeeping gene). The array was processed in the CFX96 Touch™ Real-Time PCR cycler equipped with CFX Manager™ Software and automatic threshold cycle (C_T) calculation (Bio-Rad Laboratories, Hercules, CA). The cycling conditions used were 10 minutes at 95 °C for polymerase activation, followed by 40 cycles of annealing for 15 s at 95 °C and elongation for 1 min at 60°C with fluorescence data collection. A melting curve (65 to 95 °C at a rate of 0.5 °C per 5 s) was also performed at the end of the cycling program to verify PCR specificity, evident by a single peak appearing in each reaction at temperatures greater than 80°C. C_T values were processed with the SABiosciences PCR Array Data Analysis Web-based software. The software calculates ΔC_T based on the housekeeping gene (GAPDH), then calculates the normalized gene expression in the samples using the formula $2^{-\Delta C_T}$. The normalized gene expression of the sample is divided by the control (PA) to obtain the fold change, which is then converted to fold regulation.[45] Fold regulation equals fold change for

values greater than 1, fold regulation is the negative inverse of fold change for values less than 1. The software also performs a cluster analysis of the dataset to generate a heat map with dendograms to indicate co-regulated genes across samples.

3.2.5 Statistical Analysis

Results are presented as mean ± SE. All tests were carried out in triplicate. Statistical analysis was performed using one-way analysis of variance (ANOVA). Separation of means was performed by the Tukey's Studentized Test (SAS®, version 9.2). Differences were considered significant at $p \leq 0.05$.

3.3 RESULTS AND DISCUSSION

Berry anthocyanins are widely recognized for their antioxidant properties; however their effect on the inflammatory pathway has not been explored in detail. In this study we evaluated how blueberry and black raspberry anthocyanin-rich pomace extracts and their phenolic acid products derived from colonic fermentation of pomaces affect the expression of various inflammatory biomarkers.

IL-6 levels in HCASM cells (Figure 3.1, see A) were unchanged in all the treatments except for BRB in the intervention approach, where IL-6 dose-dependently increased to levels significantly higher than control ($p<0.05$). IL-8 in HCASM cells (Figure 3.1, see A) also showed dose-dependent increases for BRB in the intervention approach and FBRB in the prevention approach; however overall IL-8 levels were higher for all treatments in the prevention approach as compared to intervention. In contrast, IL-8 levels in HCAE cells (Fig 4.1B) were overall lower

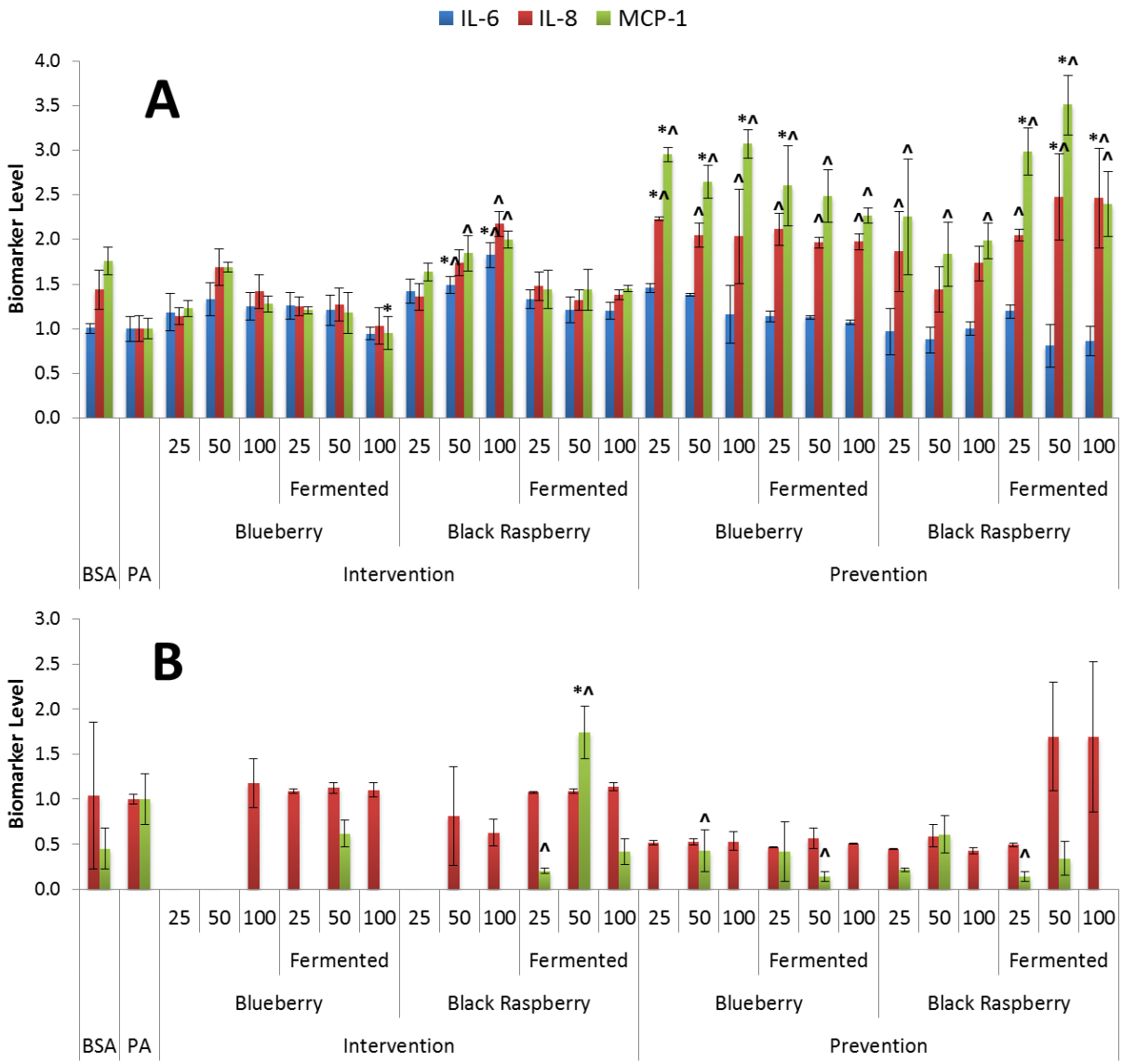

Figure 3.1. Changes in levels of IL-6, IL-8, and MCP-1 in human coronary arterial smooth muscle HCASM (A) and endothelial HCAE (B) cell supernatants as influenced by berry anthocyanin-rich extracts or phenolic acid mix (fermented) in the intervention and prevention approaches. * Treatment is significantly different ($P<0.05$) from BSA control. ^ Treatment is significantly different ($P<0.05$) from PA control. Bars that are not displayed correspond to levels too low to be detected in the supernatant.

in the prevention approach as compared to intervention, and no clear dose-dependent responses were observed for any of the treatments. MCP-1 behaved similarly to IL-8 in HCASM cells (Figure 3.1, see A), with overall higher levels for all treatments in the prevention approach and increasing dose-dependent response for BRB in the intervention approach, but also

decreasing dose-dependent responses for FBB in both approaches. MCP-1 in HCAE cells (Figure 3.1, see B) was reduced to levels similar to the BSA control only by FBRB at 25nM in both approaches, and BB and FBB at 50nM in the prevention approach.

Supernatant levels of TNFα in HCASM cells (Figure 3.2) showed dose-dependent decreases for BB in both approaches and FBRB in the prevention approach, however the greatest reductions in TNFα (to levels similar to the BSA control) were observed for all levels of FBRB in the intervention approach and for FBB and BRB in the prevention approach, independent of dose. Overall, the prevention approach resulted in lower levels of TNFα in HCASM cell supernatants. In contrast, IL-1β levels were overall higher for all treatments in the prevention approach. IL-1β also displayed dose-dependent decreases in BB and FBB in both approaches, and FBRB in the prevention approach. A slight dose-dependent increase in IL-1β

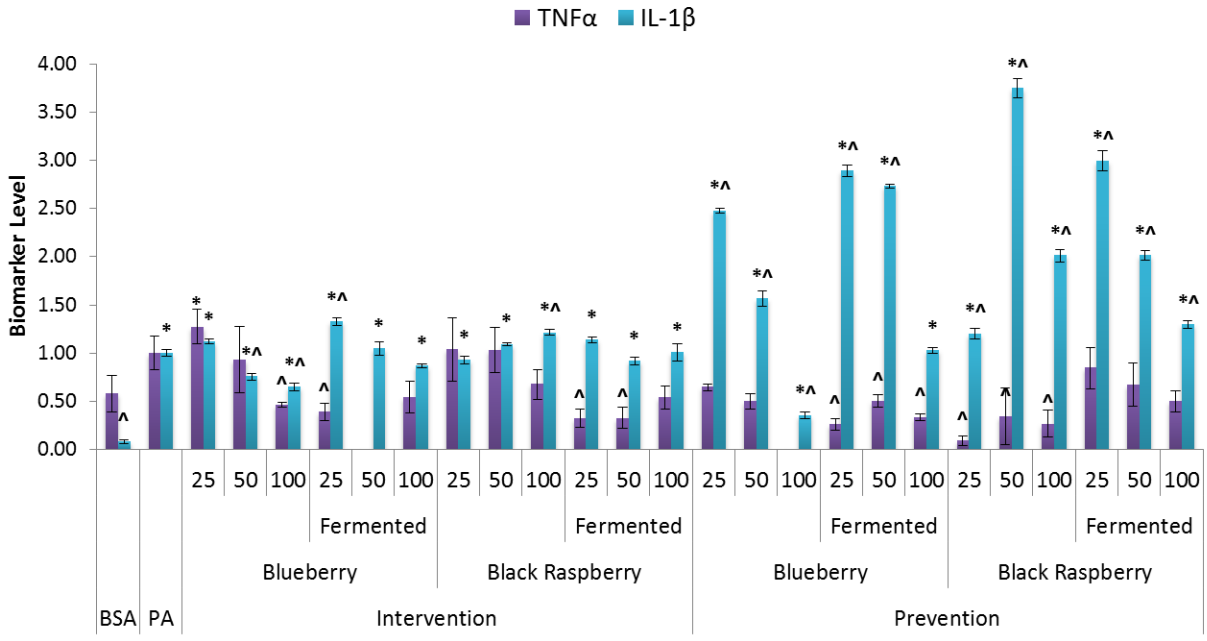

Figure 3.2. Changes in levels of IL-1β and TNFα in human coronary arterial smooth muscle cell supernatants as influenced by berry anthocyanin-rich extracts or phenolic acid mix (fermented) in the intervention and prevention approaches. * Treatment is significantly different ($P<0.05$) from BSA control. ^ Treatment is significantly different ($P<0.05$) from PA control.

was observed for BRB in the intervention approach.

Supernatant levels of proposed anti-inflammatory cytokines IL-1ra and IL-10 were also evaluated in the cell culture supernatants (Figure 3.3). Dose-dependent decreases of IL-1ra were observed only in HCASM cells for BB in both approaches, and FBB and FBRB in the prevention approach; however both cell lines displayed overall lower IL-1ra levels comparable to the BSA control in treatments performed in the prevention approach. IL-10 dose-dependent reductions to levels similar to the controls were only observed for BB in the intervention approach, and FBB and FBRB in the prevention approach. In contrast, FBRB in the intervention approach resulted in a dose-dependent increase; however a difference between overall approaches for IL-10 was not clearly evident. IL-10 was not detected in the supernatants of HCAE cells in this study.

Gene expression of inflammation related genes in HCASM cells is shown in Figure 3.4. Only those genes that resulted in fold regulation values higher than 2 or lower than -2 are depicted. Fold regulation of IL6, IL8, IL1B, NFKB1, NFKB2, TLR4, and MAPK1 remained between 2 and -2 for all treatments, while significantly being down-regulated for BSA, BB, and BRB controls at values between -2 and -12 (not shown). TLR2 was down regulated by all treatments except FBRB 25nM in the intervention approach, and this down regulation was overall more effective in the prevention approach. TNF significantly differed from both controls only with BRB 100nM and FBRB 25nM in the prevention approach, where it was up-regulated. CCL2 was more effectively down-regulated by all treatments in the prevention approach, however no treatment achieved down regulation similar to the control. ICAM1 was up regulated exclusively

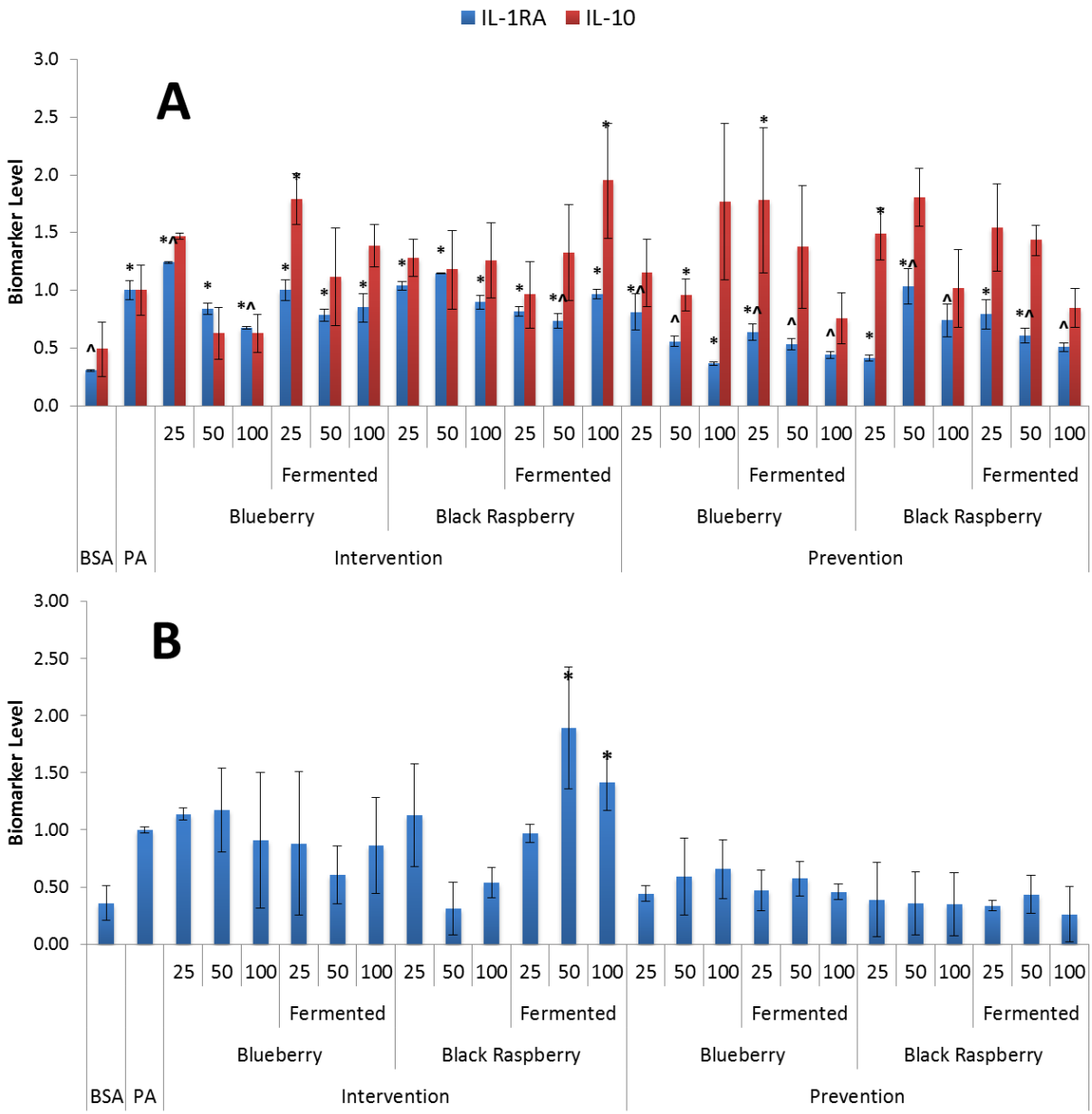

Figure 3.3. Changes in levels of IL-1ra and IL-10 in human coronary arterial smooth muscle (A) and endothelial (B) cell supernatants as influenced by berry anthocyanin-rich extracts or phenolic acid mix (fermented) in the intervention and prevention approaches. * Treatment is significantly different (P<0.05) from BSA control. ^ Treatment is significantly different (P<0.05) from PA control.

by FBB 25nM in the prevention approach, while VCAM1 was up regulated exclusively by BB 25nM in the prevention approach. PTGS2 was best down regulated by BRB 25nM in the intervention approach. Noticeably, none of the tested treatments achieved a down regulation

of the ICAM1, VCAM1 or PTGS2 genes to levels comparable to the BSA control; however, the entire observed gene up regulations in SMC were less than 3-fold.

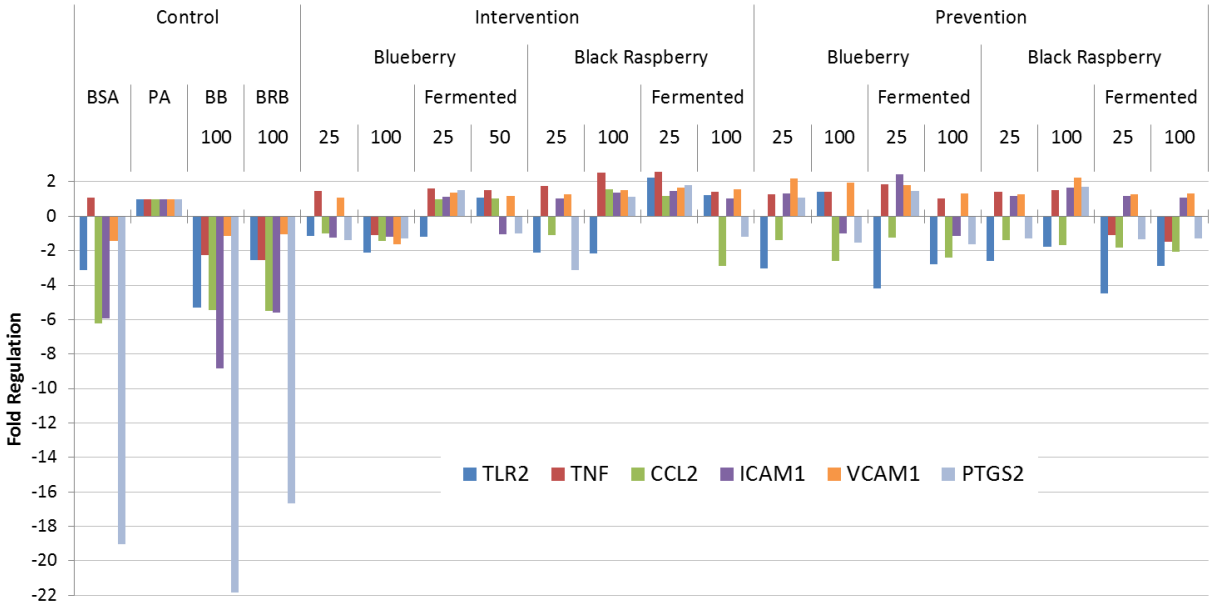

Figure 3.4. Changes in fold regulation in human coronary arterial smooth muscle cell gene expression as influenced by berry anthocyanin-rich extracts or phenolic acid mix (fermented) in the intervention and prevention approaches.

Gene regulation in HCAE cells is shown in Figure 3.5. For IL6 and PTGS2 (Figure 3.5, see A), none of the individual treatments was able to down regulate the genes to the BSA control levels except PTGS2 with BRB 50 nM in the prevention approach, in fact most treatments up regulated the genes up to 12-fold, with apparent dose-dependent up regulation for both FBRB treatments and down regulation for BB in the prevention approach. CCL2 (Figure 3.5, see A) was down regulated by most treatments, although values similar to control were only achieved by FBB 100nM in both approaches. Dose-dependent up regulation was also observed for both FBRB treatments. NFKB1 (Figure 3.5, see A) was up regulated to levels similar or higher than the BSA control, except for FBB and BRB in the prevention approach, both of which slightly down regulated the NFKB1 expression. NFKB2 (Figure 3.5, see B) was down regulated by all the tested

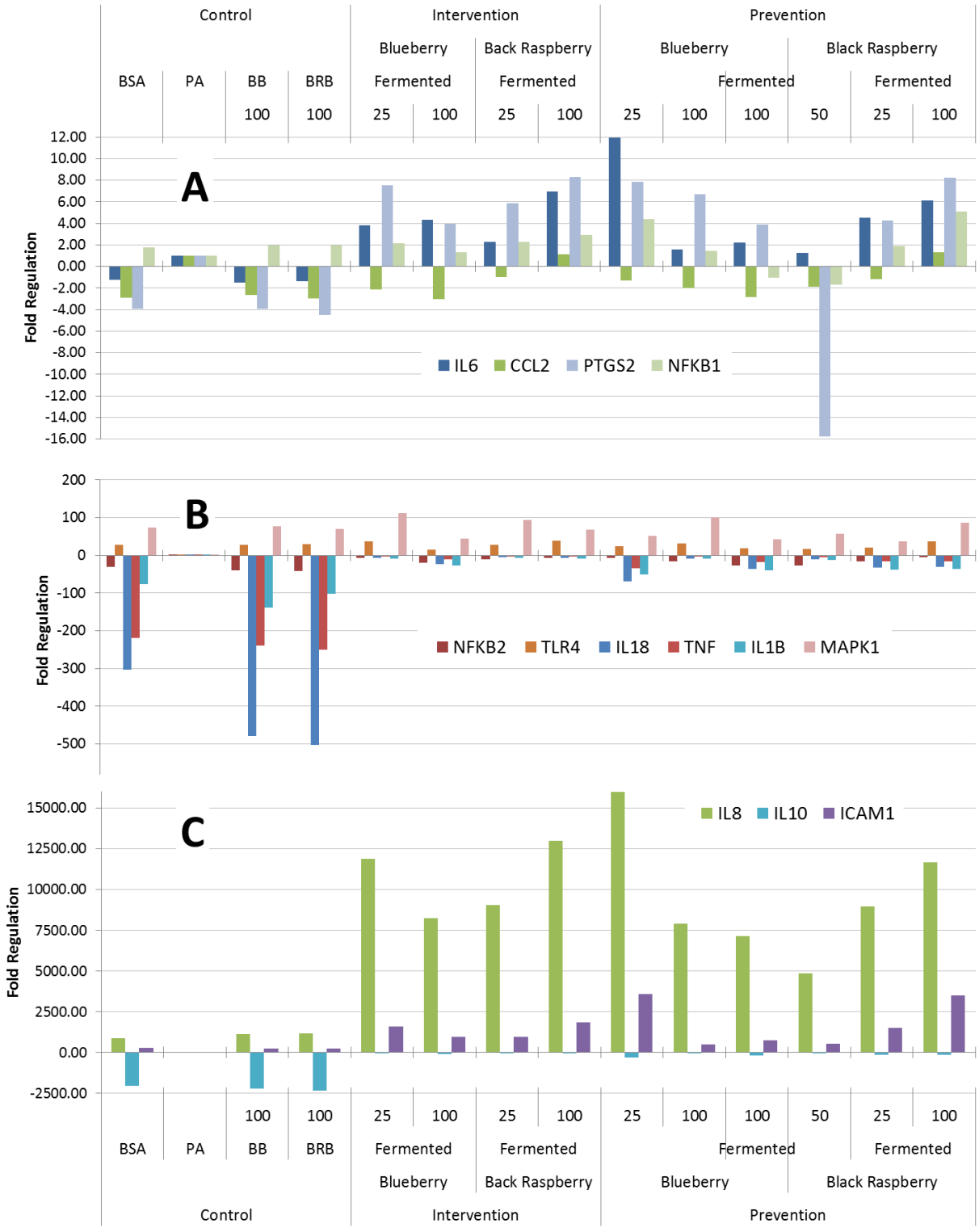

Figure 3.5. Changes in fold regulation in human coronary arterial endothelial cell gene expression as influenced by berry anthocyanin-rich extracts or phenolic acid mix (fermented) in the intervention and prevention approaches.

treatments; however levels similar to the BSA control were achieved by FBB and BRB in the prevention approach. Similarly, the gene expression of IL18, TNF and IL1B was down regulated by all the treatments evaluated, however the down regulation was not as low as the BSA control except IL1B for BB 25nM in the prevention approach. TLR4 and MAPK1 (Fig 4.5B) remained up regulated both in the BSA controls and the treatments at similar levels, thus all treatments were effective in overcoming the effects of PA on the expression of these two genes. Gene expression of IL8 and ICAM1 (Figure 3.5, see C) was up regulated both in the BSA control and the treatments, however all treatments showed an up regulation at a much higher fold than the BSA control (up to 16-fold as compared to BSA). Additionally, this up regulation appeared to be dose-dependent for FBRB in both approaches. Interestingly, the lower up regulations observed for ICAM1 were for treatments in the prevention approach. Finally, the gene expression of IL10 (Figure 3.5, see C) was down regulated in a similar magnitude by all treatments (28- to 318-fold), however these down regulations were not comparable to that observed in the BSA control (2029-fold), thus the treatments had limited effect over this gene.

The cluster analysis of the gene expression in HCASM cells (Figure 3.6, see A) displays co-regulation of genes NFKB1 and NFKB2, IL8 and PTGS2, ICAM1 and IL1B, TNF and CCL2, TLR4 and MAPK1. Additionally, the heat map allows us to graphically identify the most effective treatments in regulating the gene expression similar to BSA control. Overall, black raspberry treatments were the most effective in HCASM cells, resulting in gene expression similar to BSA control when the prevention approach was used with either original extract or after fermentation. In the tests with blueberry extracts and fermentation products it is difficult to differentiate which of the two approaches was the most effective, however it was observed

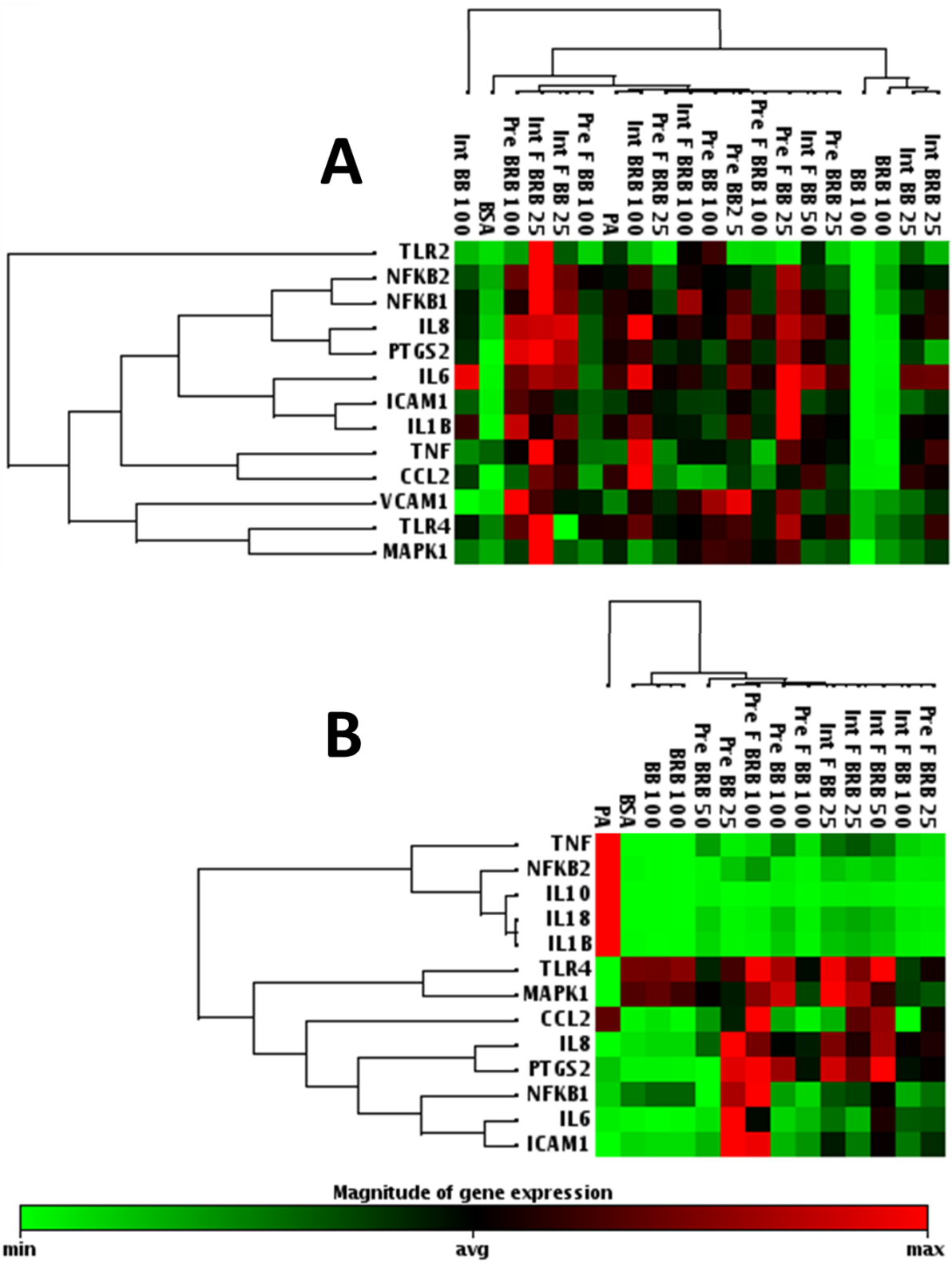

Figure 3.6. Gene expression clustergram displaying heat map indicating co-regulated genes across individual samples of human coronary arterial smooth muscle (A) and endothelial (B) cells.

that in the prevention approach the fermented blueberry pomace resuted in overall lower gene expression, while in the intervention approach the non-fermented extract was more effective. Gene co-regulation in HCAE cells (Figure 3.6, see B) displayed more distinct clustering than in HCASM cells. Strong co-regulation was observed for TNF, NFKB2, IL10, IL18 and IL1B, while co-regulation of TLR4-MAPK1 and IL8-PTGS2 occured in the same way as the corresponding genes in HCASM cells. Co-regulation of IL6 and ICAM1 was also observed in EC. Overall, treatments in the prevention approach were the most effective in modulating gene expression similarly to BSA control in HCAE cells. Within the evaluated treatments in HCAE cells, BRB at 50nM in the prevention approach was the dose that modulated all the genes closer to the BSA control than any other dose. Interestingly, though, BB at 25 nm and FBRB at 100 nM in the prevention approach resulted in high overall gene expression, suggesting a hormetic effect in the prevention approach for HCAE cells.

We hypothesize that the reduction of inflammatory biomarkers was mainly due to an effect of the berry anthocyanins or phenolic acids on the free fatty acid-induced proinflammatory transcription of NF-κB. In chronic heart failure NF-κB is activated by increased circulating TNF-α and other pro-inflammatory stimuli which in our study were substituted by PA. Activation of NF-κB promotes gene transcription of proinflammatory cytokines (IL-6, TNF-α), chemokines (IL-8, MCP-1) and adhesion molecules (ICAM-1).[28] Circulating cytokines IL-6 and TNF-α have shown significant increases in patients with heart failure.[46] Chemokines such as IL-8 in turn promote the generation of free radicals which can lead to cell oxidative stress.[47] This hypothesis is supported by our results on the protein levels and gene expression of IL-8, IL-6 and MCP-1 (CCL2); these three biomarkers were reduced in similar ratios, especially as

observed on the response of smooth muscle cells. Similar results have been observed in studies using endothelioma and human gastric cancer cells.[48] Inhibition of UV-induced NF-κB activation by black raspberry methanol extracts has also been observed in mouse epithelial cells.[49] Additionally, in the current study up- or down-regulation changes in the gene expression of NFKB1 and NFKB2, which correspond to the p-50 and p-52 subunits of NF-κB respectively, were generally accompanied by corresponding changes in various biomarkers including IL-8, PTGS2 (COX-2), IL-6, and ICAM-1 in both cell lines (Figure 3.6). This may further confirm our hypothesis that anthocyanin and their degradation products have an effect on NF-κB transcription. Additionally, similar effects of both anthocyanin and their degradation products may suggest anthocyanin degradation by the coronary cells.

Reduction in the expression of COX-2 in HCASM is in agreement with previous studies of the anti-inflammatory properties of anthocyanins.[50,51] Cyanidin from tart cherries at concentrations of 60-90 μM have shown to inhibit COX-2 synthesis from arachidonic acid by reducing the activity of prostaglandin endoperoxide H synthase-1 and -2 (PGHS-1, and -2) as compared to aspirin, ibuprofen, and naproxen; however pure anthocyanins do not exhibit this inhibitory effect and at high concentrations even increase the enzyme activity.[50] This suggests that free aglycons in our berry extracts were responsible COX-2 inhibition by this specific mechanism. The greatest inhibition of COX-2 expression observed in cells treated with black raspberry extract, which is richer in cyanidins, suggests that this was the mechanism of action. Macrophage expression of PGE_2 has also been shown to be inhibited by raspberry anthocyanins; however, anthocyanin rich extracts, especially black raspberry, promoted greater inhibition of COX-2 in the prevention approach of our study, also suggesting that the cyanidins

in black raspberry exhibit stronger anti-inflammatory potential than the malvidins in blueberry.[51]

Similar dose-dependent reductions of IL-1β and IL-1ra in most treatments, especially in the prevention approach for both cell lines indicate an effect in the activation of the IL-1 system, since levels of IL-1ra have been shown to better correlate with the activation of immune and inflamatory systems than IL-1 (α and β) which generally appears in very low levels.[52,53] High IL-1ra levels observed mostly in the intervention approach with both cell lines may indicate that the cells had initiated a process to counteract the inflammation, since IL-1ra has been proposed as an anti-inflammatory protein that is inducible by IL-6.[54] Similar supernatant protein levels of IL-6 and IL-1ra in HCASM support the previous author's views. Additionally, TNFα has been identified as the main activator of the IL-1 system, which in our study was evidenced by similar trends in TNFα and IL-1β supernatant protein levels in HCASM cells and gene expression in both cell lines.[28]

IL-18 has been proposed as an activator of interferon (IFN)-γ in atherogenesis, and has been shown to be activated by stimuli including IL-1β, TNFα and LPS.[55] In our study, IL-18 gene expression was closely co-regulated with IL-1β, supporting the proposed activation mechanism; however the gene expression of IFN-γ was not evaluated in our study.

MAPK (p38) has been shown to regulate the production of TNFα, IL-1β and COX-2 as well as some of the effects of TNFα.[56] However, gene expression of MAPK1 (p38) and its co-regulated gene TLR4 in our study was not significantly affected by the evaluated treatments, similarly to a previous study reporting no p38 changes in arteries displaying diabetes-induced inflammation as evidenced by elevated levels of IL-6, TNFα and MCP-1.[57] This further supports

our hypothesis that the modulation of inflammation in this study was due to an effect in the NF-κB cascade. In contrast, the down regulation of TLR2 in HCASM cells observed for all the treatments in the prevention approach suggests that pre-treatment with berry extract or their correspondent phenolic acids inhibited the pattern recognition of TLR2 towards cytokines or PA as a pro-inflammatory stimuli. It has been shown that TLR2 expression in vascular cells increases with inflammatory cytokines stimulation and in turn results in increased production of cytokines and chemokines.[58,59]

A hormetic effect of berry anthocyanins and their fermentation products was observed in the current study. For almost all biomarkers a clear preconditioning effect was observed in the prevention approach, where the inflammatory response to PA was significantly lower to that observed in the intervention approach. Additionally, high inflammatory responses at low phenolic doses in some of the treatments are also indicative of a hormetic effect. Induction of inflammation for the highest phenolic doses in some of the treatments may suggest low-grade toxicity (pro-inflammatory effect) as observed before with black carrot and strawberry extracts.[45,46] These characteristics are typical of hormesis and have been observed in other phytochemicals such as chalcone, ferulic acid, green tea polyphenols, and others.[60]

The results of this study suggest that a preventive approach is generally more effective than intervention when using physiological levels of anthocyanin-rich berry extracts or their fermentation products to reduce the free fatty acid-induced expression of proinflammatory biomarkers in human arterial coronary cells. Additionally, we have shown that the phenolic acids deriving from the fermentation of blueberry and black raspberry pomaces are equally or more effective in modulating the inflammatory response in human coronary arterial cells, thus

suggesting that these phenolic acids may be responsible for the beneficial effects commonly attributed purely to anthocyanins deriving from the consumption of berries *in vivo*. Although we recommend further studies be carried out to evaluate the correspondence of our model to an *in vivo* setting, the results of our study suggest that blueberry and black raspberry pomaces have the potential to be cardio protective by reducing low grade inflammation induced by circulating palmitic acid, thus the supplementation of these byproducts in foods could represent a cost-efficient approach to delivering phenolic compounds that may prevent or delay the progression of inflammation to cardiovascular disease.

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CHAPTER 4
CONCLUSIONS AND FUTURE STUDIES

4.1 CONCLUSIONS

An *in vitro* model has been developed and utilized to characterize the products of colonic fermentation and absorption of blueberry and black raspberry pomace phenolics, and evaluate the potential efficacy of these products compared to anthocyanin-rich extracts as potential anti-inflammatory compounds in fatty acid-induced inflammation. Careful review of the literature provides a rational foundation justifying this work. Circulating free fatty acids have been identified as a risk factor for inflammation and resulting cardiovascular disease. It has been previously established that berry anthocyanins are very poorly absorbed, and undergo transformation in the intestine. A model of colonic fermentation together with absorption through Caco-2 cells allowed us to profile the phenolics deriving from the fermentation of berry pomaces, as well as the absorption products. Finally, there is evidence that anthocyanins from berries can be cardioprotective by reducing inflammation. The use of cardiovascular cells allowed us to demonstrate that the phenolic acids deriving from the fermentation and absorption of berry anthocyanins may be responsible for the inflammation-lowering properties attributed to berries.

Chapter 2 explored the transformation of blueberry and black raspberry pomace phenolics through an *in vitro* model of digestion and colonic fermentation using fecal inocula accompanied by absorption through Caco-2 monolayers. HPLC characterization revealed that antocyanins, the predominant phenolic in berry pomaces, are fully degraded into anthocyanidins and phenolic acids after 12 h of colonic fermentation. Anthocyanin-rich extracts

or the anthocyanidins and phenolic acids deriving from fermentation are further degraded and transformed in the absorption process through Caco-2 cells. The study allowed us to profile the phenolic acids that could potentially be present in the bloodstream after berry pomace digestion and absorption.

Chapter 3 evaluated the potential anti-inflammatory effect of phenolic acids deriving from the fermentation and absorption of blueberry and black raspberry pomaces as compared to anthocyanin-rich extracts. Studies were carried out in two approaches, intervention and prevention, in human coronary arterial endothelial and smooth muscle cells stimulated with palmitic acid. Inflammation was assessed by the expression of supernatant proteins as well as gene expression in the RNA. The prevention approach showed to be the most effective in maintaining inflammation markers at levels close to baseline. Additionally, the fermentation and absorption products of both blueberry and black raspberry pomaces were equally or more effective than the anthocyanin-rich extracts. Gene expression analysis allowed us to propose that the mechanism of action is through the effect on NF-κB transcription. This study showed that berry pomaces, previously considered a waste product, can be used in foods to deliver phenolic compounds that may prevent inflammation in cardiovascular cells.

4.2 FUTURE STUDIES

The food bioactive screening model than we have proposed in this dissertation could represent a time and cost-efficient approach to *in vivo* studies to explore the anti-inflammatory potential of foods in a small scale. The model of fermentation and absorption could be applied to evaluate other fruit pomaces or food byproducts for the delivery of bioactive compounds

and thus reduction of waste in the food industry. Additionally, further application studies should be carried out to explore the use of blue and black raspberry pomaces in food products such as breakfast cereals, nutritional bars, and drinks.

We also suggest the further development of a model where endothelial and smooth muscle cells are co-cultured. This model would provide a more time-efficient approach at screening food bioactives in cell culture. At the same time, the cell co-culture may represent a more biologically relevant model.

VITA

Adriana Soto Rodriguez Gil (Adriana Soto-Vaca) was born in Mexico City, Mexico and raised in Mexico City and San Pedro Sula, Honduras to Carlos Soto and Adriana Rodriguez Gil. The oldest of two siblings, she attended Instituto Experimental La Salle where she graduated second of her class of 100 students (December 2000). Adriana graduated from Zamorano University with a B.S. in Agroindustrial Engineering in 2004 at the top 3% of her class. Her thesis entitled *Development and evaluation of maize and low fat soy flour based dry masa flour* was completed under the guidance of Dr. Javier Bueso and received a mention of honor. This work was also the first to represent Zamorano University at an IFT meeting in 2005. Adriana has 3 years of experience in the food industry as Plant Manager at the Zamorano Meat Processing Plant, Head of Microbiology at Quimifar Laboratories, and Senior Quality Analyst of Raw Materials and Packaging for Carbonated Soft Drinks at Cerveceria Hondureña, a SAB-Miller subsidiary. After being granted an internship at the LSU AgCenter as a research scholar in the summer of 2008, Adriana joined the Food Science graduate program at LSU in the fall of the same year. She carried out her research under the mentorship of Dr. John W. Finley and currently has two peer reviewed publications and numerous national and international presentations. Adriana will receive a dual degree of Master's and Doctor of Philosophy in Food Science during the August 2013 commencement ceremony. She is married to Franklin F. Vaca, a Ph.D. candidate in the Department of Agricultural Economics and Agribusiness at LSU.