Bioavailability and Bioactivity of Polyphenols and Their Microbial Metabolites Following Simulated Dynamic Gastrointestinal Digestion

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This thesis is dedicated to my beloved family whose unconditional love and support made this possible.

ABSTRACT

This doctoral dissertation describes the first combined use of a human simulated gut digestion model together with intestinal and hepatic cell cultures to examine the profiles, bioactivities and bioavailabilities of microbial metabolites generated from polyphenols commonly present in the human diet (chlorogenic acid, caffeic acid, ferulic acid and rutin). Study 1 involved digestion of the above polyphenols using a continuous multi-reactor Computer Controlled Dynamic Human Gastrointestinal (GI) Model representing digestion in the stomach, the small intestine and the three colonic segments. The profiles of the microbial phenolic metabolites and short chain fatty acids generated by microbiota varied greatly according to the colonic compartment. Prolonged microbial metabolism over a 16 h period generated antioxidant capacity that matched that produced by the parent polyphenols, which differs from previous short-term batch fermentation studies showing low antioxidant potential of the metabolites. Study 2 utilized the human colorectal adenocarcinoma Caco-2 cell line to explore the anti-colon cancer effects of chlorogenic acid and its major microbial metabolites (caffeic, 3-phenylpropionic and benzoic acids) at physiologically relevant concentrations of each compound and an equimolar mixture of the compounds. The combination of chlorogenic acid and its metabolites enhanced the anti-cancer efficacy as anti-proliferative, apoptotic and cell cycle arrest effects were seen at several-fold lower concentrations of those compounds within the equimolar mixture than when they were provided singly. Alterations in mitochondrial DNA were not associated with the apoptotic events for chlorogenic acid and caffeic acid but a reduction of mitochondrial DNA content was involved in 3-phenylpropionic-mediated apoptosis. In Study 3, the digests from the human simulated gut digestion system were coupled with a co-culture of human intestinal Caco-2 and hepatic HepG2 cells to investigate the digestion and first pass metabolism of a polyphenol-rich potato extract (PRPE) containing chlorogenic acid, caffeic acid, ferulic acid and rutin as the major polyphenolic

constituents. Digestion of PRPE in the GI model led to generation of the microbial-derived metabolites of the polyphenols (dihydrocaffeic, dihydroferulic, 3-hydroxybenzoic, 3-hydroxyphenylpropionic, coumaric, 3-hydroxyphenylacetic, phenylpropanoic and cinnamic acids). Following a 2 h incubation of the colonic digesta with Caco-2 cells, ferulic acid and dihydrocaffeic, dihydroferulic, 3-hydroxyphenylpropionic, 3-hydroxybenzoic and coumaric acids were noted to be poorly transported (3-15%). A two- to three-fold increase in the concentrations of ferulic, dihydrocaffeic, 3-hydroxyphenylpropionic and coumaric acids after 3 h incubation with HepG2 cells demonstrated a major contribution of hepatic metabolism in the generation of those compounds despite their poor Caco-2 cellular transport.

Overall, the combined approach using simulated gut digestion and cell culture systems applied in the current work provides a unique platform for the detailed study of mechanisms involved in biotransformation, bioavailability and bioactivity of polyphenols and their metabolites, which is otherwise difficult to perform in vivo.

RESUMÉ

Cette thèse de doctorat décrit le premier emploi combiné d'un modèle de digestion intestinale humaine simulée en association avec des cultures cellulaires intestinales et hépatiques afin d'examiner les profils, les bioactivités et les biodisponibilités des métabolites microbiens générés à partir de polyphénols communément présents dans l'alimentation humaine (l'acide chlorogénique [CGA], l'acide caféique, l'acide férulique et la rutine). L'étude 1 a porté sur la digestion des polyphénols ci-dessus à l'aide d'un modèle continu et dynamique gastro-intestinal (GI) humain contrôlé par ordinateur et à plusieurs réacteurs représentants la digestion dans l'estomac, l'intestin grêle et les trois segments du colon. Les profils des métabolites phénoliques et des acides gras à chaine courte générés par le microbiote ont varié considérablement en fonction du compartiment du colon. Le métabolisme microbien prolongé sur une période de 16 heures a généré une capacité antioxydante similaire à celle produite par les polyphénols parents; ceci diffère des études précédentes de fermentation discontinue à court terme montrant un faible potentiel antioxydant des métabolites. L'étude 2 a utilisé la lignée cellulaire d'adénocarcinome colorectal humain Caco-2 pour étudier les effets de l'acide chlorogénique et de ses principaux métabolites microbiens (les acides caféique, 3-phenylpropionique et benzoïque) sur le cancer du côlon à des concentrations physiologiquement pertinentes d'un mélange équimolaire des composés. La combinaison de CGA et de ses métabolites a amélioré l'efficacité antiproliférative de ces composés, puisque des effets antiprolifératifs, apoptotiques et d'arrêt du cycle cellulaire ont été observés à des concentrations plusieurs fois plus faibles dans le mélange équimolaire que lorsqu'ils étaient administrés séparément. Des altérations de l'ADN mitochondrial n'étaient pas impliquées dans les mécanismes d'action des évènements apoptotiques du CGA et de l'acide caféique, mais une réduction du contenu en ADN mitochondrial était impliquée dans l'apoptose médiée par l'acide 3-phenylpropionique. Dans l'étude 3, les digestats obtenus du système de digestion

intestinale humain simulé ont été soumis à une coculture de cellules intestinales humaines Caco-2 et hépatiques HepG2 pour examiner la digestion et l'effet de premier passage d'un extrait de pomme de terre (PRPE) contenant les acides chlorogénique, caféique et férulique et la rutine comme principaux constituants polyphénoliques. La digestion du PRPE par le modèle GI a mené à l'apparition de métabolites générés par les microbes (les acides dihydrocaféique, dihydroférulique, 3-hydroxybenzoïque, 3-hydroxyphénylpropanoïque, coumarique, 3-hydroxyphénylacétique, phénylpropanoïque et cinnamique). Après 2 heures d'incubation du digestat colique avec les cellules Caco-2, l'acide férulique et les acides dihydrocaféique, dihydroférulique, 3-hydroxyphénylpropionique, 3-hydroxybenzoïque et coumarique ont été mal transportés à travers la monocouche de cellules Caco-2 (3 – 15%). Une augmentation de deux à trois fois supérieure des concentrations d'acides férulique, dihydrocaféique, 3-hydroxyphénylpropionique et coumarique après 3 heures d'incubation avec les cellules HepG2 a démontré une contribution majeure du métabolisme hépatique à la génération de ces composés malgré leur faible transport à travers les cellules Caco-2.

Globalement, l'approche combinée utilisant les systèmes de digestion intestinale simulée et de culture cellulaire, développée dans le cadre des travaux actuels, offre une plate-forme unique pour l'étude détaillée des mécanismes impliqués dans la biotransformation, la biodisponibilité et la bioactivité des polyphénols et de leurs métabolites, ce qui est par ailleurs difficile à réaliser in vivo.

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CONTRIBUTION OF AUTHORS

Dr. Stan Kubow, the candidate's supervisor, initiated the original idea of the research project and was involved in the study design, provided ongoing guidance and feedback and supervised all the aspects of the study. Dr. Kubow also provided extensive feedback and input to all the manuscripts and sections of the thesis.

Shima Sadeghi Ekbatan (Candidate) was responsible for developing the protocols and carrying out all the experiments including in vitro digestions, the antioxidant assay, all the cell culture experiments, the MTT assay, the LDH assay, flow cytometry analysis, DNA extraction and preparation for qPCR analysis, protein extraction and preparation for Western blot analysis. The candidate also prepared the samples for HPLC, GC and LC-MS analyses and collected and interpreted the data. The candidate performed all the statistical analyses and produced all the tables and figures. The candidate wrote all the chapters of this thesis.

Ms. Joelle Khairallah supported the in vitro digestion experiments.

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Dr. Michele Iskandar was involved in developing the co-culture methodology. She provided ongoing guidance and support in every aspect of the lab experiments. Dr. Iskandar also helped with translating the abstract into French and provided valuable editorial input to this thesis.

STATEMENT OF ORIGINALITY

I. Claims of Original Research

The aim of this doctoral dissertation was to utilize a Computer Controlled Dynamic Human Gastrointestinal (GI) Model involving reactors representing the stomach, small intestine and three colonic compartments to study the bioavailability and bioactivities of polyphenols commonly present in the human diet (chlorogenic acid [CGA], caffeic acid [CA], ferulic acid [FA] and rutin [RU]). In Study 1, the impact of upper gastrointestinal enzymatic digestion and colonic microbial metabolism of a mixture of these above polyphenols in the absence of a matrix was studied using the GI model. The results demonstrated that the profiles of the microbial phenolic metabolites and short chain fatty acids generated from digestive processes of the polyphenols were greatly dependent on the colonic compartment. Prolonged microbial catabolism of the polyphenols in the colonic reactors after 16 h led to similar antioxidant capacity to the parent compounds in the upper intestinal vessels, which contrasts the low antioxidant potential of microbial phenolic metabolites previously noted in shorter-term batch culture studies. In Study 2, CGA together with its major microbial-derived metabolites (CA, 3-phenylpropionic and benzoic acids) noted in Study 1, were studied for their anti-colon cancer effects using human colonic Caco-2 tumor cells. The study provided the novel observation that an equimolar mixture of CGA and its microbial metabolites exerted anti-cancer activities involving cell cycle arrest, proliferation and cytotoxicity at 2- to 8-fold lower concentrations than when the compounds were tested singly. Another original finding was that the microbial metabolite 3-PPA had anti-tumorigenic properties and that this treatment was associated with depletion of mitochondrial DNA as a possible anti-cancer mechanism. Study 3 involved investigation of the catabolism of CGA, CA, FA and RU within a polyphenol-rich potato extract to microbial metabolites following digestion of the extract in the GI model and the assessment of the transport and metabolism of the microbial phenolic metabolites using a co-

culture of intestinal and hepatic cells (Caco-2/HepG2). This study demonstrated that FA and phenolic microbial metabolites were poorly transported across the intestinal Caco-2 cells after 2 h but subsequent 3 h metabolism in HepG2 cells led to 1.6 to 3.7-fold increases in ferulic, dihydrocaffeic, 3-hydroxyphenylpropionic and coumaric acids. This observation provides the first evidence that hepatic metabolism can explain previous in vivo observations of a major secondary plasma increase at 4 h in those metabolites following their earlier gradual post-prandial appearance in plasma. Taken together, findings from this thesis provide detailed new information regarding the biotransformation of common dietary polyphenols during digestion and new evidence regarding the bioactivity and bioavailability of the secondary microbial phenolic metabolites.

II. Research Publications in Peer-Reviewed Scientific Journals

- **Sadeghi Ekbatan S**, Li X-Q, Ghorbani M, Azadi B, Kubow S. Chlorogenic acid and its microbial metabolites exert anti-proliferative effects, S phase cell cycle arrest and apoptosis in human colon cancer Caco-2 cells. International Journal of Molecular Sciences 2018, 19(3):713.
- **Sadeghi Ekbatan S**, Iskandar MM, Sleno L, Sabally K, Khairallah J, Prakash S, Kubow S. Absorption and metabolism of phenolics from digests of polyphenol rich potato extracts using the Caco-2/HepG2 co-culture system. Foods 2018, 7(1):8.
- **Sadeghi Ekbatan S**, Sleno L, Sabally K, Khairallah J, Azadi B, Rodes L, Prakash S, Donnelly DJ, Kubow S. Biotransformation of polyphenols in a dynamic multistage gastrointestinal model. Food Chemistry 2016, 204:453-462.

III. Abstracts and Presentations

- **Sadeghi Ekbatan S**, Li X-Q, Ghorbani M, Azadi B, Naqvi N, Kubow S. Chlorogenic acid and its microbial metabolites exert anti-proliferative effects, S phase cell cycle arrest and

apoptosis in human colon cancer Caco-2 cells. CNS Conference, May 2017, Montreal, Canada.

- **Sadeghi Ekbatan S**, Khairallah J, Sabally K, Azadi B, Sleno L, Rodes L, Prakash S, Donnelly DJ, Kubow S. Biotransformation of polyphenols and their antioxidant activity after digestion in the computer controlled dynamic human gastrointestinal model. 10th International ISSX meeting, September 2013, Toronto, Canada.

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LIST OF ABBREVIATIONS

ABTS	2,2′-Azino-bis-(3-ethylbenzothiazoline sulphonic acid)
AC	Ascending colon
AMPK	5' Adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
AOM	Azoxymethane
Apo2L	Tumor necrosis-factor related apoptosis-inducing ligand 2
Apo3L	Tumor necrosis-factor related apoptosis-inducing ligand 3
ARE	Antioxidant response element
BA	Benzoic acid
Bax	Apoptosis regulator (Bcl-2-associated X protein)
Bcl-2	Apoptosis regulator (B-cell lymphoma 2)
BHT	Butylated hydroxytoluene
CA	Caffeic acid
Caco-2	Human epithelial colorectal adenocarcinoma cells
CDKs	Cyclin-dependent kinases
CDKIs	Cyclin-dependent kinase inhibitors
CGA	Chlorogenic acid
Cip1/p21	Cyclin-dependent kinase inhibitor 1
COX-2	Cyclooxygenase-2
COXIII	Cytochrome c oxidase subunit III gene
C_{max}	Maximal plasma concentration
DC	Descending colon
DIO	Diet-induced obesity

DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DR3	Death receptor 3
DR4	Death receptor 4
DR5	Death receptor 5
DSS	Dextran sulfate sodium
EC50	Half maximal effective concentration
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular signal–regulated kinase
ESI-TOF	Electrospray ionization time-of-flight
FA	Ferulic acid
Fas (FasR)	Fas receptor (death receptor)
FasL	Fas ligand
FBS	Fetal bovine serum
G1 phase	Cell cycle gap 1
G2 phase	Cell cycle gap 2
GCL	Glutamate cysteine ligase
GI	Gastrointestinal
GI Model	Computer Controlled Dynamic Human Gastrointestinal Model
GLP-1	Glucagon-like peptide 1
GPx	Glutathione peroxidase
GST	Glutathione *S*-transferase
HCl	Hydrochloric acid

HepG2	Human hepatoma G2 (HepG2) cells
HO-1	Heme oxygenase-1
HPLC	High performance liquid chromatography
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-1β	Interleukin 1 beta
iNOS	Inducible nitric oxide synthase
JNK	c-JunN-terminal kinases
Kip1/p27	Cyclin-dependent kinase inhibitor 1B
LC-MS	Liquid chromatography mass spectrometry
LDH	Lactate dehydrogenase
LOX	Lipooxygenase
LPS	Lipopolysaccharide
M phase	Cell cycle mitotic phase
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant Protein-1
MCT	Monocarboxylic acid transporter
MEM	Minimum essential medium Eagle
MRPs	Multidrug-resistant proteins
mtDNA	Mitochondrial DNA
MTT	Mitochondrial succinate dehydrogenase
Nad4	NADH dehydrogenase subunit 4 gene
NaOH	Sodium hydroxide
NF-κB	Nuclear factor kappa B

NQO1	NAD(P)H:quinone oxidoreductase 1
Nrf2	Nuclear factor erythroid 2-related factor 2
p21	CDK-interacting protein 1
p27	Cell cycle regulatory protein
p53	Tumor suppressor p53
PBS	Phosphate buffered Saline
PE	Potato extract
PGE2	Prostaglandin E2
P-gp	P-glycoprotein
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-kinase
3-PPA	3-Phenylpropionic acid
PPARs	Peroxisome proliferator-activated receptors
pRb	Phosphorylated retinoblastoma
PRPE	Polyphenol-rich potato extract
PYY	Pancreatic peptide YY
qPCR	Quantitative polymerase chain reaction
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RU	Rutin
S phase	Cell cycle synthesis phase
SCFA	Short chain fatty acid
SHIME	Simulated Human Intestinal Microbial Ecosystem

SI	Small intestine
SOD	Superoxide dismutase
TC	Transcending colon
TEER	Transepithelial electrical resistance
TNBS	2,4,6-Trinitrobenzene sulfonic acid
TNF-α	Tumor necrosis factor alpha

CHAPTER 1: INTRODUCTION

1.1. Rationale and Statement of Purpose

Polyphenols are among the most abundant antioxidants in the human diet [1] and have been indicated to exert protective effects against a number of chronic diseases such as cancer, cardiovascular disease and neurodegenerative disorders [2]. Phenolic acids and flavonoids are prevalent in fruits and vegetables including food staples such as potatoes [1, 3]. Potatoes are a good source of phenolic acids and flavonoids, particularly hydroxycinnamic acids 3-caffeoylquinic acid (chlorogenic acid: CGA), 3,4-dihydroxycinnamic acid (caffeic acid: CA), 3-(4-hydroxy-3-methoxy-phenyl)prop-2-enoic acid (ferulic acid: FA) and the flavonoid quercetin-3-O-rutinoside (rutin: RU) [4, 5]. The above compounds are major representatives of common dietary polyphenols [6]. A number of health-promoting properties have been attributed to such polyphenols including antioxidant and anti-cancer activities [7-11]. The co-occurrence of polyphenols with varying structures in plant foods has led to suggestions that this can lead to synergistic antioxidant and anti-proliferative effects [12-14]. Such synergisms have been largely based on in vitro studies using the native polyphenols found in foods that are generally poorly absorbable [14] rather than the more absorbable smaller molecular weight secondary polyphenol metabolites generated by gut microbiota [10] as there is limited modification of native polyphenols by upper intestinal digestive processes [15].

The chemical structure of the polyphenols within plant foods is the main determinant of the pattern of their intestinal absorption as these compounds have limited absorptivity in their native format that is ester-linked with sugars or within cell walls in association with polysaccharides, proteins or lipids [15]. It has been shown that a large amount of CGA CA, FA and RU enter the colon undigested where they are metabolized by gut microbiota [16-19]. The colonic microflora plays a key role in the biotransformation and metabolism of undigested

polyphenols in humans. The colonic microflora is the only source of esterases and some of the glycosidase enzymes such as xylanases and α-rhamnosidases that release polyphenols from the food matrix and further microbial catabolism [15]. In addition, hydroxycinnamic acids such as *p*-coumaric and caffeic acids can undergo hydrolysis by lactic acid bacterial decarboxylases [20, 21]. CGA, which is the ester of CA and quinic acid, is hydrolyzed by esterases produced by colonic microflora [15]. Certain gut bacteria belonging to the *Bifidobacterium* and *Lactobacillus* genera with esterase-producing activity have been identified to be involved in the release of hydroxycinnamic acids such as CGA and FA from the food matrix in the human colon [22]. Also, free CA is more readily absorbed than CGA following its release by bacterial esterases and subsequent catabolism via microbial decarboxylase [23]. FA is linked to hemicellulose through an ester bond [15]. It can enter the systemic circulation following its release from plant cell walls via microbial esterases [23]. RU, a glycoside of quercetin, has shown poor bioavailability in its intact form in vivo [15]. The rutinose moiety of rutin hinders its absorption in the small intestine [24]. Thus, the phenolic moiety of RU is absorbed only after hydrolysis of the sugar by gut microbiota [15]. Different bacteria species such as *Bacteroides distasonis, B. uniformis and B. ovatus* with α-rhamnosidase and β-glucosidase activities have been found to be responsible for hydrolysis of RU [15]. Further breakdown of CA, FA and quercetin generates phenolics with structures containing from zero to two hydroxyl groups such as 3-(3-hydroxyphenyl)propionic, 3-(4-hydroxyphenyl)-propionic, 2-(3,4-dihydroxyphenyl)acetic, 2-(3-hydroxyphenyl)acetic, 4-hydroxybenzoic, benzoic and protocatechuic acids and vanillin [25, 26]. 3-(3-Hydroxyphenyl) propionic acid is formed from CA following reduction of a double bond and dihydroxylation at the C4 position. Its further metabolism via β-oxidation leads to generation of benzoic acid [25].

The digestive process taking place following polyphenol ingestion exerts a significant impact on antioxidant bioactivity, which has been related to the generation of microbial

metabolites of polyphenols with antioxidant properties [27, 28]. Additionally, polyphenols have been indicated to exert prebiotic-like effects by modulating gut bacterial composition, which can enhance colonic short chain fatty acid (SCFA) profiles [29]. Numerous health promoting effects have been attributed to SCFAs including the regulation of energy metabolism [30], anti-colon cancer and anti-inflammatory activities [31]. It has been suggested that the induction of SCFA generation by dietary fiber reduces the pH in the colonic segments which may, in turn, inhibit the metabolic activity of some of the bacteria involved in polyphenolic catabolism [32]. To date, however, there is no information on the effects of pure polyphenols on SCFA profiles in different segments of the GI tract as the modulatory effects of polyphenols on SCFAs have only been studied utilizing the static batch-culture fermentation system [33].

Despite the functional significance of the phenolic microbial metabolites, there is a paucity of knowledge with regards to microbial biotransformation of polyphenols and the bioactivity of the generated metabolites. In vivo studies have demonstrated the metabolism of dietary polyphenols by gastrointestinal tract (GI) by measuring their bioactive metabolites in plasma or urine [17, 19]. It has been shown that following ingestion of a polyphenol-rich diet there is a rise in the plasma and urine levels of the phenolic microbial metabolites [10, 34]. Although the in vivo models are physiologically more relevant to study polyphenol metabolism, they are not well suited for detailed study of the pathways of biotransformation of polyphenols occurring at different GI segments. In addition, there are some differences in polyphenol metabolism between humans and animals, which has been noted when using animal models to investigate the metabolic pathways and bioavailability of polyphenols. For example, in contrast to rats, rhamnosides are not metabolized by human tissues [15]. Due to the high complexity of the process involved in the digestion and metabolism of polyphenols, various in vitro models have been designed to simulate the GI tract condition to study the digestion and biotransformation of food compounds including

polyphenols [35]. However, most of the in vitro digestion studies have only assessed the enzymatic digestion of the polyphenols without consideration of the importance of the gut microflora in digestion and metabolism of polyphenols [28, 36, 37]. In addition, the microbial biotransformation of polyphenols has been mainly evaluated via the use of basic batch fermentors containing human fecal suspension rather than more physiologically relevant continuous multistage gastrointestinal models [37, 38]. Batch models are limited by depletion of nutrients and accumulation of the bacterial end products. In addition, the absence of automatic pH control causes continuous changes in pH which affect the stability of the bacterial community during experimentation. These confounding factors limit the operational time of batch fermentations to a few hours [39]. To more closely simulate the in vivo conditions, several factors are taken into account in continuous digestion models including the transit time in different GI segments including the ascending, transverse and descending colon [40]. The pH in colonic vessels is adjusted according to the in vivo conditions to simulate the different colonic segments and corresponding bacterial profiles. In addition, in continuous multistage systems, the gut microflora is cultured for a longer period of 2 weeks before experimentation to allow the adaptation and stability of the microflora in the colonic vessels [41]. The Computer Controlled Dynamic Human Gastrointestinal Model is composed of five double-jacketed vessels representing the stomach, the small intestine, the ascending colon, the transverse colon, and the descending colon [41] and has been validated by measuring the pattern of SCFA production, enzymatic activities and gas production [41]. However, in spite of the advances in the in vitro digestion systems, these systems have been rarely used to study the biotransformation of polyphenols in different GI segments and their effects on antioxidant capacity and SCFA profile of the colonic compartments. Two previous studies have utilized the dynamic, continuous in vitro GI digestion model to show enzymatic and gut microbial catabolism of polyphenols as constituents in foods and beverages such as black tea, red wine/grape juice [42]

and purple-fleshed sweet potato [11]. The continuous in vitro GI digestion model, however, has not been applied to study gut-mediated biotransformation of pure polyphenol supplements, which could be substantively different when provided in the absence of a food matrix [32]. More detailed knowledge regarding generation of microbial phenolic metabolites, antioxidant capacity and SCFA production following digestion of pure polyphenols is pertinent as most human and animal studies examining polyphenol biotransformation and bioavailability involve the administration of pure polyphenol compounds. Moreover, pure phenolic compounds such as CGA are sold as nutraceutical supplements, which makes information regarding their digestive processes nutritionally relevant.

Prior to their systemic absorption, the presence of polyphenols in the gut lumen has been suggested to exert protection against various colonic disorders such as colorectal cancer [43, 44]. A large body of evidence has indicated a reduced risk of colon cancer in association with diets rich in fruits and vegetables [45], which could be partly mediated through their ubiquitous content of phenolics having anti-cancer properties [46]. The high concentration of polyphenols in the gut could partly explain implications for their anti-colon cancer effects [15]. Phenolics have been indicated to exert anti-proliferative effects on colonic tumor cells via various mechanisms including antioxidant activity [44], gene expression regulation [44, 47], cell cycle arrest [44, 48], caspase-3-mediated apoptosis [44] and modulation of mitochondrial DNA content [49]. Although CGA has shown promising anti-cancer activity in colon cancer cell lines [50], this compound undergoes extensive colonic microbial biotransformation to generate CA, 3-phenylpropionic acid (3-PPA) and benzoic acid (BA) as predominant metabolites present in the fecal water of human subjects [16, 51]. Despite the low systemic circulating concentrations of the microbial metabolites, their levels in the gut lumen may be great enough to modulate molecular pathways of carcinogenesis in gut cells [25]. Human fecal water containing the polyphenol metabolites has

been shown to inhibit the inducible enzyme cyclooxygenase-2 (COX-2) in HT-29 cells, which is involved in various carcinogenesis processes such as apoptosis and tumor invasiveness [52]. In the latter study, microbial metabolites including 3-phenylpropionic acid, 3-hydroxyphenylacetic acid, and 3-(4-hydroxyphenyl)-propionic acid were shown to decrease the protein levels of COX-2 in HT-29 cells [52]. Apart from one study demonstrating anti-colon cancer cell activities associated with CA, there is no information about the anti-cancer effects of other common phenolic-derived microbial metabolites either singly or in combination or their possible anti-cancer mechanisms of action in human Caco-2 colon cancer cells. The co-occurrence of CGA and its predominant microbial metabolites is of particular interest as it is unclear whether such a combination in fecal fluid can lead to either synergistic or antagonistic effects on anti-cancer activities [53, 54]. Therefore, evaluation of the anti-cancer effects of physiologically relevant concentrations of CGA and its metabolites individually and in combination using human Caco-2 colon cancer cells together with the study of possible mode of action can provide insights regarding the impact of these compounds on anti-colon cancer activities.

The diverse range of biofunctional activities of polyphenols has led to the development of polyphenol-rich nutraceutical extracts derived from polyphenol-containing foods [55, 56]. In that regard, potatoes have gained attention as their intake has been associated with antioxidant and anti-inflammatory effects in human and animal studies [3]. Moreover, a polyphenol-rich potato extract (PRPE) with an enriched content of CGA, CA, FA and RU was demonstrated to attenuate weight gain and improve glucose control in the diet-induced obesity (DIO) animal model [57, 58]. The beneficial effects from the intake of PRPE could be related to bioavailable microbial compounds generated via digestive and absorptive processes involving gut microbial, intestinal and hepatic metabolism [15]. One of the challenges in identifying the bioactive metabolites of polyphenols is the lack of detailed knowledge regarding their absorption and biotransformation. Following their

digestion, further biotransformation of polyphenol metabolites occurs at intestinal and hepatic tissues as these are two barriers that ingested compounds need to first pass before entering the systemic circulation [1]. This is a complex process involving intestinal metabolic enzymes and transporters, as well as intestinal and hepatic phase II metabolism [59]. In human subjects, following ingestion of polyphenol rich-foods, there is post-prandial appearance in plasma and urine of a variety of polyphenol metabolites depending on the polyphenol source, which includes vanillic acid, isoferulic acid as well as derivatives of phenylpropionic, hippuric, benzoic and phenylacetic acids [19, 34]. In vivo human and animal bioavailability studies usually administer a certain dose and measure the changes in plasma metabolite concentrations over time [60]. This approach, however, has led to an extensive variation (up to ten-fold) in the maximal plasma concentration (C_{max}) values for most phenolic metabolites [60]. Additionally, such studies are costly and the complexity of the factors affecting digestive and absorptive processes has made determination of the role of intestinal and hepatic metabolism difficult to perform.

To assess the bioavailability of parent polyphenols and their secondary metabolites, human intestinal Caco-2 and hepatic HepG-2 cell co-cultures can be applied as this approach has been shown to accurately predict in vivo oral bioavailability of ingested compounds in animal models [61] and humans [62]. This system showed enhanced basolateral permeation of the major flavonoids present in combined thyme and olive extracts after simulated stomach and small intestine digestion [63]. However, such an approach has not been applied to examine the bioavailability of microbial metabolites of polyphenols generated via digestive processes. The use of the Caco-2/HepG2 co-culture system for testing digests generated from both upper intestinal digestion and colonic microbial catabolism provides a novel opportunity to identify absorbable lower molecular weight microbial phenolic compounds generated from digestion of polyphenol-rich foods as well as phytochemical extracts such as PRPE. In particular, the combination of in

vitro digestion and absorption models allows the study of biotransformation and cellular uptake of polyphenol metabolites in a controlled setting that permits sampling at different time points to identify intestinal- and hepatic-mediated absorption and metabolism processes [64].

1.2. Research Objectives

1. To identify the microbial polyphenol-derived metabolites generated from the digestion of a synthetic mixture of chlorogenic acid, caffeic acid, ferulic acid and rutin using the Computer Controlled Dynamic Human Gastrointestinal (GI) Model.
2. To evaluate the antioxidant capacities and short chain fatty acid profiles of digests generated from the above polyphenol synthetic mixture in the GI model.
3. To assess the anti-proliferative and cytotoxic effects of chlorogenic acid, its microbial metabolites caffeic acid, 3-phenylpropionic acid and benzoic acid and the combined effects of an equimolar mixture of the above four compounds following their exposure to the human colonic Caco-2 cancer cell line.
4. To investigate possible cellular markers involved in the anti-cancer activities of chlorogenic acid and its microbial metabolites by measuring their effects on cell cycle events, caspase-3 activation and mitochondrial DNA content.
5. To implement the Caco-2/HepG2 system to accommodate colonic microbial digests in order to provide an evaluation of the intestinal and hepatic-mediated absorption and biotransformation processes involving polyphenols and their microbial metabolites generated from digestion of PRPE in the GI model.

1.3. Hypotheses

1. In vitro digestion and microbial metabolism of polyphenols will result in polyphenol metabolites in the colonic fecal water that are associated with varying antioxidant capacities and short chain fatty acid profiles of the digesta in different segments of the GI model.
2. Chlorogenic acid and its microbial metabolites will exert their anti-cancer activities on cell proliferation on a dose-dependent basis in concentrations that do not cause cytotoxicity.
3. The combination of chlorogenic acid with its microbial metabolites in an equimolar mixture will enhance the anti-cancer efficacy beyond what is exerted by each individual compound.
4. Chlorogenic acid and its microbial metabolites exert anti-proliferative effects via induction of cell cycle arrest and apoptosis and modulation of mitochondrial DNA content.
5. The exposure of PRPE digests to the Caco-2/HepG2 cell culture system will show differential degrees of absorption and biotransformation of the parent polyphenols and their microbial metabolites.

CHAPTER 2: LITERATURE REVIEW

2.1. Polyphenols: Classification and Chemical Structure

Dietary polyphenols are plant-based compounds and the predominant antioxidants in the human diet. Fruits, vegetables, legumes, grains, herbs, tea and spices are the main sources of polyphenols [1]. To date, several thousand molecules with polyphenol structure have been identified [1]. Polyphenols act as a defense system in plants to protect them against ultraviolet radiation or aggression by pathogens [1]. Polyphenols may also contribute to the organoleptic properties of foods such as bitterness, astringency, color, flavor, odor and oxidative stability [65]. The total daily intake of polyphenols could reach 1 g/d in subjects consuming a polyphenol-rich diet [15]. The polyphenolic content of fruits such as grapes, apple, pear and cherries could reach up to 200 – 300 mg polyphenols per 100 grams fresh mass [65].

Polyphenols are characterized as compounds with phenolic structure and are divided into several sub-groups depending on the number of phenol rings and the structural elements binding phenol rings to one another [1]. The majority of polyphenols are present in plants as glycosides with different sugar or acylated sugars attached to different positions on the polyphenol structure [66]. The main classes of polyphenols include phenolic acids, phenolic amides, flavonoid, stilbenes and lignans and each group may be divided into different subclasses [1, 66].

Phenolic acids are widespread in foods and are divided into the two main classes of cinnamic acid and benzoic acid derivatives [1]. The cinnamic acid derivatives are more common than benzoic acid derivatives. Caffeic (CA), ferulic (FA) and *p*-coumaric acids are some of the known cinnamic acid derivatives and gallic, vanillic, syringic and protocatechuic acids are among the main benzoic acid derivatives [66]. Phenolic acids present in fruits and vegetables are mainly in the free form while they are often found in the bound form in grains [66]. For example, FA is mainly linked to hemicelluloses through ester bond linkages in cereals [1]. In wheat bran, one of

the main sources of FA, only 10% of FA is found in the soluble free form. The bound forms are released upon acid or alkaline hydrolysis or by the action of enzymes in the GI tract [66]. CA is found in the ester form in combination with quinic acid to form chlorogenic acid (CGA). CA does not typically occur in the free form except as a dietary supplement or if a polyphenol-containing food is exposed to processing such as fermentation [1]. CGA is the main caffeoyl ester present in fruits, vegetables and coffee. One cup of coffee may contain 70 – 350 mg CGA [1].

Polyphenol amides contain N-functional substitution. Capsaicinoids in chili peppers and avenanthramides in oats are among the main known compounds in this group. These phenolic compounds have strong antioxidant and anti-inflammatory activities [66].

Flavonoids are the most common group of phenolic compounds consisting of phenolic and pyran rings. Flavonoids vary based on the arrangements of hydroxyl, methoxy, and glycosidic side groups and the conjugation between the A- and B- rings. According to their substitutions flavonoids can be divided into six subclasses including flavonols, flavones, flavanones, flavanols, anthocyanins and isoflavones [67]. Flavonols are the most abundant flavonoids in plant foods [1]. Isoflavones are classified as phytoestrogen due to their structural similarities to estrogen and their ability to bind to estrogen receptors. Quercetin, genistein, myricetin, catechins, kaempferol and naringenin are some of the most common flavonoids in the human diet [1].

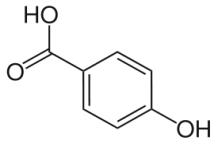
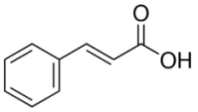
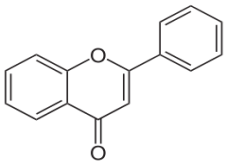
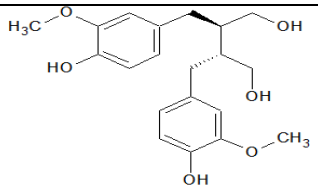
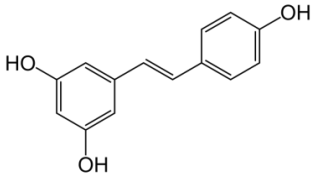
Flavonoids mainly occur in foods as glycosides. For instance, rutin (RU), a rhamnoglucoside of quercetin, is one of the most common glycosides of quercetin found in high concentration in tea [68].

Lignans and stilbenes are found in low quantities in the human diet and are less studied among the polyphenolic compounds. Stilbenes consist of two phenyl rings, which are connected by a two-carbon methylene bridge. The stilbene resveratrol is present in significant amounts in grapes and red wine and has been suggested to provide many beneficial health effects [65]. The

basic structure of lignans is 2,3-dibenzylbutane and their richest dietary sources are flaxseed, sesame seed and many grains [65]. A number of health benefits have been shown to be associated with lignan consumption such as reduced risk of various types of cancer [69].

The polyphenol content of foods is influenced by several factors such as environmental factors, degree of ripeness, processing and storage conditions [65]. In addition, the phenolic compounds are not equally distributed in different parts of the plants. The phenolic content in the outer (epidermal) layers of plants are generally greater than the inner parts [65]. Although some polyphenols may be specifically found in certain types of food, most plants contain a complex mixture of polyphenols so that their interaction to one another can determine their ultimate biological activities [70, 71]. Table 2.1 presents a summary of polyphenol classification and their major food sources.

Table 2.1. Classification, chemical structure and food sources of polyphenols [1, 66].

Polyphenols	Subclasses	Food source	Chemical structure
Phenolic acids			
Hydroxybenzoic acids	Protocatechuic acid, Gallic acid	Berries	
Hydroxycinnamic acids	Caffeic acid, Coumaric acid, Ferulic acid, Sinapic acid	Apple, Potato, Coffee, Kiwi, Aubergine	
Flavonoids			
Flavonols	Kaempferol, Quercetin,	Onion, Kale, Leek	
Flavones	Apigenin, Luteolin	Parsley, Celery, Broccoli	
Flavanones	Naringenin, Hesperidin	Grapefruit, Orange juice	
Flavanols	Catechin, Epicatechin	Tea, Chocolate, Beans	
Anthocyanins	Cyanidin, Delphinidin, Malvidin	Berries, Cherry	
Isoflavones	Genistein, Daidzein	Soy flour, Soybeans, Tofu	
Lignans	Secoisolariciresinol	Flax seed, Flax seed oil	
	Matairesinol	Flax seed, Flax seed oil	
Stilbenes	Resveratrol	Grapes, Wine	

2. 2. Potatoes: A Good Source of Polyphenols and Antioxidants

Potatoes are a good dietary source of antioxidants and polyphenols and are a food staple consumed in large amounts by many populations [4, 5]. Potatoes provide an inexpensive source of energy and protein [72] and play an important role in food security especially in developing countries [73]. Polyphenols, ascorbic acid, tocopherols, selenium and α-lipoic acid are the main antioxidants in potatoes [74].

CGA, CA and FA are the most abundant phenolic compounds present in potato with CGA constituting up to 90% of the potato's phenolic content [75]. The phenolic content of potatoes can vary greatly among cultivars [76, 77]. Potatoes have been promoted as a healthy food item and a potential functional food because of their abundant antioxidant content and their contribution to human health [4, 74]. Polyphenol-rich potato extracts have shown antioxidant and anti-inflammatory activities both in vivo [7] and in vitro [8, 78]. In addition, the beneficial health effects of the above polyphenols present in potatoes have been reported previously [10]. For example, CGA has been shown to exert many potential health promoting effects including antioxidant, anti-inflammatory, anti-cancer, anti-lipidemic, anti-diabetic, anti-hypertensive, and anti-neurodegenerative activities [79]. CGA has exhibited anti-diabetic and anti-lipidemic effects in mice, which was shown to be partly mediated via activation of AMP-activated protein kinase (AMPK) [80]. CGA has also been associated with beneficial effects on hepatic and gastrointestinal health through anti-inflammatory activities by reducing the pro-inflammatory cytokines, tumor necrosis factor-α (TNF-α), inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) protein and nuclear factor-κB (NF-κB)–activation [81]. In addition, CGA has shown protective effects against lipopolysaccharide (LPS)-induced hepatotoxicity [82] and LPS-induced lung injury [9] in mice.

CA shows antioxidant and anti-inflammatory properties [83, 84]. CA has been shown to diminish glucose-induced endothelial dysfunction in primary human umbilical-vein endothelial cells through modulation of NF-κB and nuclear factor erythroid 2–related factor 2 (Nrf2) pathways [85]. CA treatment was also shown to prevent diet-induced obesity and hyperlipidemia in mice [86] and to exert anti-cancer effects in various tumor cells [50].

FA has been indicated to exert antioxidant and anti-inflammatory effects [87] and has been associated with potential health benefits against various diseases such as cancer, diabetes, cardiovascular and neurodegenerative disorders [88]. Some of the beneficial health effects of FA were associated with its strong antioxidant activity. The phenolic hydroxyl group in FA structure is the key element in its radical scavenging activity [88]. FA has shown anti-cancer effects in various cell lines by inhibiting cell proliferation and inducing cell cycle arrest and apoptosis [89, 90]. Furthermore, FA has shown pulmonary protective effects against oxidative damage induced by nicotine and reversed nicotine-induced lung damage by enhancing the endogenous antioxidant defense system [88].

RU is a strong antioxidant and has been associated with possible beneficial health effects in various disease such as diabetes, cardiovascular disorders and cancers [91]. RU has been shown to exert antioxidant activity by reducing the generation of reactive oxygen species (ROS) and malondialdehyde in HepG2 cells [92]. RU has also been shown to protect mice against carbon tetrachloride-induced kidney damage by inhibiting oxidative stress, inflammation and apoptosis [93]. In the latter study, RU was shown to exert its effects via reducing ROS and ceramide levels, suppressing the p53, TNF-α, IL-1β activities and mitogen-activated protein kinase (MAPK) phosphorylation and inhibiting the release of cytochrome c from mitochondria in kidneys [93].

The presence of significant amounts of polyphenols in potatoes especially CGA and FA may act synergistically to provide antioxidant and anti-inflammatory benefits [57]. A significant

inverse correlation between potato polyphenol content and the glycemic index of potatoes has been noted [94]. In that regard, CGA has been shown to inhibit the digestion of potato starch [95]. On the other hand, some studies have reported an association between potato consumption and an increased risk of obesity and type 2 diabetes that was linked to a high glycemic index of some potato cultivars [4]. In contrast, other studies have reported no association between potato consumption and incidence of diabetes, even showing a lower risk of developing type-2 diabetes in some populations [4]. Such contradictory results among different populations could be due to different preparation methods (boiling vs. frying) and phytochemical differences in the types of cultivars commonly consumed [96]. Interestingly, polyphenol-rich potato extracts (PRPE) were demonstrated in the diet-induced obesity (DIO) mouse model to exert significantly greater anti-obesity effects and improvement in blood glucose control than supplements of CGA and FA that were provided at the same amounts as in the PRPE [57]. Since the major polyphenols present in PRPE have limited bioavailability, it is conceivable that the health promoting activities of PRPE could be partly exerted by their post-digest and post-absorbed microbial metabolites. There is a knowledge gap, however, regarding the bioaccessibility and bioavailability of polyphenols and their microbial metabolites that could play an important role in the health benefits of PRPE.

2.3. Bioaccessibility and Bioavailability of Polyphenols

Bioaccessibility is defined as the amount of food component that is released from the food matrix upon digestion and becomes available for absorption and further metabolism by intestinal cells [97]. Bioavailability refers to the proportion of a nutrient that is digested, absorbed and metabolized by normal systemic metabolic pathways [98]. The health effects of dietary polyphenols depend on their bioavailability and metabolism in the gastrointestinal tract [1]. Polyphenols and their metabolites have varying degrees of bioavailability. A number of factors have been recognized to affect the bioavailability of polyphenols and their subsequent biological

effects including chemical structure, molecular linkage, type of sugar in the glycoside, food matrix characteristic, food processing, interaction with other compounds, and host related factors [65, 99]. The digestion and absorption of certain polyphenols can begin in the stomach. For example, quercetin and some anthocyanins can be absorbed from the stomach [65]. Most polyphenols, however, are present in foods as esters, glycosides or polymers that are resistant to stomach digestion and so must be hydrolyzed by intestinal enzymes or colonic microflora before absorption [100]. A large amount of dietary polyphenols bypass upper intestinal absorption to reach colonic regions where they are biotransformed by the enzymatic activity of the intestinal microflora to generate more absorbable smaller molecular weight compounds [100].

Polyphenol catabolism by colonic microflora has been considered an important factor related to the health benefits of polyphenols as bioactive microbial metabolites are generated [98]. In that context, low molecular weight phenolic metabolites have been indicated to be more readily absorbed as compared to the higher molecular weight parent compounds [10]. Polyphenol microbial metabolites such as dihydroferulic acid, dihydrocaffeic acid and their sulfated and glucuronidated derivatives have been identified in plasma [17] as well as human urine following consumption of a polyphenol-rich diet [19]. The urinary excretion of simple microbial phenolics such as hydroxyhippuric, hydroxyphenylacetic and 3-(hydroxyphenyl)-propionic acids has been associated with intake of a high flavonoid diet [101].

Animal and human studies have shown that a large amount of CGA CA, FA and RU reach the large intestine and are metabolized by the gut microflora [16-18, 23, 102]. Only one-third of the ingested CGA is absorbed in the upper GI tract while the major part reaches the colon where it is hydrolyzed by the esterase activity of the microflora [23, 101]. Following cleavage of the ester bond of CGA by colonic microbiota, there is release of CA and quinic acid. CA undergoes further microbial biotransformation producing metabolites such as dihydrocaffeic acid, *m*-coumaric acid,

hippuric acid, and derivatives of phenylpropionic and benzoic acids [16, 18, 103]. About half of the ingested RU has been shown to be catabolized by gut microbiota [51] to generate metabolites such as 3-hydroxylphenylacetic acid [104]. The absence of the microbial RU- and CGA-derived metabolites in the urine of ileostomy patients also indicates their production upon microbial action [51]. It has been shown that over 95% of the FA release from the food matrix take place in the colon by the action of several hydrolytic enzymes such as xylanase and esterase whereas only small amounts of FA are released following upper GI digestion [105].

Polyphenols and their metabolites undergo further intestinal and hepatic modification by phase I and phase II enzymes that results in conjugated, methylated, sulfated and glucuronidated compounds [65]. Glucuronidation occurs in both the intestine and liver whereas sulfation takes place mainly in the liver [65]. On the other hand, *O*-methylation of hydroxycinnamic acids can occur by the action of gut microflora [19]. The conjugation process is highly efficient as polyphenol metabolites are found in plasma mainly in the conjugated form with relatively smaller concentrations present as aglycones [65]. Polyphenol metabolites circulate in the blood bound to proteins such as albumin, which plays an important role in polyphenol bioavailability. Binding to albumin may affect the clearance rate and cellular uptake of the metabolites, which is not fully understood. Excretion of polyphenols and their metabolites occurs through urine and bile [65]. Figure 2.1 illustrates a summary of possible metabolic routes of ingested polyphenols in humans.

Overall, considering the extensive metabolism of polyphenols in the gastrointestinal tract, more detailed knowledge of the metabolic fate of polyphenols and their metabolites is required for better understanding of their biological effects. The existing knowledge about polyphenol digestion and biotransformation in the gastrointestinal tract is based on the in vivo studies that measured polyphenols and their metabolites in plasma or urine following consumption of polyphenol-rich foods [17, 19]. In addition, a number of recent studies have used in vitro digestion

models to monitor the biotransformation of polyphenols in different GI segments and to study the microbial biotransformation of polyphenols [38, 106].

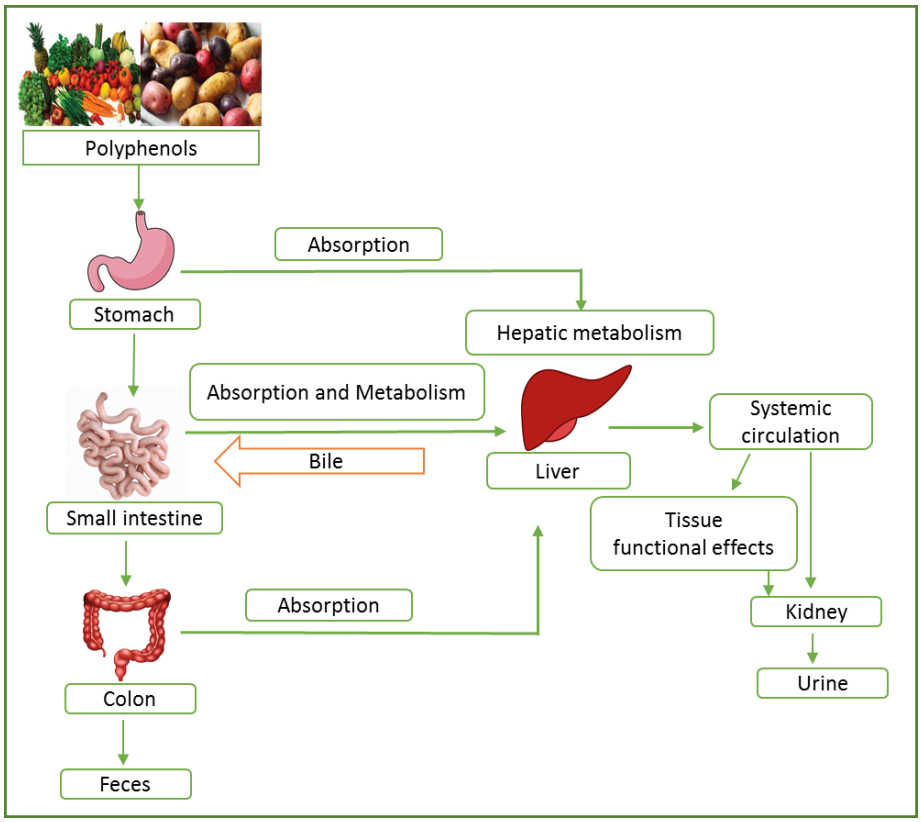


Figure 2.1. Schematic diagram of the metabolism of dietary polyphenols (Adapted from Scalbert and Williamson 2000). Polyphenols, mainly aglycones, are partially absorbed from the stomach and small intestine, transported to the liver. After phase I and phase II modification, they may enter circulation and reach different organs, or are excreted with bile back into the small intestine and pass to the colon. A large amount of polyphenols reach the colon undigested where they are metabolized by the colonic microbiota. In the colon, polyphenols are deconjugated and hydrolyzed by the action of colonic bacterial enzymes such as α-rhamnosidase, β-glucosidase and esterase. The absorbed polyphenols and their microbial metabolites undergo phase I and II hepatic metabolism to form methylated, sulfated and glucuronidated metabolites and then they enter the

circulation in both the conjugated and aglycone forms to exert their biological activities in target organs or are excreted via urine.

2. 4. In Vitro Digestion of Polyphenols

In recent years, various in vitro gastrointestinal (GI) models have been developed to simulate the digestion and biotransformation of dietary compounds in the human GI tract. Most of the in vitro models that evaluated the effects of digestion on polyphenols have either not included gut microflora or have only used basic fermentors containing human fecal suspension to examine the effects of gut microflora on polyphenol digestion [18, 105, 107]. In such models, the dynamics of transit during digestion or the varying microbial and digestive conditions in different segments of the GI tract have not been considered. Although the static systems are useful for short-term fermentation studies they are limited by factors such as substrate availability and weakness in microbiological control [39].

Brown *et al.* (2014) coupled the upper GI digestion with batch culture fermentation to compare the in vitro digestion of lingonberry polyphenols to their in vivo digestion using ileostomy subjects. They reported similar in vitro and in vivo results for the post-digestion modification in the polyphenolic composition of lingonberries [108]. The in vitro digestion of blueberry demonstrated high stability of polyphenols and anthocyanins during gastric digestion. However, extensive degradation occurred during colonic digestion leading to generation of phenolic compounds such as syringic, cinnamic, caffeic and protocatechuic acids [109]. In the latter study, metabolites of the colon digest exhibited lower free radical scavenging activity as well as decreased anti-proliferative activity on a human colonic epithelial cell line (CRL 1790) and a human colorectal cancer cell line (HT 29) than their precursors in the stomach and intestinal digests [109]. In another study, the tannase-mediated enzymatic biotransformation of green tea extract and CGA led to a significant increase in their antioxidant capacities [110]. Tannases are known to hydrolyse

both the “ester” and “depside” bonds of polyphenols such as tannic acid, epicatechin gallate, epigallocatechin gallate, and CGA [110]. Stepwise pepsin and pancreatic digestion and batch fermentation of grape and chokeberry wines demonstrated bacterial degradation of resveratrol and CGA and the generation of CA and other bacteria-derived phenolic metabolites [107]. In that study, the greatest phenolic content and antioxidant activity was described following stomach digestion, which then decreased significantly during the 2 h small and 18 h large intestine digestion [107]. The initial increase in the antioxidant activity following gastric digestion could be due to the hydrolysis of the glycosidic bond and generation of aglycones with greater antioxidant activity than the bound glycosidic forms. Further enzymatic and bacterial degradation of the phenolic compounds in the small and large intestine resulted in generation of new metabolites with decreased antioxidant capacity [107].

To simulate the complexity of human digestion processes, more sophisticated in vitro digestion systems have been developed by increasing the number of simultaneously operated reactors to mimic the digestion and biotransformation of different GI segments [39]. Such systems include continuous fermentation models and more complex artificial digestive systems that couple upper GI digestion models with continuous fermentation systems. The continuous flow of substrate and fecal solutions in these systems in a well-controlled environment mimics in vivo conditions more closely [39]. In contrast to static systems, the continuous replenishment of nutrients and removal of the metabolic by-products of digestion in these systems allow for long-term experimentation [39]. These systems have begun to be widely applied for the study of the modulatory effects of various prebiotics and probiotics on gut microbiota profiles and to assess the bioaccessibility of phytochemicals and environmental contaminants from foods [39]. Only a few studies have used dynamic multi-compartment GI models that incorporate the upper GI digestion together with colonic compartments that simulate the biotransformation of polyphenols in different

segments of the human GI tract [11, 111]. Multistage GI models are computer-controlled continuous digestion systems composed of five double-jacketed vessels representing the stomach, the small intestine, the ascending colon, the transverse colon, and the descending colon. The pH is automatically controlled by the addition of acid and base solutions to simulate the in vivo conditions in different GI segments of the human gastrointestinal tract and the growth of corresponding microbial communities in the colonic segments. The temperature is kept at 37^{o}C and anaerobic conditions are maintained in the fermentation vessels by flushing nitrogen gas into the airspace. This system has been validated by monitoring the changes in SCFA profiles, gas production and enzymatic activity [41].

The computer-controlled dynamic human GI model has been used to study the biotransformation of anthocyanins from two purple-fleshed sweet potatoes and demonstrated an accession-dependent pattern for anthocyanin bioaccessibility and degradation in different segments of the GI system [11]. An increase in antioxidant capacity in the colonic reactors corresponded to the pattern of anthocyanin release for each accession [11]. In another study, Cueva *et al*. (2015) reported a good correlation between microbial phenolic metabolite profiles of the digesta obtained from the in vitro GI digestion of wine with the fecal phenolic profiles from human subjects fed wine [112]. Table 2.2 summarize the list of studies of polyphenols using different in vitro digestion models. These in vitro studies have demonstrated the utility of the multi-stage in vitro digestion system as a valuable model to study the enzymatic and microbial metabolism of polyphenols taking place in different colonic regions. Although such systems may not completely mimic in vivo conditions in terms of absorption due to the absence of host intestinal epithelial cells, they are a useful and cost-effective approach to explore the impact of digestion on bioaccessibility of food components in a controlled environment [39].

Table 2.2. Summary of in vitro digestion studies on polyphenol biotransformation.

Food/polyphenols	In vitro digestion models	Key findings	References
Elderberry extract	Mouth, stomach and small intestine	81.8% decrease in polyphenol content following simulated enzymatic digestion Reduction in antioxidant activity of the digested extract	[28]
Dietary supplements: pomegranate, milk thistle, green tea, grape seed and acai	Mouth, stomach and small intestine	Reduction in antioxidant activity of green tea and grape seeds following enzymatic digestion Increase in antioxidant activity of pomegranate, milk thistle, resveratrol, goji and acai after enzymatic digestion	[113]
Cinnamon-fortified yogurt Cinnamon water extract	Gastric and pancreatic digestion	Significant increase in total polyphenol content of cinnamon-fortified yogurt after pepsin digestion with no change following pancreatic digestion Significant increase in radical scavenging activity of post-pancreatic digest Significant decrease in total polyphenol content following pepsin digestion of cinnamon water extract which was unchanged after pancreatic digestion No significant change in radical scavenging activity of the digesta	[36]
Red grape pomace	Gastric and pancreatic digestion	Significant decrease in total polyphenol content, phenolic acids, flavonols and anthocyanins of the digested samples Reduction in the antioxidant activity of the digesta	[37]
Cocoa (water-insoluble fraction)	Sequential in vitro digestion with pepsin, pancreatin, pronase, viscozyme L followed by three-stage culture system (proximal, transverse and distal colon)	Increase in antioxidant activity following enzymatic digestion Conversion of flavanols into phenolic acids by the microbiota resulting in high concentrations of 3-hydroxyphenylpropionic acid in the last gut compartment Significant increase in *Bifidobacteria* in all colonic vessels and *Lactobacilli* in proximal and distal compartments as well as butyrate production in all colonic segments	[114]

Table 2.2- Continued

Food/polyphenols	In vitro digestion models	Key findings	References
Mulberry anthocyanins	Gastro intestinal digestion	Decrease in bioaccessibilty of anthocyanins after intestinal digestion Generation of small molecular flavonoids and phenolic acids following gastro-intestinal digestion The gastric digest showed greater antioxidant activity than intestinal digest	[106]
Date seed polyphenols	Gastric, small intestine and colonic fermentation	Stability of catechin, epicatechin and procyanidin during gastro-intestinal digestion Generation of colonic metabolites such as FA, 3-hydroxyphenylacetic, 3-phenylpropionic and 3-(4-hydroxyphenyl) propionic acids following fermentation with fecal slurry	[38]
Grape pomace extracts	Stomach, small intestine, ascending, transverse and descending colon	Release of benzoic, phenylacetic and phenylpropionic acid derivatives in the colonic compartments Increase in SCFA levels in all colonic compartments Significant increase in *Lactobacillus*, *Bifidobacterium* and *Bacteroides* levels in all the colonic compartments	[115]
Anthocyanins from two purple-fleshed sweet potato accessions	Stomach, small intestine, ascending, transverse and descending colon	Release of anthocyanins in the small intestine and ascending colon Identification of varying amounts of pelargonidin-feruloyl-rutinoside in all vessels for two accessions Increase in ferric reducing antioxidant power in the small intestine and ascending colon segments	[11]

2. 5. Polyphenol Transport and Metabolism in Intestinal and Hepatic Cell Cultures

The intestinal wall and liver are two barriers that any compound needs to first pass through before entering the systemic circulation via the gastrointestinal tract. Various cell culture models have been used to study the bioavailability and metabolism of dietary compounds including polyphenols [116].

Epithelial cells from the intestine are very difficult to grow and maitain in vitro as primary cells. Thus, in recent years epithelial cell lines such as Caco-2 and HT29 have been used as surrogate cell models for human intestinal cells for pharmacological studies [117].

Human colorectal adenocarcinoma Caco-2 cells are intestinal epithelial cells, which have been used for identifying the mechanisms of absorption, transport and metabolism of drugs and dietary compounds [118]. Caco-2 cells are differentiated after 21 days forming a monolayer with well-established polarity and tight junctions [62]. This cell line also expresses drug transporters similar to the human small intestine [119]. Despite their cancerous origin, their differentiation results in formation of a cell monolayer with morphological and functional characteristics of mature enterocytes [120]. Previous studies have shown significant correlations between permeability of Caco-2 cells and the in vivo percent absorption of a wide range of drugs as well as nutrients such as iron in humans [62, 116, 121]. A number of studies have applied this cell line to study the uptake and transport of polyphenols [120]. Zhang *et al*. (2013) has reported a dose-dependent absorption of RU by Caco-2 cells at the concentration range of 5 to 200 μM. A 2.5-fold increase in intracellular concentration of RU was observed after 2 h incubation with Caco-2 cells and about 33% of RU was metabolized to glucuronidated metabolites [122]. P-glycoprotein (P-gp) and multidrug-resistant proteins (MRPs) have been shown to be involved in intracellular accumulation and transport processes of RU by intestinal epithelial cells [122]. FA has shown low

permeability across Caco-2 cells via the monocarboxylic acid transporter (MCT) in a concentration-dependent manner [123]. Caco-2 cells have also been used to study the transepithelial transport of CGA, CA, and their colonic metabolites [124]. CGA and CA are mainly transported via paracellular diffusion [124]. CA also has been shown to be absorbed, to a lesser extent, via MCT [124]. The MCT-mediated absorption by Caco-2 cells has also been suggested for *m*-coumaric acid and 3-(*m*-hydroxyphenyl)propionic acid [124].

HT-29 is another human carcinoma cell line that has been used to simulate intestinal absorption and metabolism as these cells share some characteristics of normal intestinal tissue. The post-confluent cells of this cell line comprise a small portion (i.e., <5%) of mucus-secreting and absorptive cells [120]. One of the limitations of Caco-2 and HT-29 cells is their inability to produce a mucosal layer [120]. To address this issue, stable homogenous mucus-secreting cells, HT29-MTX, have been developed from HT-29 cells using methotrexate treatment [125]. The HT29-MTX, however, do not develop functional tight junction complexe to the same extent as Caco-2 cells. The transepithelial electric resistance (TEER) values of polarized Caco-2 cells is more similar to in vivo condtion than HT29. Thus, Caco-2 cells has been a preffered model of intestinal epithelium to study absorption and transepithelial trasnport of drug or dietary compounds [126].

To overcome the lack of mucus production, a co-culture of HT29-MTX and Caco-2 cells has been developed. The co-culture of HT29-MTX and Caco-2 cells has been used to study the FA transport and metabolism. The free form of FA was shown to be transported rapidly and efficiently across the Caco-2/HT29-MTX co-culture. In addition, a small portion of FA was transported as feruloyl-glucuronide, feruloyl-sulfate or as free dihydroferulic acid [127].

Other limitations of intestinal cell lines in comparison with normal epithelium are varation

in gene expression profile, the lack of other cell type and effect of non-cellular parameters such as bile acids that may influence the cellural uptake in vivo. Despite these limitations, intestinal cell lines such as Caco-2 are valuable tools for characterization of intestinal absorption and metabolism of drugs and dietary compounds [126].

Primary human hepatocytes are widely used in pharmacological and toxicological studies and serve as a "gold standard" to study drug metabolism. These cells, however, "have high variability, short life span and limited availability" [128]. On the other hand, hepatoma cell lines such as HepG2 are relatively easy to maintain and are commonly used to mimic in vitro hepatic metabolism of a variety of xenobiotic compounds. Other advantages of using cell lines such as HepG2 are the lower cost, higher reproducibility and stability of gene expression profile. The most important disadvantage of cell lines in comparison with primary human hepatocytes is lower activities of some of the drug-metabolizing enzymes. Although there are some limitations in the use of hepatic cell lines, they are still considered as a valuable in vitro model for pharmacological and toxicological studies [128].

HepG2 cells express many functional characteristics of normal human hepatocytes such as plasma proteins, bile acid secretion and detoxification processes [120]. HepaRG is another hepatoma cell line that have shown the expression of many drug-metabolizing genes similar to primary hepatocytes and has been suggested as a reliable in vitro model to simulate xenobiotic metabolism by hepatocytes [128].

HepG2 cells has been used previously to study the metabolism of polyphenols [129-131]. CA and FA were shown to have moderate uptake and metabolism by HepG2 cells whereas CGA demonstrated poor HepG2 cellular uptake. CA was indicated to be conjugated to form methylated,

glucuronidated and sulfated metabolites while glucuronidation was demonstrated to be the only biotransformation route noted for FA [131].

A co-culture of intestinal and hepatic cell lines has been utilized to simulate first pass metabolism of an ingested dietary compound. In co-culture systems, cells are either cultured together in a single or multi-well plate [120]. In the latter approach, one of the cell lines is cultured on a porous membrane insert and can be designed for varying levels of contact with the other cultured cell line [120]. The combination of Caco-2 and liver cell lines has been shown to provide an accurate prediction of the oral bioavailability of a number of foreign compounds both in animal models [61] and in humans [62] with a very good correlation between the in vitro area under the concentration time curve and the in vivo bioavailability of the same compound. The co-culture of Caco-2/HepG2 cell model has also been used to study iron absorption [132] and the bioactivity of grape-seed derived extract on hepatic cells [133].

These data show that the co-culture system of intestinal and hepatic cells can be used as a feasible and useful in vitro model to mimic the absorption and first-pass effect in humans. Both cell types have the capacity of major biotransformation reactions including deglycosylation, glucuronidation, sulfation and desulfation [120]. One of the limitations of the existing co-culture studies, however, has been the direct addition of the polyphenol-rich plant extracts or pure polyphenols into this cellular testing system without consideration of their prior enzymatic and microbial transformation in the GI tract. Thus, coupling the co-culture of intestinal and liver cell lines with the in vitro digestion system could provide a better insight into the complex process of digestion and metabolism of polyphenols that take place in vivo.

2.6. Polyphenols: Gut Microbiota and Generation of Short Chain Fatty Acids

The human gut contains trillions of bacteria that collectively are referred to as gut microbiota.

The majority of our gut bacteria belong to two bacterial divisions (phyla): the gram-negative *Bacteroidetes* and the gram-positive *Firmicutes* [134]. An imbalance in gut bacterial composition has been associated with an increased risk for the development of a number of acute and chronic diseases [135]. The composition of gut microbiota can be affected by endogenous factors as well as exogenous factors such as diet [136]. Diet directly influences gut microbial composition and metabolic activity by providing substrates available in the form of undigested dietary residues that are resistant to digestive enzymes and the digestion process [137]. It has been shown that even short term dietary plant- and animal-based interventions could have significant effects on gut microbial community structure and microbiome-associated metabolic gene expression [138]. Dietary polyphenols and gut microbiota are known to influence each other in a two-way interaction [139]. In that regard, gut bacteria affect polyphenol metabolism, which leads to generation of new polyphenol-derived metabolites with varying physiological properties and polyphenols can also influence the composition of gut microbiota [29]. Metabolism of polyphenols by gut microflora involves the cleavage of glycosidic linkages and opening of the heterocyclic ring at different points, which can lead to metabolites that are further metabolized into various aromatic acids [98]. Glycans, generated by glycosidic cleavage, are required for the survival of the gut microbiota and provide immunomodulatory properties to the host [140]. Individual differences in microbiota composition influence the biotransformation and metabolism of the polyphenols [141].

Gut microbiota has been proposed as a factor contributing to energy balance and weight regulation [142]. One of the mechanisms of particular interest is the role of gut microbiota in production of SCFAs such as acetate, butyrate and propionate by fermenting nutrients such as dietary carbohydrates that escape digestion in the upper GI [143]. The SCFAs are absorbed by intestinal tissue where they can be utilized for glucose or lipid synthesis and serve as an energy

source for the host [139]. The SCFAs also may act as signaling molecules [139]. An increased gut production of SCFAs has been associated with diverse health promoting effects including enhanced growth of probiotic bacteria and anti-inflammatory properties [136]. Butyrate has been shown to exert multiple biological effects with regards to gut and systemic health, which includes gene expression, cell differentiation, gut tissue development, immune modulation, oxidative stress reduction and diarrhea control [144]. Butyrate mediates its anti-inflammatory activities via several mechanisms including the reduction of pro-inflammatory cytokine expression, TNF-α, IL-1ß, IL-6, IL-8, and most importantly via inhibition of the NF-κB pathway [144]. Butyrate has been indicated to play a protective role against colon cancer following its generation via increased consumption of dietary fiber [144] and has been suggested to enhance the effects of anti-cancer drugs [144]. Propionic acid has been shown to exert anti-lipogenic, cholesterol-lowering and anti-proliferative effects [145]. Acetate has been demonstrated to exert anti-inflammatory effects in colonic cells via the inhibition of NF-κB but to a lesser extent than propionate and butyrate [31].

Polyphenols have recently gained interest as a dietary intervention strategy to stimulate SCFA production via their prebiotic-like properties. Certain bacteria species such as *Bifidobacteria* have been demonstrated to be involved in generation of SCFAs [146]. Polyphenols were shown in batch culture fermentation studies to induce SCFA production by modulating the gut microbiota composition [33]. An improvement in SCFA production has been observed after 24 h batch culture fermentation of different flavonoids including quercetin, icraiin, luteolin, amygdalin and naringin [33]. In another study, batch culture fermentation of RU, quercetin, CGA and CA was shown to improve bacterial profiles by stimulating the proliferation of *Bifidobacteria* and reducing the ratio of *Firmicutes* to *Bacteroidetes* while increasing the generation of SCFAs [104]. The observed increase in SCFAs in the latter study was associated with co-generation of

bacterial metabolites as parent polyphenolic compounds were rapidly biotransformed to bacterial metabolites such as 3-hydroxyphenylacetic, 4-hydroxyphenylpropionic, 3-hydroxyphenylpropionic and 3-phenylpropionic acids after 0.5 h incubation [104]. One of the limitations of such studies, however, is that they are a substrate-limited batch culture as opposed to the continuous flow of nutrients that is provided in vivo. The substrate depletion and accumulation of the fermentation products in the batch culture models causes a drop in pH, leading to alteration in microbial composition and the pattern of SCFA profiles. For example, the rate of butyrate production was shown to be considerably higher in batch culture models compared to continuous fermentors [147]. The accumulation of fermentation products in batch culture may also affect the bacterial activity [148].

A few in vivo studies have also investigated the effects of different types of polyphenols and polyphenol-rich plant extracts on SCFA production. In human subjects, a red wine-grape juice extract, but not grape juice extract, reduced the fecal concentrations of isobutyric acid [149]. Administration of tea polyphenols to pigs for 2 weeks was shown to increase fecal acetic and lactic acids [150]. A 5-week treatment with polyphenol-rich extract from passion fruit leaves led to a decrease in fecal SCFA levels, although it promoted the growth of probiotic bacteria [151]. In another study, supplementation with trans-resveratrol and quercetin in high-fat sucrose diet-fed rats showed no significant effects on fecal SCFA levels [152]. It was suggested that the unchanged levels of SCFAs after polyphenol treatment in the latter study could have been due to the measurement of SCFAs in feces while a large amount of these compounds are absorbed in the large intestine in rats [152]. Overall, the above research has indicated that the modulatory effects of polyphenols on SCFAs can depend upon the nature of the polyphenol and the associated food matrix. In addition, in human studies, the effects of polyphenols on SCFA production could be

influenced by other dietary components that are difficult to control during the dietary interventions.

An alternative in vitro approach that more closely simulates the in vivo conditions than provided by batch cultures is the use of continuous multistage GI models to monitor the changes in SCFA profiles in different GI sections in a controlled environment. In a recent study, the continuous multistage GI model was used to assess the effects of grape pomace extract, which is rich in fiber and polyphenols, on microbiota composition and SCFA production. The grape pomace extract improved the gut bacterial composition via increasing the probiotic *Lactobacillus* and *Bacteroides* species and increased the SCFAs in the colonic vessels after 14 days of continuous feeding [115]. The effects of polyphenols on SCFA production was shown to be dependent on both colonic location and polyphenol source [42]. This new methodological approach can be applied to evaluate the alterations of other food components and their SCFA products following gastrointestinal transit. Such studies can involve pure polyphenol supplements that might lead to differing biotransformation and SCFA modulating patterns when provided in the absence of other bacteria-modulating components such as fiber.

2.7. Synergistic Interactions Among Polyphenols

The health promoting activities associated with polyphenol-rich fruits and vegetables are likely to be attributed to the synergistic interactions among the mixture of polyphenolic compounds found in these foods as well as their derived post-digest and post-absorbed metabolites. The synergistic interactions among polyphenols could provide health benefits by the targeting of multiple pathways involved in pathogenesis of various chronic diseases such as cancer [153]. Such synergisms among various polyphenols have been demonstrated by a number of in vivo and in vitro studies [153]. For example, the antioxidant effect of the combination of curcumin and resveratrol was found to be 15% greater than when the individual antioxidant activities of these

two compounds were tested [154]. The combination of chlorogenic, gallic, protocatechuic and vanillic acids was shown to exert synergistic antioxidant effects as measured by their capacity in scavenging the 2,2-diphenyl-1-picrylhydrazy (DPPH) radical [155]. The combination of CGA, apigenin and CA was demonstrated to induce synergistic protective effects against carbon tetrachloride-induced hepatotoxicity in mice via downregulation of expression of inflammatory mediators including TNF-α, iNOS, and COX-2 [53]. In an in vitro study, the combined treatment of quercetin and kaemferol exhibited synergistic anti-proliferative effects in human adenocarcinoma HuTu-80 and Caco-2 cells and PMC42 breast cancer cells [156]. Curcumin was shown to enhance the anti-proliferative effects of epigallocatechin gallate on PC3 prostate cancer cells by up-regulation of p21-induced cell cycle arrest [157]. The anti-cancer synergy between several other phenolic compounds have also been reported in various cancer cell lines [14]. Polyphenols or polyphenol-rich extracts also have been used to enhance the anti-cancer efficacy of chemotherapeutic drugs in different human cancer cell lines as well as in animal models [14]. The above studies are generally limited via the exposure of cells to the parent polyphenol compounds but not to their more absorbable post-digested metabolites. Hence, although much has been reported on the synergistic interaction between native polyphenols, evidence is lacking regarding anti-cancer effects of the combination polyphenols and their post-digest microbial metabolites that have been detected in the fecal content and lumen of the human colon [23, 158-160]. Additionally, inter-individual variability in the human gut microbiota can lead to variations in phenolic microbial metabolite profiles [161], which could result in inter-individual variations in polyphenol-mediated chemoprevention.

2.8. Health Benefits of Polyphenols

2.8.1. Antioxidant Activity

Free radicals are defined as atoms or molecules with unpaired electrons, which make them unstable and highly reactive. Due to their high reactivity, they attack other compounds to abstract electrons and so become stable [162]. The attacked molecule then loses its electron and becomes a free radical itself, which begins a cascade of chain reactions causing damage to living cells [163]. Free radicals are mainly produced from oxygen (reactive oxygen species; ROS) and nitrogen (reactive nitrogen species; RNS), which both contain reactive radical and non-radical species [163]. A moderate or low level of ROS and RNS can exert beneficial biofunctional effects. However, an imbalanced redox status between the production of ROS/RNS, and the antioxidant system to remove them, can cause oxidative stress [163]. The cellular antioxidant defense systems include enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase as well as non-enzymatic antioxidants such as vitamin E, vitamin C, β-carotene, glutathione and coenzyme Q [164]. Additionally, the antioxidant benefits of phytochemicals such as polyphenols have been studied towards the prevention and treatment of various oxidative stress-related metabolic disorders [165]. Polyphenols can exert their antioxidant activity either directly by scavenging the reactive species such as ROS or indirectly through inducing the activity of antioxidant enzymes as mediated by the Nrf2/ antioxidant response element (ARE) system [166].

Polyphenols may directly disrupt the free radical chain oxidation reactions in cellular components by donating an electron or hydrogen atom to neutralize free radicals or to generate less reactive radicals [167]. The radical-scavenging capacities of polyphenols have been shown to be more effective than vitamin C and E on a molar basis [167]. Detoxification of ROS by polyphenols has been associated with prevention of oxidative damage to DNA, proteins and lipids and protection against ROS-mediated diseases [168, 169]. CA and its related hydroxycinnamic compounds have shown significant antioxidant and free radical scavenging activities as measured

by the Rancimat test and the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) assay. Among all the tested compounds in the latter study, CA showed the greatest antioxidant activity that was equal to α-tocopherol and rosmarinic acid that have been shown to exert strong DPPH scavenging activity [170]. The hydroxyl groups of polyphenol structures are the main determinants of their antioxidant activity [171]. Therefore, the presence of more hydroxyl groups in CA and rosmarinic acid could explain their greater antioxidant activity compared with FA and ferulic acid phenyl ester [170]. CGA and CA have shown to have protective effects against ischemia/reperfusion (I/R) injury in rat small intestines that was indicated to be mediated partly by their ROS scavenging activities [83]. FA is a strong antioxidant and free radical scavenger and is used as a food additive to prevent lipid peroxidation [88]. The antioxidant activity of RU has been shown to be comparable to standard antioxidants such as butylated hydroxytoluene (BHT) and ascorbic acid [172]. Polyphenols in their native form, however, are more likely to exert their direct antioxidant effects within the gastrointestinal tract, where they may come into direct contact with cells before undergoing biotransformation and metabolism by intestinal cells and gut microflora [166]. In addition, the concentrations of polyphenols in the gut are much greater than in the plasma or other organs. The concentration of polyphenols in the large intestinal lumen could reach up to the millimolar range [43]. Moreover, the increase in antioxidant capacity that occurs after consumption of polyphenol-rich diets can enhance the capacity of other endogenous antioxidants [65]. The activation of phase II detoxifying or antioxidant enzymes is a critical pathway of the cellular defense system against various stimuli and their consequent oxidative stress before toxins cause serious cell damage [173]. GPx, glutamate cysteine ligase (GCL), glutathione *S*-transferase (GST), and NAD(P)H:quinone oxidoreductase 1 (NQO1) are some of the known phase II detoxifying enzymes with expression regulated by Nrf2 activity [173]. Nrf2 is a transcription

factor necessary for regulation of the detoxification and antioxidant pathway(s) against oxidative stress and plays a significant role in cell survival [174]. Nrf2 activity is controlled by Keap1 in the cytoplasm. Under normal conditions, protein Keap1 keeps Nrf2 in its inactive form. Signals or stimuli that activate the signaling of ARE will disrupt the Nrf2-Keap1 complex leading to nuclear translocation of Nrf2 [175]. ARE is essential for regulating the expression of genes encoding phase II detoxification enzymes and antioxidant proteins. Activated Nrf2 binds to ARE sites in the promoter regions of many detoxification and antioxidant genes leading to up-regulation in expression of enzymes involved in the cellular detoxification processes and antioxidant defense system [175]. Polyphenols may act through disruption of the Keap1/Nrf2 complex by enhancing intracellular oxidative stress at non-toxic levels. Nrf2 will then be translocated to the nucleus to interact with ARE to regulate the expression of downstream detoxifying genes [168, 173].

The induction of the phase II enzymes and Nrf2 activity through ARE has been reported as a protective mechanism of anthocyanins and CGA against oxidative stress-induced cytotoxicity in vitro [176, 177]. CA was shown to protect hepatocytes against acetaminophen-induced oxidative stress by reducing the cellular ROS level and enhancing the Nrf2 activity, which led to increased expression of antioxidative signals such as heme oxygenase-1(HO-1) and NQO1 [178]. Likewise, CGA has been indicated as a potent inducer of Nrf2 translocation [179]. The indirect antioxidant effects of polyphenols through induction of antioxidant enzymes via the Nrf2/ARE system may be more efficient than their direct antioxidant properties [166].

2.8.2. Anti-Colon Cancer Effects

Cancer is one of the main causes of morbidity and mortality in the world and is influenced by multiple environmental and behavioral factors including diet. Colorectal cancer is among the most common causes of cancer deaths worldwide [180]. The World Health Organization has

reported that 30% of cancer mortality is related to behavioral and dietary factors including low fruit and vegetable intake [180]. Colorectal cancer is one of the most common diet-related cancers and one of the most preventable forms of cancer [181]. The epidemiology of colon cancer among populations with different dietary patterns indicates the important role of dietary factors in development of colon cancer [181]. Diets high in red meat and saturated fats and low in fruits and vegetables are associated with increased risk of colorectal cancer [182]. The beneficial effects of fruits and vegetables in reducing the risk of colon cancer may be partly attributed to their polyphenolic content [182]. Polyphenols can interfere with different stages of cancer development from initiation to progression, which has led to growing research interest on the anti-cancer effects of various polyphenol-rich foods [183] as well as individual synthetic polyphenols [184, 185].

Despite all the progress in cancer diagnosis and treatment, the global burden of cancer is still on the rise especially in low-and middle-income countries [186]. Due to the complex nature of cancer, more in-depth understanding in all areas of cancer research such as the underlying mechanisms and preventive and therapeutic strategies are still required to advance new tools and treatments for cancer therapy [187]. Natural compounds like polyphenols have potential for development of safe and effective chemopreventive and therapeutic agents that can work individually or in combination with anti-cancer drugs to strengthen their effects [14].

In vivo and in vitro studies have been used as screening tools to study the anti-cancer effects of dietary polyphenols and to elucidate their mechanisms of action using various types of cancer cell lines and have shown promising results [188]. One of the major limitations of such in vitro studies has been the testing of native forms of polyphenols for anti-cancer activities despite the extensive digestion-mediated biotransformation and metabolism of the polyphenols, which are known to occur and significantly affect their biological activity. In a recent study, an extract of

urinary phenolic metabolites obtained from subjects following acute intake of a polyphenol-rich juice showed anti-proliferative effects on human breast cancer cells [189]. It is conceivable that these anti-proliferative outcomes were partly mediated by synergistic effects generated from the combination of phenolic metabolites present in the urine.

To date, there has been no systematic study regarding testing the anti-colon cancer effects resulting from combinations of specific polyphenols and their microbial metabolites. Information is lacking as to whether such combinations can exert anti-proliferative effects via modulation of cell cycle regulation, inducing apoptosis or alteration of the mitochondrial DNA (mtDNA) content of tumorigenic cells as possible mode of action.

2.8.2.1. Regulation of Cell Cycle Progression

Cell cycle progression is regulated by cyclin-dependent kinases (CDKs), which are controlled by cyclin proteins and CDK inhibitors (CDKIs). The CDKs govern the initiation, progression, and completion of cell cycle events and the transition between cell cycle phases in association with cell cycle regulators [190]. The cell cycle regulators include cyclins (A, B, D, and E), CDKs (CDK 1, 2, 4 and 6), CDKIs such as p21, p27, p53 and phosphorylated retinoblastoma (pRb) [191]. Figure 2.2 represents the different phases of the cell cycle and associated regulatory proteins and enzymes.

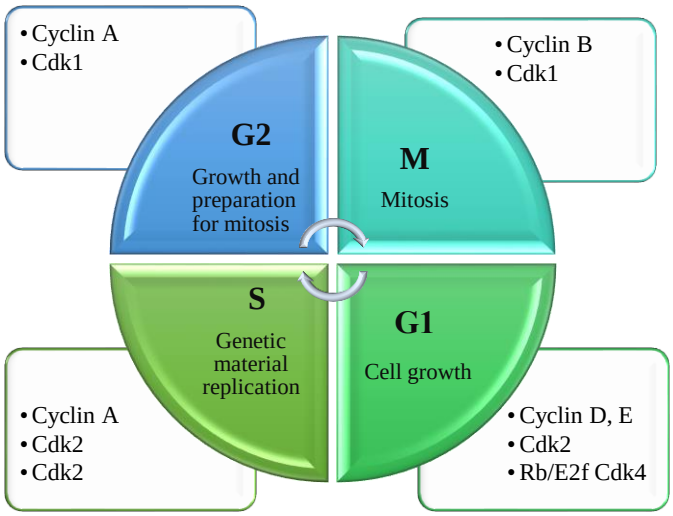


Figure 2.2. Schematic of cell cycle and corresponding regulators of each stage. The cell cycle consists of three sequential phases in which cells undergo growth and development processes followed by the mitotic phase in which the cell divides into two daughter cells. Each phase is regulated by cyclin proteins and Cdks [192].

Dysregulation of the cell cycle process leads to uncontrolled cell growth and is associated with the pathogenesis of different types of cancer [193]. Unlike normal cells, tumor cells are not able to control their cell cycle at predetermined checkpoints, which could be due to inactivation of critical CDKIs or overexpression of the activators [194]. Thus, modulation of the cell cycle regulators and checkpoints is one of the target pathways of anti-cancer compounds [190, 195]. In this regard, induction of cell cycle arrest by various natural food-derived compounds including polyphenols have been investigated as a possible mechanism of their anti-cancer effects [194]. Cell cycle arrest most frequently occurs at the G1/S or G2/M checkpoints [190]. Cell cycle arrest by polyphenols has been reported in various cancer cell lines such as Caco-2 cells [196], human acute promyelocytic leukemis HL-60 cells and human sporadic colon cancer cell lines (HCT116 and SW480) [197, 198]. Polyphenols may induce cell cycle arrest by modulating the cell cycle

regulators such as cyclin proteins [184, 199]. Sulforaphane has induced cell cycle arrest at the G2/M phase and increased expression of G2/M phase-related cyclins A and B1 in HT-29 cells [184]. Cyclins A and B1 regulate Cdc2 kinase activity at G2/M phase [184]. Resveratrol has been shown to upregulate the cyclins A, E and B1 and induce cell cycle arrest at S-phase in melanoma cells [199]. Pomegranate punicalagin and its hydrolysis product ellagic acid have been shown to down-regulate the cyclins A and B1, and upregulate cyclin E leading to S-phase cell cycle arrest in Caco-2 cells [200].

CDKs inhibitors are also among the main candidates for developing novel anti-cancer drugs [190]. For example, flavopiridol is an anti-cancer agent that directly binds to CDKs (1, 2, 4 and 6), inhibits their activity and so induces cell cycle arrest in a number of cell lines [194]. Polyphenols also may act as CDKs inhibitors and induce cell cycle arrest [201, 202]. Resveratrol has induced G2/M cell cycle arrest in colon carcinoma HT29 cells via inhibition of CDK7 [201]. Polyphenols may inhibit the activity of the CDKs by increasing the expression of Cdk inhibitory (Cdki) proteins such as Cip1/p21, Kip1/p27 and the binding of Cdki and Cdks [202]. The (-)-epigallocatechin-3-gallate compound was shown to inhibit the activity of cyclin-dependent kinases 2 and 4 and induce the activity of Cdk inhibitors p21 and p27 during growth arrest of human breast carcinoma cells [203]. In a recent study, CGA induced S-phase arrest via increasing the levels of phosphorylated p53, and inactivation of the extracellular signal–regulated kinase (ERK) in human colon cancer cells [185]. The above studies indicate that some polyphenols can act as potent inducers of cell cycle arrest through various molecular pathways and at different stages of cell growth. Therefore, due to the complex nature of cell cycle regulation, understanding the specific molecular mechanisms of phenolic compounds in target cell lines is important for developing new anti-cancer agents. In addition, it is essential to understand how these pathways could be affected

by microbial-derived secondary phenolic metabolites generated via digestive processes as our knowledge to date has been limited to the modulatory effects of parent polyphenols on cell cycle events.

2.8.2.2. Apoptosis

Apoptosis is programmed cell death, which plays an essential role in normal cell growth and development [204]. Apoptosis is a cascade of complex events involving the activation of a group of protease enzymes called caspases that lead cellular events towards cell death [204]. Among the ten major identified caspases, caspase-2, -8, -9, and -10 are categorized as initiators; caspase-3,-6, and -7 are known as effectors or executioners; and caspase-1, -4, and -5 are inflammatory [204]. Apoptosis occurs through two main pathways, which are termed the extrinsic pathway and the intrinsic pathway. The extrinsic pathway is mediated by death receptors such as FasL/FasR, TNF-α/TNFR1, Apo3L/DR3, Apo2L/DR4 and Apo2L/DR5. The intrinsic pathway is mediated through mitochondrial apoptotic signals affecting the ratio between pro-apoptotic (Bax, Bad, Bak, Bid, Bcl-XS) and anti-apoptotic (Bcl-2, Bcl-XL, Bag-1, Bcl-W) proteins [205]. The two pathways are linked and molecules in each pathway can influence the other [204]. The activation of both pathways culminates in cleavage of caspase-3, which results in the appearance of biochemical features of apoptosis such as DNA fragmentation, degradation of proteins and formation of apoptotic bodies that are eventually up taken by phagocytic cells [204]. Caspase-3 activation is a confirmatory marker for apoptosis [206].

Apoptosis works as a protective mechanism against cancer by removing the damaged cells [207]. Dysregulation of apoptosis has been linked to pathogenesis of a variety of diseases including cancers [207]. In this regard, many of chemo-preventive or therapeutic agents including pharmaceutical products [208] as well as naturally occurring compounds such as polyphenols have

been indicated to exert their anti-cancer effects through activation of apoptotic pathways [207, 209].

CA has been shown to induce apoptosis and inhibit cancer cell proliferation in the HT-1080 human fibrosarcoma cell line [210] and human leukaemia cells [211]. CGA was demonstrated to induced apoptosis in leukemia HL-60 cells [198]. In the above studies, apoptosis was confirmed by the presence of apoptotic morphological changes, but the molecular mechanisms of apoptosis were not investigated.

A number of studies have investigated the effects of native polyphenols on modulation of molecular pathways of apoptosis in different cell lines. Caffeoylquinic acid (CQA) derivatives were shown to induce apoptosis in human colon cancer cells (CRL-2577 (RKO) and HT-29) via activation of caspase-8 and caspase-3 [212]. CQA also increased the Bax:Bcl-2 ratio in HT-29 cells [212]. In the latter study, the increased levels of Bcl-2-associated X protein (Bax) induced cytochrome c release from mitochondria to the cytosol leading to activation of caspase-3. Likewise, butein was shown to induce apoptosis in HL-60 cells by increasing the expression of Bax and caspase activity and decreasing Bcl-2 expression [213]. In another study, ellagic acid induced apoptosis via Fas-independent and caspase 8-independent pathways through down-regulation of Bcl-XL and mitochondrial release of cytochrome c and subsequent activation of caspase 9 and caspase 3 [199]. Sulforaphane has been shown to induce apoptosis in HT-29 human colon cancer cells via a Bax-dependent pathway [184]. In Caco-2 cells, grape seed extract induced apoptosis through both caspase-dependent and caspase-independent pathways [214]. Extracts of polyphenol-rich sweet potato greens were shown to induce apoptosis through inactivation of Bcl-2, upregulation of Bax, release of cytochrome c and activation of downstream apoptotic signaling pathway [215]. CGA induced apoptosis in U937 leukemia cells by disruption of mitochondrial

membrane potentials (reduction of $\Delta\Psi m$), ROS production and activation of caspase-3, -7, -8 and -9 [216]. In a recent study, a CGA mixture extracted from green coffee beans induced apoptotic cell death in colon cancer HCT-116 cells [217]. The cleavage of PARP-1, activation of caspase-9, down regulation of Bcl-2 and up regulation of Bax were molecular features of apoptosis induced by the CGA mixture [217]. CA has been shown to induce apoptosis in human cervical cancer cells (HeLa cells) via the mitochondrial apoptotic pathway [218]. In the latter study, upregulation of p53 and inhibition of the Bcl-2 activity by CA caused release of mitochondrial cytochrome c and caspase-3 activation [218]. In contrast, in T47D human breast cancer cells, CA induced apoptosis via the Fas/FasL pathway [219]. FA has been shown to induce apoptosis in various cancer cell lines via modulating the expression of different apoptotic genes [220, 221].

In an in vivo study, Volate *et al*. (2004) investigated the effects of four flavonoids (quercetin, curcumin, silymari and rutin) and ginseng powder on molecular mechanisms of apoptosis in an azoxymethane (AOM)-induced rat colon cancer model [222]. Among all the treatments, only quercetin and curcumin were shown to increase protein levels of active caspase-9 and Bax [222]. In this study, Bcl-2 levels were unaffected, however, the ratio of Bax: Bcl-2 protein levels was 7% and 13% greater in the quercetin- and ginseng-fed groups, respectively. These results demonstrated the involvement of mitochondrial pathways in the apoptotic effects of quercetin and curcumin. The low Bax:Bcl-2 ratio, however, indicated that other Bcl-2 family members might be responsible for activation of mitochondrial pathways by curcumin [222]. In summary, phenolic compounds may exert anti-cancer effects by inducing apoptosis through modulation of different molecular pathways, which were all mediated through activation of caspase-3 [205]. Figure 2.3 briefly illustrates the major apoptotic signaling pathways and possible mechanisms that can be affected by polyphenols.

The effects of microbial phenolic metabolites on these pathways, however, have been rarely studied. It is conceivable that apoptosis induction by polyphenols reported by in vivo studies could be partly attributed to their secondary phenolic metabolites and so this possibility requires further investigation.

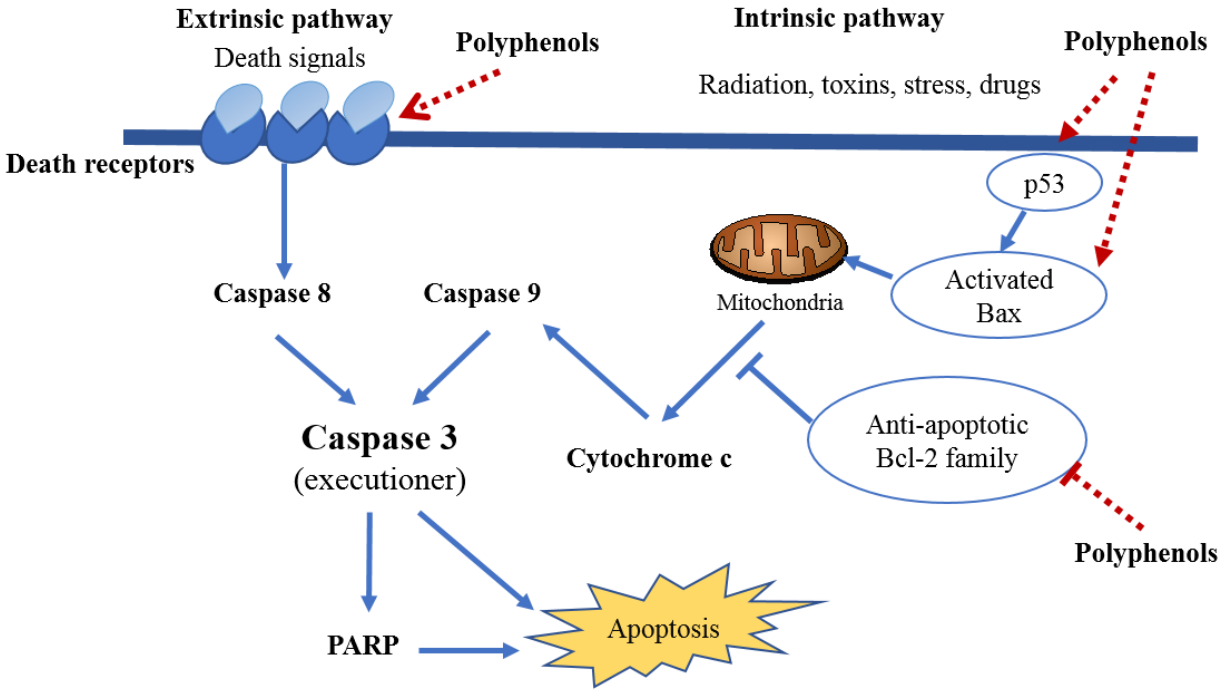


Figure 2.3. Summary of extrinsic and intrinsic pathways leading to apoptosis and involvement of polyphenols. Apoptosis can be triggered in a cell through the intrinsic or extrinsic pathways. The intrinsic pathway is initiated in response to cellular stress within the cell and the extrinsic pathway triggers apoptosis in response to external stimuli binding to the death receptor on the cell membrane such as Fas receptors. Both pathways can lead to activation of the executioner caspase-3, which induces apoptosis either directly or via poly(ADP-ribose) polymerase (PARP) activation. p53: tumor suppressor p53; Bax: apoptosis regulator (Bcl-2-associated X protein); Bcl-2: apoptosis regulator (B-cell lymphoma 2). Adapted from D'Archivio *et al.*, 2008 [223].

2.8.2.3. Modulation of Mitochondrial DNA Content by Polyphenols

Mitochondria is one of the key targets for anti-cancer agents including polyphenols [49]. It has been demonstrated that the mutations in mtDNA and an imbalance in the mtDNA content are associated with various types of cancer [224]. The mtDNA content is tissue specific and its level can be influenced by a number of internal or external factors. Increased and decreased levels of mtDNA content have both been reported in different types of cancer [225]. Decreased levels of mtDNA content could inhibit apoptosis leading to cancer progression and resistance to anti-cancer agents [224]. On the other hand, elevated levels of mtDNA content are also associated with resistance to anti-cancer drugs [224]. Therefore, it is suggested that maintaining the balance in mtDNA content could have beneficial effects in cancer therapy [224].

Very few studies have investigated alterations in the mtDNA content as a possible mechanism of anti-cancer effects of phenolic compounds. Resveratrol was shown to decrease mtDNA in MDA-MB231 breast cancer cells after 24 h at 50 and 100 µM concentrations [226] whereas at lower dose (25 µM) resveratrol induced mitochondrial biogenesis and increased mtDNA content in the murine skeletal muscle cell line (C2C12) [227]. Likewise, doxorubicin, a known anti-cancer agent, increased the mtDNA content and induced caspase-dependent apoptosis in colon cancer cells [228]. It has been suggested that higher doses of resveratrol damage and deplete mtDNA through induction of ROS production [226]. Removal of damaged mitochondria by autophagy enables survival of cancer cells exposed to resveratrol treatment, which delays apoptosis and is effectively a survival response. In addition, mtDNA depletion may inhibit apoptosis through upregulation of phosphatidylinositol 3-kinase (PI3K)/Akt2 signaling and so can promote survival of cancer cells [224]. The involvement of mtDNA content in apoptosis can vary depending on the

dose and duration of resveratrol treatment and the type of cell lines tested [226, 227], which involves mechanisms that have not yet been fully understood.

CONNECTING STATEMENT I

A review of the literature described evidence showing that the bioactivity of polyphenols depends on their bioaccessibility and bioavailability from the gastrointestinal (GI) tract after undergoing digestion and microbial biotransformation processes [15]. Biotransformation involving colonic microbiota alters the chemical structure of polyphenols and their biological properties such as antioxidant activities [15]. Polyphenol-derived microbial metabolites have been indicated as the major contributors to enhanced antioxidant capacity of plasma after consumption of polyphenol-rich foods [15]. In addition, polyphenols have been shown to modulate the composition of gut microbiota by inhibiting the growth of pathogenic bacteria while enhancing the growth of beneficial bacteria [229]. The prebiotic effects of polyphenols can also improve the short chain fatty acid profile which has not been fully studied [29]. Most in vitro digestion studies have focused upon upper intestinal digestion of polyphenols and polyphenol-rich foods and extracts [28, 106]. The limited number of in vitro studies that have investigated the microbiota-mediated biotransformation of polyphenols have typically used static batch culture systems [18, 107]. The static models are limited as they do not consider the dynamics of transit during digestion and are constrained by several factors such as substrate availability and excessive accumulation of end products that lead to feedback inhibition on microbial metabolism [39]. Continuous dynamic multi-reactor gastrointestinal simulators are more physiologically relevant models for evaluating the digestion and microbial biotransformation of polyphenols as these systems allow for monitoring of bioactivity of the generated secondary metabolites such as antioxidant capacity and short chain fatty acid profiles in different colonic regions. Such dynamic gastrointestinal simulators have been used in a few studies evaluating the impact of polyphenols on gut microbiota [230] and more recently have assessed the bioaccessibility of polyphenols from grape pomace

extracts following colonic microbial metabolism [115]. Nevertheless, the presence of other food components such as fiber in the extract in the latter study could have influenced the observed pattern of polyphenol biotransformation in the colonic reactors [32]. There is a knowledge gap regarding the enzymatic and microbial biotransformation of pure polyphenol compound supplements in the absence of other food components using a dynamic gastrointestinal simulator. Chlorogenic acid, caffeic acid, ferulic and rutin are abundant in human diets and co-occur in plant foods such as potatoes [57]. The following study utilized a dynamic gastrointestinal simulator (Computer Controlled Dynamic Human Gastrointestinal Model) to assess the enzymatic digestion and microbial biotransformation of a mixture of the above polyphenols and evaluated subsequent alterations in the antioxidant capacity and short chain fatty acid profiles of the GI regions.

CHAPTER 3: PAPER 1

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Biotransformation of polyphenols in a dynamic multistage gastrointestinal model

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3.1. Abstract

A multi-reactor gastrointestinal model was used to digest a mixture of pure polyphenol compounds including non-flavonoid phenolic acids (chlorogenic acid, caffeic acid, ferulic acid) and a flavonoid (rutin) to identify phenolic metabolites and short chain fatty acids (SCFAs) and compare relative antioxidant capacity following 24 h digestion. Biotransformation of these polyphenols occurred in the colonic compartments generating phenylpropionic, benzoic, phenylacetic and cinnamic acids. Total SCFAs increased in all colonic vessels with a rise in the proportion of propionic to acetic acid. Antioxidant capacity increased significantly in all compartments; first in the stomach, small intestine and ascending colon. After 24 h, the colonic vessels without parent polyphenols but containing new metabolites had a similar antioxidant capacity as the stomach and small intestine containing parent compounds. Biotransformation of pure polyphenols resulted in different phenolic metabolite and SCFAs profiles in each colonic segment, with important health implications for these colonic compartments.

Keywords: antioxidant capacity, caffeic acid, chlorogenic acid, digestion, ferulic acid, rutin, short chain fatty acids

3.2. Introduction

Protective health benefits from the intake of polyphenols widespread in fruits, vegetables, leguminous plants, cereal grains and beverages such as tea and coffee have been reported in many epidemiologic and clinical studies against a wide range of degenerative diseases including cardiovascular and neurodegenerative disorders and some cancers [65]. A major issue in the evaluation of the health benefits of polyphenols is the limited information concerning their biotransformation in the gastrointestinal (GI) tract. Polyphenols are generally poorly absorbed during digestion as they undergo extensive metabolism by digestive enzymes and gut microflora to generate a variety of smaller molecular weight compounds. Previous animal and human studies have consistently shown that large amounts of the polyphenols present in plant foods such as chlorogenic acid, caffeic acid, ferulic acid and rutin reach colonic regions to be metabolized by gut microflora and so generate a variety of simpler absorbable phenolic compounds [17, 23]. The microbial-derived phenolic metabolites have varying physiological properties that could account for many of the biological properties of dietary polyphenols [35]. Although polyphenols are considered to generate most of the antioxidant capacity from plant foods [231], the antioxidant activities that could be attributed to microbial-derived phenolic metabolites have not been well explored. The antioxidant activities of phenolic metabolites could be especially relevant towards gut health in view of their relatively high concentrations in colonic regions [232]. Dietary polyphenols also induce increased colonic generation of short chain fatty acids (SCFAs) implicated in the promotion of colonic health [233]. In general, however, while gut metabolism of polyphenols as constituents in foods and beverages has been primarily examined much less is known regarding the gut mediated biotransformation of pure polyphenol compounds when provided in the absence of a food matrix.

In vitro GI models have been developed to simulate the digestion and biotransformation of dietary components throughout the human GI tract. The majority of GI model studies evaluating polyphenol metabolism have used static models that do not consider the dynamics of transit during digestion or the varying microbial and digestive conditions in different segments of the GI tract [18, 105, 107, 234, 235]. On the other hand, multistage, dynamic in vitro models comprised of stomach, small intestine and the three colonic compartments that simulate both upper and lower GI digestion have been developed and validated by monitoring changes in SCFA profiles, gas production and enzymatic activity [41, 236]. Such models have been used to investigate the effect of prebiotics on the human intestinal microbial ecosystem [237, 238]. There has been limited study of polyphenol biotransformation with a dynamic multistage GI simulator apart from a recent study showing good correlation between phenolic metabolite profiles obtained from in vitro GI digestion of wine in comparison to the phenolic profiles of fecal samples obtained from human subjects fed wine [112]. To date, in vitro GI model studies have focused on the biotransformation of polyphenols in foods as opposed to pure polyphenol compounds. One substrate-limited batch culture study of caffeic acid, chlorogenic acid, quercetin and rutin showed their rapid breakdown to phenolic by-products within 0.5 h [104]. To our knowledge, however, the biotransformation of pure phenolic compounds has not been investigated using a continuous dynamic multi-reactor gastrointestinal simulator.

In the current study, we characterized the biotransformation of a mixture of four common dietary polyphenols including: non-flavonoids (chlorogenic acid, caffeic acid, ferulic acid) and the flavonoid rutin, to their microbial phenolic metabolites after 24 h using a dynamic multi-reactor gastrointestinal simulator of the human intestinal microbial ecosystem. This system enabled us to monitor the biotransformation of the pure polyphenol compounds in different segments of the GI

tract as it has been shown that the pattern of degradation of polyphenols from foods can depend upon gut microbial profiles [239]. This approach also allowed determination of the antioxidant capacity and production of SCFAs at different stages of the simulated digestion.

3.3. Materials and Methods

3.3.1. In vitro Digestion of Polyphenols

The GI model setup consisted of five consecutive reactors representing the stomach, the small intestine (SI), the ascending colon (AC), the transverse colon (TC) and the descending colon (DC) interconnected by plastic tubing and peristaltic pumps (Figure 3.1), as previously described by Molly *et al.* (1993) [41]. The unit was fully computer controlled (LabVIEW® software) for the addition of: (a) food to the stomach; (b) buffers to adjust pH of all compartments; and (c) pancreatic juice to the SI. The transit time of the flow of intestinal content between reactors was also automatically computer controlled. Temperature-controlled water flowed between the double glass jacketed reactors to keep the temperature at 37 ºC. The pH was automatically controlled by addition of 0.2 M HCl (AC423795000, Fisher Scientific, Ottawa, ON, Canada) and 0.5 M NaOH (415413, Sigma-Aldrich, Oakville, ON, Canada) into the stomach vessel and SI to keep a pH of 2.0 in the stomach and 6.5 in the SI. The pH ranges in the reactors of the AC, TC and DC were maintained at: 5.60-5.80, 6.20-6.40 and 6.60-6.80, respectively. The volume in the upper GI reactors was 200 mL and the AC, TC and DC compartments had volumes of 500, 800 and 600 mL, respectively. The passage of food in the stomach was simulated by the addition of gastric solution including 0.1 M HCl and pepsin (P7125, Sigma–Aldrich) as described by Gumienna *et al*. (2011) [107]. The SI was simulated by the addition of a pancreatic solution containing 12 g/L $NaHCO_3$ (S 7277, Sigma–Aldrich), 6 g/L Oxgall (DF 0128-17-8, Fisher Scientific) and 0.9 g/L pancreatin (P 1750, Sigma–Aldrich) suspended in sterile water. The transit time was 2 h in the

stomach and SI compartments followed by 4 h digestion in the colonic vessels. A 2-week stabilization period in which fresh fecal slurry obtained from 5 healthy volunteers with no history of GI disease or antibiotic use in the previous 6 months was inoculated into the 3 colonic reactors. During the stabilization period, the system was continuously fed three times a day with a nutritive medium composed of (g/L): arabinogalactan (1), pectin (2), xylan (1), starch (3), glucose (0.4), yeast extracts (3), peptone (1), mucin (4) and cysteine powders (0.5); a composition previously shown to stabilize the microbial community in the colonic vessels [41]. The anaerobic milieu was maintained by a daily flushing of nitrogen for 20 min into the air space. The nutritive medium was stored at 4 ºC during the experiment. After the 2-week stabilization period, the polyphenol treatment was started, which consisted of feeding a polyphenol mixture including chlorogenic acid, caffeic acid, ferulic acid and rutin dissolved in the nutritive medium. Chlorogenic acid, caffeic acid, ferulic acid and rutin (Sigma-Aldrich, Oakville, Ontario, Canada) were prepared at concentrations of (mg/L): 1139.2, 76.8, 25.6, and 153.6, respectively. The formulation was based on the polyphenol proportion associated with prevention of diet-induced obesity in an animal model [57]. A 330 mL volume of the polyphenol meal was fed every 8 h over a 24 h period and corresponded to an intake of approximately 1.139, 0.076, 0.025 and 0.153 g/d for chlorogenic acid, caffeic acid, ferulic acid and rutin, respectively. The experiments were carried out in triplicate. Thirty mL aliquots were withdrawn from each of the reactors at time 0 before addition of the polyphenol mixture and then after 8, 16 and 24 h. The aliquots were centrifuged at 1000 x g for 20 min and stored in 15 mL Falcon tubes at -80 ºC. Containers were wrapped in aluminum foil to protect contents against photodegradation.

3.3.2. Short Chain Fatty Acid Analysis

Samples (3 mL) were centrifuged and filtered using 25 mm syringe filters (0.2 μm, MCE, sterile) (09-719C, Fisher Scientific) and 1 μL was directly injected into a 6890 series gas chromatograph (GC) system equipped with a flame ionization detector (Agilent Technologies, Santa Clara, CA, USA) based on a modified method [240]. The SCFAs were separated using a HP-INNOWAS 30 m fused capillary column (Agilent Technologies, Santa Clara, CA, USA), 250 μm internal diameter, with a film thickness of 0.25 μm. Helium was used as the carrier gas at a flow rate of 1.0 mL/min. The inlet and detector temperatures were set at 220 ºC and 230 ºC, respectively. The oven temperature was initially 150 ºC held for 10 min and increased by 10 ºC/min from 150 ºC to 180 ºC and then held for 5 min. SCFAs were identified and quantified based on their retention time compared to that of pure standards (Nu-Check Prep, Inc., Waterville, MN). Samples were analyzed in duplicate from three independent experiments. The concentration of SCFA was calculated in mM.

3.3.3. Targeted Analysis of Phenolic Metabolites

After thawing, samples were vortexed, filtered with 25 mm syringe filters (0.2 μm, MCE, sterile; 09-719C, Fisher Scientific) and transferred to vials for liquid chromatography mass spectrometry (LC-MS) analysis. Phenolic compounds and metabolites were separated based on the modified method of Shakya and Navarre (2006), using a reverse phase high-performance liquid chromatography (HPLC) Gemini-NX (5 μm, 100 mm × 4.6 mm) column (Phenomenex, Torrance, CA, USA) and a 4.6 mm × 2.0 mm guard column. Elution was achieved using solvent A (10 mM formic acid, pH 3.5) and B (5 mM ammonium formate solution in 100 % methanol). Gradient conditions were: 0 min 5 % B, 2 min 5 % B, 5 min 30 % B, 7 min 70 % B, 9 min 100 % B and 12 min 100 % B with a flow rate of 1.0 mL/min and 20 μL of sample was injected. Accurate mass

data were obtained using an Agilent 1200 series HPLC system equipped with an Agilent 6210 electrospray ionization, time-of-flight (ESI-TOF) mass spectrometer (Agilent, Santa Clara, CA, USA), with internal mass calibration. The analyses were conducted using electrospray ionization in both positive and negative modes (injected separately): data was acquired over a mass range of *m/z* 100-1000. The source was operated with the following parameters: temperature 350 ºC, gas flow 12 L/min, nebulizer 50 psi (344.74 kPa), capillary voltage (+/-) 4000 V, fragmentor 100 V, skimmer voltage 60 V. Reference masses (internal calibration of high resolution spectra) were, for positive mode: *m/z* 121.050873, 922.009798; negative mode: *m/z* 119.03632, 966.000725. The data was processed using Agilent Mass Hunter software version B.04.00. Extracted ion chromatograms of accurate masses for deprotonated (MH-) or protonated (MH+) ions were used for confirmation of presence of parent polyphenolic compounds as well as metabolites within 20 ppm.

3.3.4. ABTS Radical Scavenging Capacity Assay

The Trolox equivalent antioxidant capacity 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) assay was used based on the method described by Gao, Ohlander, Jeppsson, Björk, and Trajkovski (2000) [241]. The ABTS radical cation ($ABTS^{\cdot+}$) was generated by reacting 5 mL of 7 mM ABTS solution mixed with 88 µL of 140 mM potassium persulfate ($K_2S_2O_8$). The $ABTS^{\cdot+}$ solution was diluted with 95% ethanol (radical solution) to an absorbance of 0.70 ± 0.05 at 734 nm. Filtered sample (100 µL) was then added to 1200 µL ABTS radical solution, vortexed and the decrease in absorbance at 734 nm was measured after 2 min. All measurements were carried out in duplicate. Trolox (Sigma-Aldrich, Oakville, Ontario, Canada), an analogue of vitamin E, was used as an external standard with a calibration curve range of 2.5-785 µM. The results were expressed as Trolox equivalent antioxidant capacity (TEAC).

3.3.5. Statistical Analysis

All data are reported as mean ± standard error (SE). For multiple comparisons, one-way ANOVA was carried out followed by Tukey's *post-hoc* tests after checking for potential outliers and verification of normality of distribution using scatter plots. The difference between baseline and endpoint measurements were assessed by paired t-test. Statistical significance was set at $p < 0.05$ and all statistical analyses were performed using SPSS 22.0 (IBM Corp., 2013)[242].

3.4. Results and Discussion

3.4.1. Polyphenol Profiles

Table 3.1 presents phenolic metabolite profiles at baseline and after 24 h digestion of polyphenols in the five vessels of the GI model. The relative abundance of the compounds was calculated relative to the quantification of benzoic acid as the reference peak, as benzoic acid was present in all the vessels at the same concentration at all time points. The polyphenolic substrates were detected in the stomach, SI and AC vessels after the phenolic treatment. The appearance of microbial metabolites began in the AC and continued through the last two colonic vessels along with the disappearance of parent polyphenol compounds. A total of 13 microbial-derived metabolites were detected in the three colonic vessels that showed unique phenolic profiles although some of these metabolites did not seem to be generated from metabolism of the phenolic mixture. The extracted ion chromatogram from the first colonic vessel before and after digestion is presented in Figure 3.2a and Figure 3.2b respectively, as a representative compartment that contains both parent polyphenols as well as microbial metabolites. In the case of the presence of two peaks with similar mass and different retention time, confirmation was based on the exact mass of the compound identified in the mass spectra. As the pattern of polyphenol degradation depends on gut microbial profiles [239], the phenolic profile differences among the colonic vessels

could be attributable to different microbial communities noted in the AC, TC and DC culture reactors of computer-controlled dynamic GI models after a 2-week inoculation with human fecal slurry [243]. Microbial metabolites were not detected in the stomach and SI vessels apart from benzoic acid derivatives that were found in all vessels and coumaric acid in the SI, which indicated their production from the growth medium rather than the administered polyphenol mixture. All of the four parent polyphenols were present in the AC reactor after the 24 h treatment. The presence of parent polyphenols in the AC differs from human fecal batch culture studies showing rapid biotransformation of pure chlorogenic acid, caffeic acid and rutin compounds to phenolic acid products within 30 min, with only trace levels of the parent compounds remaining [104]. The contrasting results are likely related to extensive rapid metabolism associated with batch cultures as opposed to the dynamics of metabolism associated with the continuous flow of substrate in the GI model. Caffeic acid and 3-(3-hydroxyphenyl)propionic acid were both detected in the AC while disappearance of chlorogenic acid in the last two colonic vessels coincided with the appearance of quinic acid. Gut microbial metabolism of chlorogenic acid causes cleavage of the ester bond between caffeic acid and quinic acid by microbial esterase activity and subsequent generation of 3-(3-hydroxyphenyl)propionic acid within 24 h [101]. Rutin and its bacterial breakdown product, quercetin, were also detected in the AC reactor. Batch culture fermentation of rutin was reported to be metabolized to form 3-hydroxylphenylacetic acid [104], and this was detected in the AC (Table 3.1). The other microbial metabolites detected in the AC were dihydrocaffeic acid, dihydroferulic acid, 3-hydroxybenzoic acid, coumaric acid and cinnamic acid as well as benzoic acid and protocatechuic acid. Further microbial metabolism of caffeic acid leads to the formation of dihydrocaffeic acid, 3-(3-hydroxyphenyl)propionic acid and *m*-coumaric acid (3-hydroxycinnamic acid) [124], which has been described previously via in vitro fermentation

studies [101, 124] and in human fecal water [25]. 3-Hydroxyphenylpropionic acid has been reported as a major metabolite detected after incubation of chlorogenic acid and caffeic acid with human fecal microbiota in batch culture fermentations [18]. Caffeic acid can be *O*-methylated and biotransformed to ferulic and dihydroferulic acids [19] and these were both detected in the AC compartment (Table 3.1). Both dihydroferulic acid and dihydrocaffeic acid have been identified in the plasma of human subjects 4 h after drinking chlorogenic acid-containing coffee [17]. Protocatechuic acid, which can be derived from caffeic acid by the action of gut microflora [244], was detected in the AC reactor. Ferulic acid ingested from dietary sources or generated from caffeic acid methylation can be further metabolized to dihydroferulic acid and vanillic acid [101]. In the current study, the vanillic acid was detected in the TC and DC after polyphenol treatment that coincided with the disappearance of ferulic acid. None of the four parent polyphenolic substrates were detectable in the TC and DC; coumaric acid was no longer present in the TC and cinnamic acid was barely detectable. In addition to the presence of quercetin and 3-(3-hydroxyphenyl)propionic acid in the TC, an increase in dihydrocaffeic acid, dihydroferulic acid, 3-hydroxyphenylacetic acid, protocatechuic acid and the appearance of vanillic acid and 3-phenylpropionic acid was observed, indicating further active phenolic metabolism by microflora in this colonic compartment (Table 3.1). The microbial generation of these metabolites corresponds with an increase in the urinary excretion of ferulic, isoferulic, dihydroferulic and vanillic acids shown in subjects consuming chlorogenic acid-containing instant coffee [17]. In the present study, some microbial metabolites such as 3-hydroxyphenylacetic acid and benzoic acid were detected in the TC and DC compartments before polyphenol addition (Table 3.1), which has also been noted previously by Cueva *et al.* (2015) using a similar dynamic gut model containing five successive reactors. These authors surmised that such acids may have arisen from the microbial fermentation

of proteins and carbohydrates found in the nutrient medium. Alternatively or in addition, the presence of these metabolites may be explained by the lack of a polyphenol restricted diet prior to the collection of the fecal samples as such metabolites have been identified in human fecal water [241]. The DC compartment was marked with the disappearance of quercetin and cinnamic acid and a decrease in dihydroferulic acid indicating further microbial breakdown of those compounds in this reactor (Table 3.1). In that regard, microbial β-oxidation reactions can shorten side-chain lengths to generate derivatives of phenylpropionic, benzoic and phenylacetic acids as previously shown with the production of 3-phenylpropionic acid from dehydroxylation of 3-(3,4-dihydroxyphenyl)propionic acid [245].

3.4.2. Total Radical Scavenging Activity

Figure 3.3 shows the time course of changes in the antioxidant capacity measured by the ABTS assay in the five compartments of the GI model after polyphenol addition. The results represent the change in antioxidant capacity at 8, 16 and 24 h after polyphenol digestion in comparison to time zero (control). In general, a higher antioxidant capacity was achieved in all GI compartments upon addition of the polyphenols to the GI model. In particular, the stomach and SI antioxidant capacity increased significantly ($p < 0.05$) after 8 h of polyphenol digestion. The comparison of the antioxidant capacity between vessels at different time points showed significantly lower antioxidant capacity in the AC at time 0 h (control) compare to the other compartments. After 8 h of polyphenol digestion, the stomach, SI and AC reactors had significantly higher antioxidant capacity compare to the TC and DC ($p < 0.05$). There was no significant difference in antioxidant capacity among any of the compartments after 16 h or 24 h post-digestion of the polyphenols. The increased antioxidant capacity in the stomach and SI can be attributed to the parent polyphenols since no biotransformation of parent polyphenols was

apparent in these two compartments. A wide variety of antioxidant capacity assays have shown significant antioxidant activities of chlorogenic acid, caffeic acid, ferulic acid and rutin [83, 246, 247]. Among the colonic vessels, AC containing both the parent compounds and their microbial metabolites showed the earliest and greatest increase in antioxidant capacity following polyphenol administration as antioxidant capacity increased after 8 h ($p < 0.05$) whereas TC and DC only showed an increased antioxidant capacity after 16 h of polyphenol digestion ($p < 0.05$). It appears that metabolism of the parent polyphenols by the colonic bacteria diminished antioxidant capacity until sufficient phenolic metabolites accumulated after 24 h digestion to generate similar antioxidant capacity as the parent compounds. The simultaneous presence of both parent and microbial metabolites in AC can explain the earlier rise in antioxidant capacity in this compartment versus the TC and DC vessels. Both parent polyphenols and their secondary microbial metabolites have been indicated to contribute to in vivo antioxidant capacity. For example, plasma concentrations of both ellagic acid and its metabolites were associated with an increase in oxygen radical absorbance antioxidant capacity after consumption of polyphenol-rich pomegranate extract by human subjects [248]. Despite lower ABTS capacity measurements in TC and DC at earlier time points, the plateau of ABTS capacity in these vessels at 16 h was similar to the other compartments, which signifies that a more prolonged fermentation time was needed to accumulate colonic metabolites with significant antioxidant activity. Since the parent polyphenols were not detectable in TC and DC, the antioxidant capacity of the digesta in those reactors can be attributed to the phenolic metabolites generated from the degradation of the polyphenolic substrates. This concept is supported by several studies [249, 250] showing effective antioxidant capacity attributable to small phenolic acid colonic digestion products including coumaric acid, vanillic acid, dihydroferulic acid, dihydrocaffeic acid and protocatechuic acid. In some cases, the

antioxidant capacity of the microbial metabolites were found to be greater than the parent compound. For example, dihydroferulic acid showed greater ABTS antioxidant capacity than the parent polyphenol chlorogenic acid and dihydrocaffeic acid demonstrated greater radical scavenging activity than caffeic acid [251]. Similarly, 3,4-dihydroxyphenylacetic acid and 4-hydroxylphenylacetic acid microbial metabolites of rutin and quercetin, respectively, exerted greater antioxidant activity than their parent compounds [246]. Our data indicates that microbial metabolites can contribute as much antioxidant capacity as their parent polyphenols after the 16 h digestion.

3.4.3. Analysis of SCFAs Using Gas Chromatography

After the stabilization period, the initial SCFA concentration was highest for acetic acid followed by propionic and butyric acid (Figure 3.4a-c), which is similar to previously published observations of colonic profiles obtained from in vivo human studies [252]. The sum total of SCFAs in all colonic vessels was increased significantly ($p < 0.05$) after polyphenol feeding indicating induction of these fermentation products of colonic microflora with the highest amounts seen in the TC and DC (Figure 3.4d). In accordance to our findings, total SCFAs were higher in the TC and DC reactors following feeding of a polyphenol-rich red wine in a dynamic GI model system [112]. To date, relatively little is known about the effects of pure polyphenols on the modulation of SCFA production by gut microbiota. Previous work has shown that incubation of pure polyphenols including chlorogenic acid, caffeic acid and rutin in a human fecal batch culture for 48 h was associated with only a modest increase in formation of the major SFCAs [104]. Our results extend these latter findings by the demonstration of a considerable elevation in SCFA concentrations in the continuous dynamic GI model system fed a polyphenol mixture for 24 h, which ranged from a two to five-fold increase depending on the specific SCFA and reactor. The

major increase in SCFA concentrations observed in the present study is likely due to a combination of factors including the lack of product inhibition associated with batch culture incubations and a possible synergism associated with a polyphenol mixture.

Following polyphenol treatment, acetic acid and propionic acid concentrations were increased significantly ($p < 0.05$) in all three colonic compartments whereas concentrations of butyric acid were increased ($p < 0.05$) only in the AC and TC compartments (Figure 3.4a-c). Logically, the SCFA production is also affected by composition of the fed polyphenols as gut model digestion of cocoa polyphenols also induced butyric acid production, but without a significant change in acetic acid and propionic acid levels [114]. Enhancement in gut production of SCFAs, particularly butyric acid, has been associated with many gut health promoting effects including enhanced growth of probiotic bacteria [253] as well as anti-inflammatory and anti-cancer properties [136].

Figure 3.4e shows changes in the pattern of the SCFA profiles in terms of percent of total SCFA within each reactor. After the stabilization period, different proportions of the major SCFAs were seen among the three colonic reactor vessels whereby a lower ratio of butyric acid to acetic acid was observed in the AC as compared to the TC and DC reactors (Figure 3.4e). In contrast, a previous study showed a fairly uniform SCFA proportion among the three colonic compartments following a 14-day stabilization period using fecal samples from two of three volunteers within a similar dynamic gut model system [112]. In the latter study, however, the proportion of acetic acid to butyric acid in the AC for the third volunteer differed in comparison to the other two reactors, which indicates that SCFA variations can occur among different subjects, which is likely due to differences in their fecal microbial populations. Polyphenol feeding in the present study was associated with an increase in the percentage of propionic acid relative to acetic acid in all colonic

vessels although to a lesser extent in DC. The percent of propionic acid to acetic acid as a percent of total fatty acids was increased significantly ($p < 0.05$) by polyphenol feeding by 2-, 1.5- and 1.2-fold in the AC, TC and DC compartments, respectively. Likewise, 24 h and 48 h batch culture fermentation of individual polyphenols including chlorogenic acid, caffeic acid and rutin resulted in a relative increase in the proportions of propionic acid to acetic acid [104]. It is noteworthy that despite a significant increase in the total SCFA concentrations in the DC, the SCFA proportions were less markedly changed by polyphenol administration in this compartment. This latter finding could be related to the degradation of polyphenol metabolites such as quercetin prior to transfer to the final DC reactor (Table 3.1) since quercetin induced proportionally greater amounts of propionic acid following human fecal bacteria batch fermentation [104].

3.5. Conclusions

To our knowledge, this is the first study to use a continuous dynamic multi-reactor gastrointestinal simulator system to evaluate phenolic metabolism and SCFA production following degradation of a polyphenol mixture by human colonic microbiota. Our results support in vitro batch fermentation culture studies involving individual polyphenols, showing that colonic microbiota metabolism on chlorogenic acid, caffeic acid, ferulic acid and rutin generate phenylpropionic acids (3-(3,4-dihydroxyphenyl)propionic acid, 3-(phenyl)propionic acid, 3-(4-hydroxy-3-methoxyphenyl)propionic acid, benzoic acids (protocatechuic acid, benzoic acid, vanillic acid, 3-hydroxybenzoic acid), phenylacetic acid (3-hydroxyphenylacetic acid) and cinnamic acids (caffeic acid, ferulic acid, coumaric acid, cinnamic acid). Previous investigations have indicated that phenolic metabolites are absorbable at the colonic level and can be detected in the plasma and urine following intake of the parent compounds [17, 19]. Unlike batch fermentation culture studies showing a rapid disappearance of the polyphenol substrates, production of phenolic

metabolites in the AC compartment coincided with the presence of the parent polyphenols. In concert with earlier work showing colonic region-dependent biotransformation by colonic microflora [42], our results using the human simulated GI model show that gut microbial production of phenolic metabolites and SCFAs from feeding the mixture of four individual polyphenols was dependent on the colonic compartment. A variety of polyphenol-rich food extracts using multistage, dynamic in vitro models have shown microbial conversion of polyphenols associated with red wine, grape juice, black tea and cranberry resulting in a greater formation of phenolic acids in the TC and DC compartments [42, 243]. In contrast, the feeding of pure polyphenols in the present study, without an associated food matrix, generated the greatest amount of phenolic metabolites in the AC and TC compartments. This latter observation is likely due to a higher exposure of these pure polyphenol substrates to microbial action in the AC in contrast to the delayed release of phenolic compounds from the food matrix of polyphenol-rich foods.

In summary, a mixture of pure polyphenol compounds was extensively biotransformed by colonic microflora leading to differing phenolic metabolite profiles in the different colonic regions. The findings also suggest that changes in the structure of polyphenols via their biotransformation during colonic digestion can result in changes in their antioxidant activities and the generation of SCFAs in the different colonic compartments. This study underlines the importance of the multi-reactor gastrointestinal model for investigation of biotransformation of phenolics towards a better understanding of the associated important health implications in these colonic compartments.

Acknowledgments

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Conflict of Interest

The authors declare that they have no conflict of interest

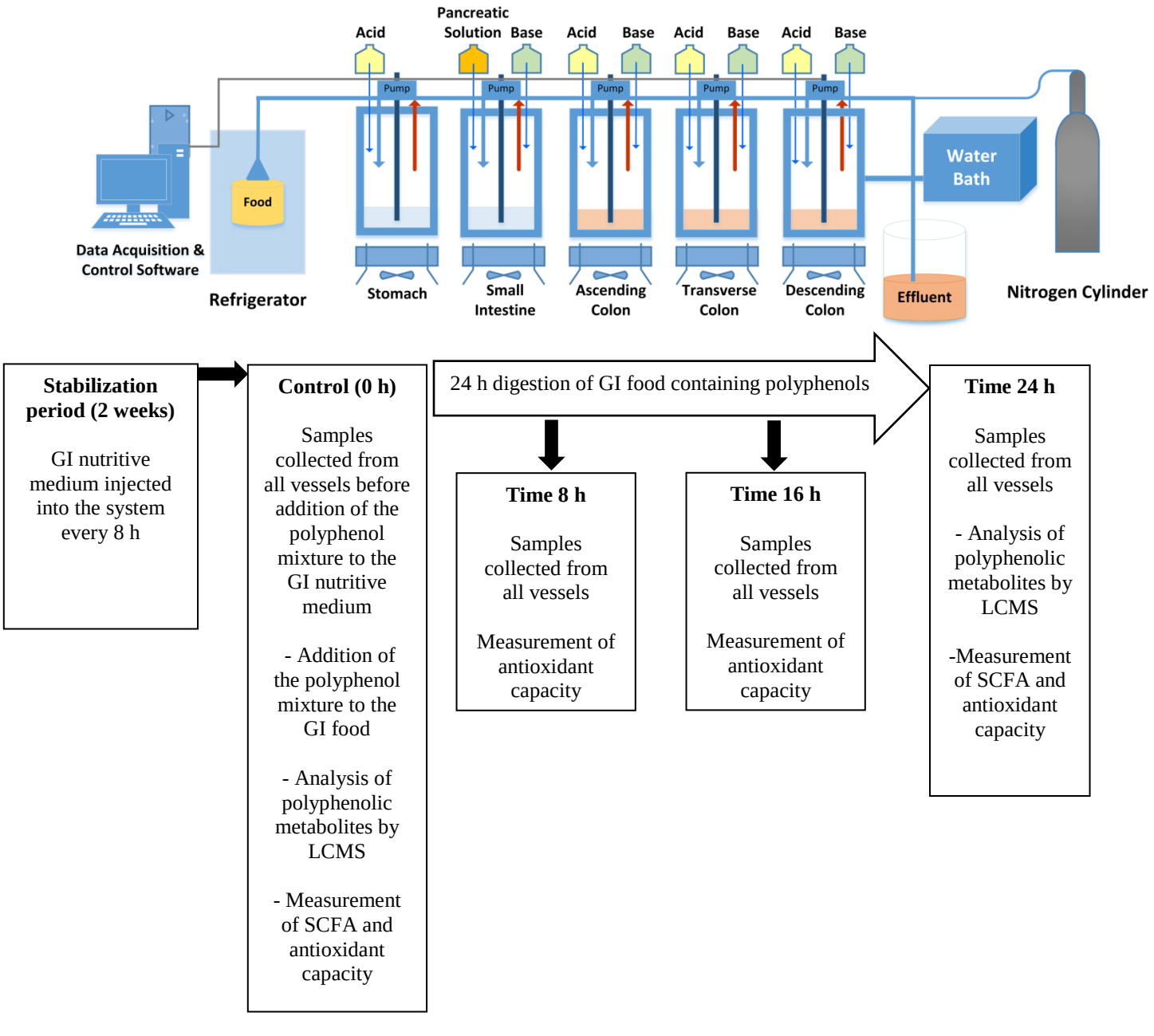


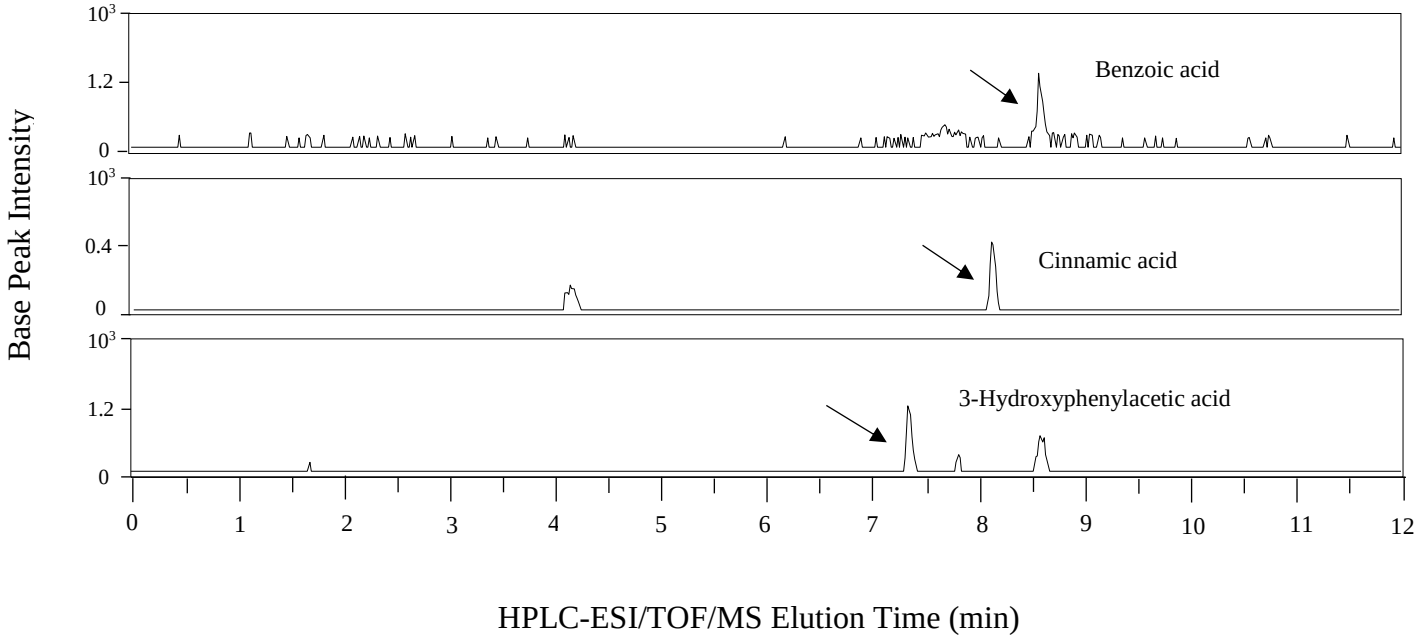
Figure 3.1. Schematic representation of the computer controlled human gastrointestinal model and the sampling procedure. Vessel 1: stomach; Vessel 2: small intestine (SI); Vessels 3, 4, and 5 represent the ascending colon (AC), transverse colon (TC), and descending colon (DC), respectively.

Table 3.1. Polyphenols and their metabolites after human simulated intestinal digestion at baseline (T0) and after 24 h (T24) post digestion[1].

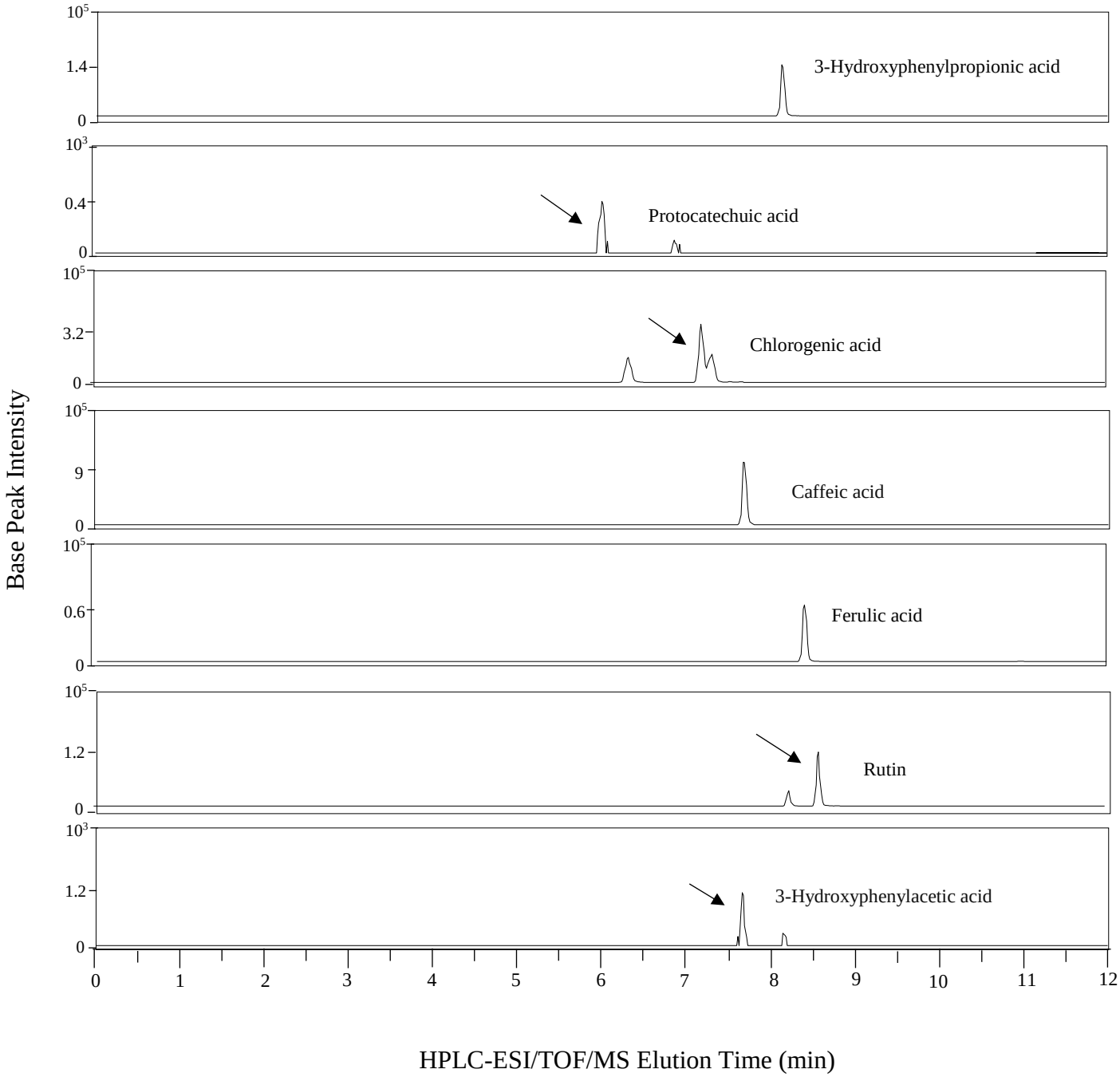
Theoretical mass (m/z)[2]	Measured mass	Mass accuracy (ppm)	Retention time (min)	Common name	Systematic name	Stomach		SI		AC		TC		DC	
						T0	T24	T0	T24	T0	T24	T0	T24	T0	T24
609.1461	**609.1422**	6.4	8.7	Rutin	Quercetin-3-*O*-rutinoside	-	160.36	-	125.80	-	83.11	-	-	-	-
353.0878	**353.0863**	4.3	7.5	Chlorogenic acid	(1*S*,3*R*,4*R*,5*R*)-3-{[(2*E*)-3-(3,4-Dihydroxyphenyl)prop-2-enoyl]oxy}-1,4,5-trihydroxycyclohexanecarboxylic acid	-	856.74	-	586.71	-	191.61	-	-	-	-
301.0354	**301.0395**	13.7	8	Quercetin	2-(3,4-Dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one	-	-	-	-	-	0.80	-	1.03	-	-
195.0663	**195.0642**	10.6	8.6	Dihydroferulic acid	3-(4-Hydroxy-3-methoxyphenyl)propionic acid	-	-	-	-	-	2.40	-	14.30	-	2.20
193.0506	**193.0499**	3.8	8.5	Ferulic acid	3-(4-Hydroxy-3-methoxy-phenyl)prop-2-enoic acid	-	83.25	-	48.82	-	28.12	-	-	-	-
191.0561	**191.0544**	2.3	1.7	Quinic acid	(1*S*,3*R*,4*S*,5*R*)-1,3,4,5-Tetrahydroxycyclohexanecarboxylic acid	-	-	-	-	-	-	-	2.05	-	1.50
181.0506	**181.0505**	0.7	7.7	Dihydrocaffeic acid	3-(3′,4′-Dihydroxyphenyl)propionic acid	-	-	-	-	-	131.17	-	500.80	-	333.95
179.0325	**179.339**	6.1	8	Caffeic acid	3,4-Dihydroxycinnamic acid	-	27.01	-	154.99	-	630.50	-	-	-	-
167.035	**167.0346**	2.2	6.6	Vanillic acid	4-Hydroxy-3-methoxybenzoic acid	-	-	-	-	-	-	-	5.40	-	3.20
165.0557	**165.0557**	0.1	8.4	3-Hydroxyphenyl propionic acid	3-(3-Hydroxyphenyl)propionic acid	-	-	-	-	14.90	87.25	5.96	22.64	3.77	2.50
163.0401	**163.0401**	0.2	8.4	Coumaric acid	The isomer is not specified from our data	-	-	1.70	1.70	-	3.74	-	-	-	-
153.0193	**153.0204**	6.9	7.2	Protocatechuic acid	3,4-Dihydroxybenzoic acid	-	-	-	-	-	1.19	0.07	1.50	0.10	1.06
151.0401	**151.0409**	5.5	7.7	3-Hydroxyphenyl acetic acid	3-Hydroxyphenylacetic acid	-	-	-	-	1.05	1.28	4.30	7.20	5.73	8.50
149.0608	**149.0604**	2.7	9.4	3-Phenylpropionic acid	3-Phenylpropanoic acid	-	-	-	-	-	-	1.90	8.50	26.70	37.80
147.0452	**147.0453**	1	8.5	Cinnamic acid	3-Phenylprop-2-enoic acid	-	-	-	-	0.20	1.40	-	0.35	-	-
137.0244	**137.0245**	0.6	7.2	3- Hydroxybenzoic acid	3-Hydroxybenzoic acid	-	34.75	-	22.82	-	30.50	-	3.50	-	1.50
121.0295	**121.0298**	2.5	9	Benzoic acid	Benzoic acid[3]	+	+	+	+	+	+	+	+	+	+

[1]Determined by LC-MS analysis.
[2]Identification based on previous literature data [18, 19, 101, 103].
[3]The quantities of the polyphenols and their metabolites are calculated relative to the concentration of benzoic acid
SI = small intestine; AC = ascending colon; TC = transverse colon; DC = descending colon, (+) present, (-) absent, masses are shown as [M-H]

a)


Base Peak Intensity
10^3
1.2
0
Benzoic acid
10^3
0.4
0
Cinnamic acid
10^3
1.2
0
3-Hydroxyphenylacetic acid
0 1 2 3 4 5 6 7 8 9 10 11 12
HPLC-ESI/TOF/MS Elution Time (min)

b)


Base Peak Intensity
3-Hydroxyphenylpropionic acid
Protocatechuic acid
Chlorogenic acid
Caffeic acid
Ferulic acid
Rutin
3-Hydroxyphenylacetic acid
HPLC-ESI/TOF/MS Elution Time (min)

b) continued

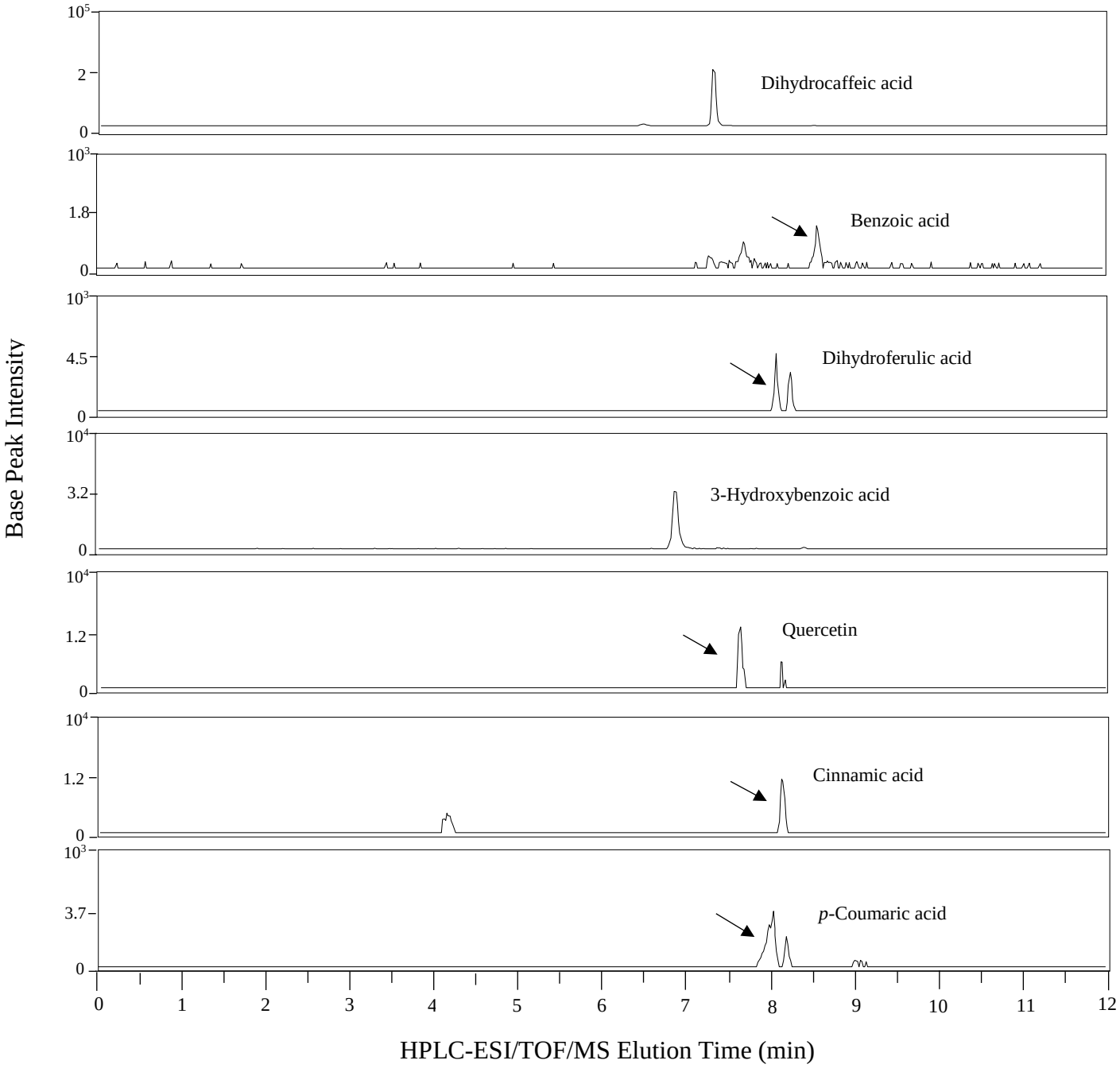


Figure 3.2. Representative extracted ion chromatograms from the first colonic vessel (AC) before (control; 0 h) and after 24 h of polyphenolic fermentation. (a) ascending colon (0 h); (b) ascending colon (24 h). The chromatogram shows the appearance of polyphenols and their metabolites after 24 h digestion in the first colonic vessel of the GI model.

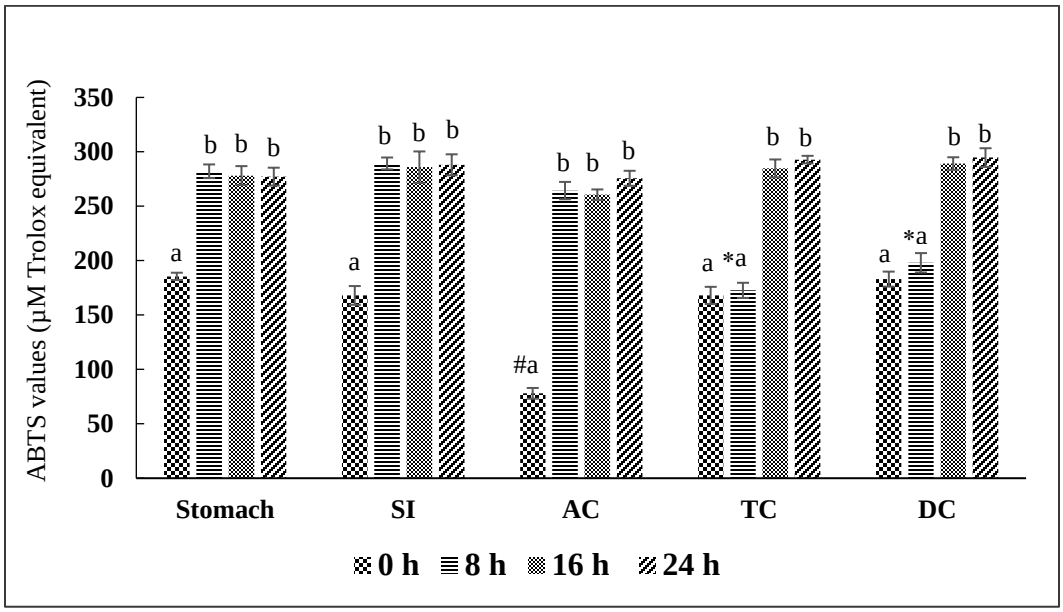


Figure 3.3. Time course in ABTS antioxidant capacity of digested samples from the 5 vessels of the GI model following feeding of a mixture of isolated polyphenols. SI = small intestine; AC = ascending colon; TC = transverse colon; DC = descending colon. Data are mean ± SE. Bars within the same vessel not sharing the same letter are significantly different from each other ($p < 0.05$). # indicates significant difference between AC and all other vessels at time 0 h (control). ∗indicates TC and DC were significantly different from other three vessels at time 8 h ($p < 0.05$).

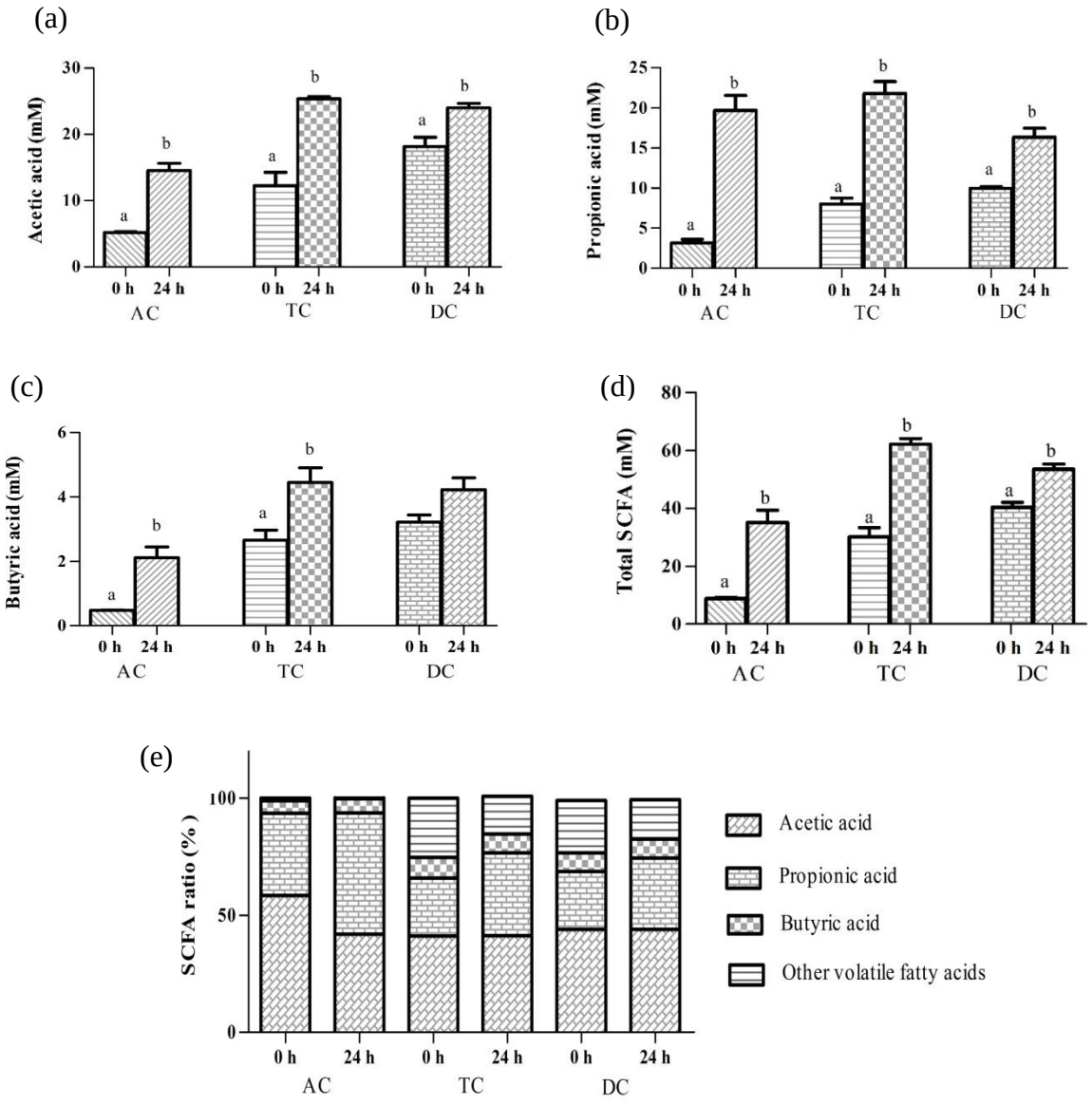


Figure 3.4. Changes in concentration (mM) of acetic acid (a), propionic acid (b) butyric acid (c), total SCFA (d) and the proportion of each SCFA (e) measured in the ascending, transverse and descending colon of the simulated gut model before (control; T0) and 24 h after polyphenol digestion (T 24). AC = ascending colon; TC = transverse colon; DC = descending colon. Data are represented as mean ± SE. Bars within the same vessel not sharing the same letter are significantly different from each other ($p < 0.05$).

CONNECTING STATEMENT II

Polyphenols have shown promising effects in prevention and treatment of colorectal cancer through various mechanisms [44]. The results from the first study demonstrated that a large amount of the tested polyphenols including CGA reach the colon undigested and undergo extensive microbial metabolism. The high concentrations of the polyphenols and their microbial metabolites in the colon [15], indicates that gut cells can be a major target for anti-cancer effects of polyphenols and their microbial-derived metabolites [25]. CGA and CA are the most abundant phenolic acids in the human diet, especially in the diet of coffee drinkers [15]. The association between diets-rich in CGA and CA with reduced risk of colon cancer has been reported previously by epidemiological studies [254, 255]. A few in vitro studies have also reported the anti-colon cancer activities of CGA and CA [44]. In addition to CA, 3-phenylpropionic and benzoic acids are among the main microbial metabolites of CGA [16, 51], as was also observed in our first study. To our knowledge, the anti-cancer activities of the latter two metabolites have not been studied previously; however, the co-presence of the high concentrations of CGA and its microbial metabolites in the gut could exert synergistic anti-cancer effects. Previous studies have indicated that combinations of parent polyphenols can lead to enhanced functional health outcomes at lower concentrations of each individual compound [155]. To date, however, there has been no evidence relating to the impact of the combination of CGA and its major microbial metabolites on colon cancer cells. In the proceeding chapter, we examined the anti-colon cancer activities of CGA and its main metabolites (CA, 3-PPA and BA) on the human colonic Caco-2 cancer cell line. In addition, the effects of an equimolar mixture of the above four compounds were tested for additive or synergistic effects. To assess the mechanisms involved in anti-cancer activities of the above phenolics, their effects on

some of the cellular features that are dysregulated in cancers including cell proliferation, cell cycle, mitochondrial DNA content, and apoptotic pathway were evaluated.

CHAPTER 4: PAPER 2

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Chlorogenic acid and its microbial metabolites exert anti-proliferative effects, S-phase cell-cycle arrest and apoptosis in human colon cancer Caco-2 cells

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4.1. Abstract

Chlorogenic acid (CGA) decreases colon cancer-cell proliferation but the combined anti-cancer effects of CGA with its major colonic microbial metabolites, caffeic acid (CA), 3-phenylpropionic acid (3-PPA) and benzoic acid (BA), needs elucidation as they occur together in colonic digesta. Caco-2 cancer cells were treated for 24 h with the four compounds individually (50–1000 μM) and as an equimolar ratio (1:1:1:1; MIX). The effective concentration to decrease cell proliferation by 50% (EC_{50}) was lower for MIX (431 ± 51.84 μM) and CA (460 ± 21.88) versus CGA (758 ± 19.09 μM). The EC_{50} for cytotoxicity measured by lactate dehydrogenase release in MIX (527 ± 75.34 μM) showed more potency than CA (740 ± 38.68 μM). Cell proliferation was decreased by 3-PPA and BA at 1000 μM with no cytotoxicity. Cell-cycle arrest was induced at the S-phase by CA (100 μM), MIX (100 μM), CGA (250 μM) and 3-PPA (500 μM) with activation of caspase-3 by CGA, CA, MIX (500 and 1000 μM). Mitochondrial DNA content was reduced by 3-PPA (1000 μM). The anti-cancer effects occurred at markedly lower concentrations of each compound within MIX than when provided singly, indicating that they function together to enhance anti-colon cancer activities.

Keywords: Caco-2 cells; chlorogenic acid; caffeic acid; 3-phenylpropionic acid; benzoic acid; cell cycle; caspase-3; apoptosis

4.2. Introduction

Colorectal cancer is among the most common cause of cancer deaths worldwide [44]. There is a large body of evidence supporting the role of fruit and vegetable consumption in reducing the risk of different types of cancer, including colorectal cancer [44]. A variety of nutrients and phytochemicals present in fruits and vegetables have been targeted as potential anti-cancer factors. Among the proposed chemopreventative food components, polyphenols have been consistently indicated to play a major anti-cancer role [44]. A diet rich in polyphenols has been indicated to contribute towards the health of the gastrointestinal tract, which is the site exposed to high concentrations of those compounds [256]. In that regard, various animal models and in vitro cell-culture studies representing different stages of colonic cancer have provided supportive evidence for the anti-carcinogenic effects of some polyphenols [44]. Polyphenols can exert anti-cancer properties via a variety of mechanisms, which are not yet fully understood. These mechanisms include induction of cell-cycle arrest and modulation of various oncogenic signaling cascades that affect cell proliferation and apoptosis [48]. Polyphenols could also exert anti-cancer activities by damaging mitochondrial DNA or via mitochondrial DNA depletion, as such effects could lead to autophagy and the induction of apoptosis [49].

Chlorogenic acid (CGA) is the major dietary polyphenol in many populations due to their high consumption of coffee, a rich source of CGA [257]. An inverse association between coffee intake and colon cancer has been reported in epidemiological studies, which has been related to a higher intake of CGA [257]. CGA, an ester of caffeic acid (CA) with quinic acid, is partly absorbed in the upper GI tract in its intact form; however, most of ingested CGA (approximately 70%) is cleaved in the lower GI by gut microflora resulting in the release of free CA and additional microbial metabolites with varying biological effects such as 3-phenypropionic acid (3-PPA) and

benzoic acid (BA) [158, 258]. Despite promising human and animal data indicating the anti-cancer effects of CGA [81], the mechanisms of action have not been studied extensively. Several investigations have treated various cultured cancer cell types with CGA and CA as single compounds at physiological and supraphysiological concentrations [44, 48, 81]. In such studies, these compounds showed anti-proliferative effects that were related to the stimulation of the expression of several apoptosis-associated genes [47] and to cell-cycle arrest [44, 48].

The aim of this study was to evaluate the anti-cancer effects of CGA and its metabolites (CA, 3-PPA and BA) on the human colonic Caco-2 cancer cell line, which is a well-utilized colorectal cancer model. The effects of an equimolar combination of the above four compounds (MIX) was tested as gut epithelial cells are exposed to both parent compounds and their microbial metabolites. For that assessment, measurements included cell proliferation, cytotoxicity, cell-cycle events, caspase-3 activation and mitochondrial DNA content. To our knowledge, this is the first study to relate the anti-cancer effects of CGA in combination with its microbial metabolites in colonic tumor cells, which could be physiologically relevant based on their concurrent presence in gut digesta.

4.3. Material and Methods

4.3.1. Cell Culture

Human colon cancer Caco-2 cells (adenocarcinoma) were obtained from the American Tissue Type Collection (ATCC, Burlington, ON, Canada) and cultured according to the company's procedures as explained briefly below. The Caco-2 cells were cultured in minimum essential medium Eagle (MEM) supplemented with 10% fetal bovine serum (FBS), 1% nonessential amino acids and 0.1% penicillin-streptomycin. Cells were incubated at 37 °C with 5% CO_2 and 90% humidity and were monitored daily. The cells were subcultured at 80% confluence with 0.25%

trypsin EDTA solution for 5–10 min and were seeded into a new flask or seeded onto appropriate plates for different experiments. Cells were treated with different concentrations (50, 100, 250, 500 and 1000 μM) of CGA, CA, 3-PPA and BA and an equimolar mixture (MIX) of the four compounds (CGA + CA + 3-PPA + BA; 1:1:1:1, respectively) for 24 h. The stock solution of the tested compounds was prepared in DMSO and the final percentage of the DMSO in the cell culture media during the treatment was less than 0.1%. The highest dose of the test compounds of 1000 μM correspond to their maximal concentrations in digesta following the consumption of CGA or CA-rich foods diluted in an intestinal volume of 600 mL [159, 259].

4.3.2. Analysis of Cell Proliferation

Cell proliferation was assessed using the MTT colorimetric assay. The MTT assay is based on the reduction of yellow tetrazolium MTT to purple formazan by the action of mitochondrial dehydrogenase in viable cells. To assess the effects of CGA and its metabolites on cell viability, a dose-response study using concentrations of 50, 100, 250, 500 and 1000 μM of the tested compounds was performed. Briefly, after each treatment the supernatant was collected and the MTT solution was dissolved in phenol red free medium, added to the cells and incubated for 3 h. The supernatant was then removed, and the blue formazan crystals were dissolved in HCl-isopropanol and the absorbance was measured at 570 nm.

4.3.3. Cell Cytotoxicity Assay

The LDH assay is a colorimetric membrane integrity assay for the quantification of cytotoxicity based on the release of cytoplasmic LDH into culture media from damaged cells. The cells were treated with the tested compounds (50, 100, 250, 500, 1000 μM) for 24 h, supernatant was collected and LDH release into media was measured using a cytotoxicity detection kit (LDH; Roche, Mississauga, ON, Canada) according to the manufacturer’s protocol.

4.3.4. Cell Cycle by Flow Cytometry Analysis

Cells were prepared for cell cycle analysis according to a modification of a previously described method [260]. Briefly, cells were seeded onto 6-well plates, and after reaching 80% confluency, treated with the test compounds for 24 h. Cells were then washed with phosphate-buffered saline (PBS), trypsinized, and 1×10^6 cells mL^{-1} were collected into 5 mL tubes. Cells were centrifuged for 5 min at 200× *g*, the supernatant was removed, and cells were fixed with 1 mL of 70% ethanol and stored at −20 °C, where they were kept until DNA staining. For DNA staining, samples were centrifuged and washed with PBS twice and centrifuged at 400× *g* for 5 min. Then, 1 mL of DNA staining buffer including 0.25 g sodium citrate, 0.75 mL Triton X-100 (1%), 0.005 g ribonuclease A, which were all purchased from Sigma-Aldrich (Sigma-Aldrich, Oakville, ON, Canada); 0.025 g propidium iodide (PI) (Fisher Scientific, Ottawa, ON, Canada) and 250 mL distilled water were added to the cell pellet. Immediately before acquisition, samples were filtered with 70 µm filters (Fisher Scientific, Ottawa, ON, Canada) to avoid any clogging. Cells were then analyzed for cell-cycle distribution by BD FACSCalibur flow cytometer (Becton Dickinson, Mississauga, ON, Canada). The PI fluorescence was collected as FL2 at 585 nm using CellQuest software (Becton Dickinson, San Jose, CA, USA). For each sample, 10,000 events were acquired. The analyses of cell-cycle distribution were performed on duplicate samples of three independent experiments using CellQuest software.

4.3.5. Western Blot Analysis of Caspase-3 Expression

Following 24 h treatment with the test compounds, the supernatant was removed and cells were washed twice with cold PBS. Thereafter, cells were lysed with radioimmunoprecipitation assay (RIPA) buffer (Fisher Scientific, Ottawa, ON, Canada) and the lysates were collected by scraping with a cold plastic cell scraper. The cell suspension was transferred into a centrifuge tube

and shaken for 15 min at 4 °C to lyse the cells. The lysate was centrifuged at 14,000× *g* in a precooled centrifuge for 15 min. The supernatant containing the cytoplasmic proteins was transferred to a new tube. The protein concentration was determined using the Coomassie (Bradford) protein assay. A 10 µg amount of protein was resolved on to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane using the wet-Western-blotting system. After blocking with 5% milk, the membrane was incubated at 4 °C overnight under shaking with the appropriate antibodies. The caspase-3 and cleaved caspase-3 bands were detected by caspase-3 (8G10) rabbit monoclonal antibody (#9665, Cell Signaling Technology, Beverly, MA, USA).

4.3.6. Mitochondrial DNA Content

To assess mitochondrial DNA content, cells were seeded onto 6-well plates and treated with 1000 µM of the test compounds for 24 h. The supernatant was collected and the cells were washed twice with cold PBS. Cells were scraped into 2 mL cold PBS using a rubber policeman and transferred to a centrifuge tube on ice. Cells were recovered by centrifugation at 1500× *g* for 10 min at 4 °C. The cell pellet was used for total genomic DNA extraction using the Qiagen Blood & Cell Culture DNA Mini Kit (Qiagen, Toronto, ON, Canada) according to the manufacturer's protocol. DNA quality and quantity were assessed at 260 and 280 nm using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Droplet Digital PCR (ddPCR) was conducted using QX200 Droplet Digital PCR System (Bio-Rad, Hercules, CA, USA) following the application guide. The PCR conditions and cycles were 95 °C for 5 min, 40 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s, then 4 °C for 5 min and 90 °C for 5 min. Two mitochondrial DNA regions are used, one is from the *coxIII* gene (U35430.1HSU35430, *Homo sapiens* cytochrome c oxidase subunit III gene), and one is from the *nad4* gene (AY063363.1,

Homo sapiens NADH dehydrogenase subunit 4 gene). For the mitochondrial *coxIII* gene, the forward primer was the Primer 6031 (our lab code) 5′-TCTCAGCCCTCCTAATGACCTC-3′ and reverse primer was primer 6032 5′-CCTTGGTATGTGCTTTCTCGTG-3′, for mitochondrial DNA gene, forward primer was Primer 6029 5′-GCCCTCGTAGTAACAGCCATTC-3′, and reverse primer was Primer 6029 5′-TGTGAGTGCGTTCGTAGTTTGA-3′. The mitochondrial coxIII and nad4 genes were designed in this study using Primer 3 (www.realtimeprimers.com). For the human nuclear genome, the forward primer (Primer 6057) was 5′-GACAGTCAGCCGCATCTTCT-3′ and reverse primer (Primer 6058) was 5′-TTAAAAGCAGCCCTGGTGAC-3′ [261]. For each treatment-gene combination, there were four biological replications and two ddPCR detection channels.

4.3.7. Statistical Analysis

All data are expressed as mean ± standard error (SE). For cell cycle analysis, one-way analysis of variance (ANOVA) was used to compare group means followed by Tukey's post-hoc test after checking for outliers and verification of normality of distribution using the *Shapiro*-Wilk test. The MTT and LDH data were analyzed by two-way ANOVA using treatment and concentration as main factors followed by Tukey's post-hoc test for multiple comparisons. Statistical significance was set at $p < 0.05$ and all statistical analyses were performed using SPSS 22.0 (IBM Corp., 2013) [242] and SigmaPlot v. 13 (Systat Software Inc., San Jose, CA, USA). The data analyses were performed on duplicate samples of three independent experiments except for the DNA analysis that was carried out on four biological repeats.

4.4. Results

4.4.1. Cell Proliferation and Cytotoxicity

Caco-2 cells have been used extensively as an in vitro model of the exposure of cancer cells to bioactive dietary components and chemotherapeutic agents. The influence of CGA and CGA metabolites on the growth of Caco-2 cells as measured by the MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay at 24 h is shown in Figure 4.1. The significant decrease ($p < 0.05$) in cell proliferation by CGA, CA and MIX treatment started at the lowest tested concentration (50 µM) (Figure 4.1). However, at the lower concentrations (50 and 100 µM), they only exerted slight (<20%) anti-proliferative effects. In terms of CGA, a substantial decrease (42.5%) in cell proliferation was noted at 500 µM ($p < 0.05$) with a further reduction (60.4%) seen at 1000 µM ($p < 0.05$). In contrast to CGA, the CA- and MIX-treated cells showed significant effects ($p < 0.05$) on proliferation starting at a lower concentration of 250 µM, with decreases of 31.2% and 38.94%, respectively. The CA and MIX treatments showed significantly lower cell proliferation ($p < 0.05$) at 250, 500 and 1000 µM relative to CGA. Treatment with CA and MIX showed dose-dependent reductions ($p < 0.05$) at 500 µM (55.9% and 56.7%) and 1000 µM (72.2% and 72.8%). Cell proliferation was affected by BA only at higher concentrations with a slight decrease in cell proliferation starting at 100 µM ($p < 0.05$) and further ($p < 0.05$) dose-related decreases at 250, 500 and 1000 µM. Relative to BA, significantly greater reductions ($p < 0.05$) in proliferation were seen at 50, 500 and 1000 µM for CGA and at 50, 250, 500 and 1000 µM for CA and MIX. Cell proliferation was affected only to a small extent ($p < 0.05$) for 3-PPA at 500 and 1000 µM. CGA, CA and MIX had significantly greater decreases ($p < 0.05$) in cell proliferation at all concentrations than 3-PPA. BA-treated cells also showed significantly greater decreases ($p < 0.05$) in proliferation than 3-PPA at 100, 250 and 1000 µM. Due to their inability to decrease cell proliferation by 50%, an EC_{50} was not obtained for 3-PPA and BA. Both 3-PPA and BA, however, appear to have contributed to the anti-proliferative effect in MIX as the

concentration to decrease cell proliferation by 50% (effective concentration; EC_{50}) for MIX was 431 ± 51.84 µM. The EC_{50} for CGA was significantly higher ($p < 0.05$) than for MIX and CA (Figure 4.2), which reflected a lower anti-proliferative potential for CGA. In that regard, the EC_{50} for MIX had a combined concentration of the two major anti-proliferative compounds of CGA and CA (215.5 µM) that was markedly lower than the EC_{50} concentrations of the two compounds individually, 758 ± 19.09 µM and 460 ± 21.88 µM, respectively.

The lactate dehydrogenase (LDH) assay is complementary to MTT as it describes the release of intracellular LDH into the culture medium, which indicates that cell-membrane damage resulted in irreversible cell death [262]. The CGA, CA and MIX treatments caused significant concentration-dependent increases in LDH release compared to control ($p < 0.05$) although only slight increases in cytotoxicity were noted at the lower concentration range of 50–250 µM (Figure 4.3). Treatment with CA and MIX showed dose-dependent increases in LDH release ($p < 0.05$) at 500 µM (46.5% and 50.4%) and 1000 µM (54% and 69.5%). The CA and MIX treatments exerted significantly greater cytotoxicity ($p < 0.05$) as compared to CGA at 250 µM (12.2%), 500 µM (22.5%) and 1000 µM (39.2%). Both BA and 3-PPA showed no significant effect on LDH release (data not shown). The MIX combination treatment (250 µM each for CGA, CA, BA, 3-PPA) showed stronger cytotoxicity (69.5%) ($p < 0.05$) relative to CGA (39.2%) and CA (54.1%) at the highest dose of 1000 µM. The obtained EC_{50} for MIX (527 ± 75.34 µM) was significantly lower ($p < 0.05$) and thus showed a higher cytotoxic potential than the EC_{50} value for CA (740 ± 38.68 µM). CGA treatment did not reach values to obtain an EC_{50} within the tested concentration range. The results thus show a combined action on cytotoxicity of CGA and its microbial-generated metabolites in MIX as these effects occurred at lower concentrations of the single compounds in MIX than when those agents were tested separately.

4.4.2. Cell-Cycle Analysis

To understand the possible mechanisms for cell death, the cell cycle was measured, as the cytotoxicity of polyphenols has been related to induction of apoptosis and cell-cycle arrest by various mechanisms [44, 197, 198]. The regulatory checkpoints of the G_1/S and G_2/M phases that control repair of damaged DNA are defective in cancer cells, which enables unregulated proliferation of these cells. Consequently, cell-cycle disruption has been a focus of anti-cancer therapies to inhibit cancer-cell growth and proliferation [194]. To determine whether the inhibitory effects of the test compounds resulted from growth arrest, the cell-cycle response of Caco-2 cells was examined by flow cytometry.

Evaluation of the disruption of cells in the G_0/G_1, S and G2/M phases of the cell cycle took place following incubation with CGA, CA, 3-PPA and MIX at doses of 100, 250 and 500 µM for 24 h (Figure 4.4). For CGA, CA, 3-PPA and MIX, a dose-related significant reduction ($p < 0.05$) of cells in the G_0/G_1 phase was noted with no significant increase in cells in the G_2/M phases. BA treatment did not show any effect on cell-cycle distribution (data not shown). CA and MIX significantly lowered ($p < 0.05$) the percentage of G_0/G_1 cells following the 100 µM treatment with a corresponding increase of cells in the S-phase. On the other hand, CGA caused significant reduction ($p < 0.05$) in the G_0/G_1 and increase in S-phases only at the higher concentration of 250 µM. Further significant reductions ($p < 0.05$) of the G_0/G_1 phase and increases in S-phase were noted following incubation of MIX and CA at 250 and 500 µM, respectively. Treatment with 3-PPA for 24 h caused significant reduction ($p < 0.05$) in the G_0/G_1 and increase in S-phases at 500 µM. The cell-cycle analyses thus signified that CGA, CA, 3-PPA and MIX caused a cell-cycle arrest at the S-phases in a dose-dependent manner. The effects of MIX on the arrest of the S-phases at 100 and 250 µM occurred with a combined content of CGA and CA (50 and 125 µM,

respectively) that was markedly lower than seen with the effects of the single compounds CGA (250 and 500 µM) and CA (100 and 500 µM). The cell cycle findings were therefore in line with the combined effects of CGA and its metabolites on cell proliferation and cytotoxicity exerted by MIX on Caco-2 cells.

4.4.3. Apoptosis

Activation of apoptosis in cancer cells is one of the pathways induced by chemopreventative or chemotherapeutic compounds including pharmaceuticals [208] as well as naturally occurring compounds such as phenolics [207]. Activation of at least one caspase is essential to induce cellular apoptosis as these enzymes annul the effect of protective factors towards cellular integrity such as the DNA-repair enzyme poly(ADP-ribose) polymerase (PARP). To confirm the induction of apoptosis, the next step was to analyze the expression of caspase-3, which is one of the major enzymes involved in the initiation of apoptosis. Activation of caspase-3 is a confirmed target of apoptosis induction as the application of active staining of caspase-3 and cleaved caspase-3 is a validated marker of apoptosis for cancer cells [263]. Therefore, we investigated the activation of caspase-3 as a possible apoptotic pathway induced by CGA, CA and MIX treatments in Caco-2 cells. To further confirm the activity, the expression level of the caspase-3 protein was studied. Detection of cleaved caspase-3 bands was performed and this indicates complete activation of caspase-3 by cleavage of pro-caspase-3. Figure 4.5 shows expression of caspase-3 after exposure to 500 and 1000 µM of CGA and CA and 500 µM of the MIX treatment. Relative to controls, only the 1000 µM dose of CA was associated with an increase in expression in caspase-3, whereas a similar level of expression was noted for CGA at 500 and 1000 µM. An elevated activity of caspase-3 was supported by an observed increase in the expression of cleaved caspase-3 bands. The MIX treatment containing a combined content of 250

μM for CGA and CA appeared to demonstrate a similar degree of expression in caspase-3 and cleaved caspase-3 to the 1000 μM concentrations of CGA and CA, which is supportive of effects exerted by the combination of compounds in MIX.

4.4.4. Mitochondrial DNA Content

The effects of CGA and its metabolites (CA, 3-PPA) on mitochondrial DNA content, in terms of the ratio of mitochondrial DNA (mtDNA) to nuclear DNA (nucDNA), was assessed since phytochemicals such as curcumin [49, 264] and certain chemotherapeutic drugs cause damage to mtDNA, which can be a mechanism for induction of apoptosis in cancer cells [264]. Two mitochondrial genes (*NAD* and *Cox*) were tested. For the *NAD* gene, treatment with 3-PPA significantly reduced ($p < 0.05$) the mtDNA/nucDNA ratio compared with the control (Figure 4.6). A similar tendency obtained for 3-PPA was seen with the mitochondrial *Cox* gene (Figure 4.6). The results are indicative of involvement of depletion of mitochondrial DNA content in the induction of apoptosis by 3-PPA in Caco-2 cells.

4.5. Discussion

The results of the current study demonstrate for the first time that treatment with a combination of CGA and its microbial metabolites CA, BA and 3-PPA exerts greater effects than the single compounds on cytotoxicity and the inhibition of human colon cancer cell proliferation, involving cell cycle arrest and apoptosis. These findings were achieved using concentrations of CGA and its microbial breakdown products detected in human fecal content [159, 160] and human digesta [23, 158]. Following ingestion of 1000 mg CGA (2.8 mmol), about 67% was recovered in ileal fluid of ileostomy subjects [23] which is higher than the highest tested concentration in the present study. Likewise, 71% of 385 μM ingested CGA (≈275 μM) was recovered in ileal effluent [158] which is in the middle of the range of tested concentrations of CGA used in the present work.

The values of CA seen in human fecal water of 52–126 µM [159] correspond to the lower range of the tested concentrations. Previous human fecal studies have shown a wide range of 3-PPA concentrations of 1747–2136 µM [160], 45–417 µM [52] and 165–440 µM [159] that corresponds to the range of the studied concentrations. Similarly, BA has shown a large range of values in human fecal water of 2060–4625 µM [159] and 51–134 µM [159].

Although such compounds are poorly absorbed [230], their relatively high concentrations in the gut lumen could exert functional anti-colon cancer effects. There are a limited number of studies regarding the anti-cancer effects of CGA and CA on colon cancer cell lines, including Caco-2 cells. Similar to the present findings, CGA treatment of human HT29 colon cancer cells showed anti-proliferative effects after 72 h at concentrations of 289.2 µM [265] and 500 µM [266]. The greater anti-proliferative and cytotoxic potency of CA as compared to CGA is also in agreement with previous work that showed more effective anti-proliferative effects on human HT29 colon cancer cells at a lower dose (EC_{50}: 235.1 µM after 48 h) compared to CGA (EC_{50}: 289 µM after 72 h) [265]. In another study, CA decreased cell viability in HT29 cells at an earlier time point than CGA (24 h vs. 96 h) [267]. The chemical structure of the polyphenols determine their anti-cancer efficacy. It has been shown that the aromatic ring and hydroxyl groups are key functional elements required for the anti-cancer activity of polyphenols [48]. The esterification of the carboxyl group of CA with quinic acid could affect the efficacy of the biological activity exerted by CGA [268].

The present investigation extends these previous findings by demonstrating that the combination of CGA with CA, BA and 3-PPA in the MIX treatment led to a net increase in bioactivity in terms of the inhibition of cell proliferation, cytotoxicity, and cell cycle arrest as compared to those compounds tested singly. The antiproliferative potential of the MIX

combination showed a EC_{50} value that contained concentrations of CGA (107.7 µM) and CA (107.7 µM) that were notably lower than the EC_{50} concentrations of the two compounds individually, 758 and 460 µM, respectively. Similarly, the evaluation of the cytotoxic effect of the compounds showed a higher EC_{50} cytotoxic index for MIX than the individual compounds. The greater antiproliferative and cytotoxic effects associated with MIX are presumably associated with the combined presence of CGA and its breakdown products. It is noteworthy that despite minimal effects of either BA or 3-PPA alone on Caco-2 cells, their presence in MIX contributed towards an additive anti-cancer effect in concert with CGA and CA. Additive or synergistic antiproliferative effects on Caco-2 cells have been observed from the combination of polyphenols in extracts of cranberries [269] and pomegranates [270] as compared to the isolated polyphenols identified in those extracts. The benefits of additive or synergistic effects between polyphenols has been indicated as a potentially important aspect towards cancer prevention and treatment [271]. Such additive or synergistic actions can be due to the combination of complementary antiproliferative mechanisms induced by the different polyphenol compounds such as modulation of cell cycle regulators like *p*53, or inhibition of molecular pathways involving mitogen-activated protein kinase, NF-κB and activator protein 1 [48].

Based on the antiproliferative effects induced by CGA and the CGA metabolites, cell cycle progression was studied. The progression of the cell cycle is regulated by cyclins, cyclin-dependent-kinases (CDKs), CDK inhibitors and check-point kinases that regulate cell proliferation at the G_1/S and G_2/M cell cycle checkpoints [194]. Dysregulated expression of these proteins can lead to tumorigenesis, and so modulating the cell cycle is a target pathway for chemoprevention [194]. Anti-cancer compounds can arrest the cell cycle at the G_0/G_1, S or G_2/M phase to stimulate apoptotic events. The significant accumulation of cells in the S-phase with a concomitant decrease

in G_0/G_1 indicated that Caco-2 cells treated with MIX, CGA, CA and 3-PPA had inhibited cell proliferation due to the blockage of the cell cycle at the S-phase. Overall, these findings indicate that these compounds at subtoxic concentrations can inhibit cell proliferation and the progression of the S-phase in the cell cycle. CGA and CA have been shown to induce cell-cycle arrest in other cell lines, including human sporadic colon-cancer cell lines (HCT116 and SW480) and human acute promyelocytic leukemia HL60 cells [197, 198]. Previous studies in Caco-2 cells have also shown inhibition of cell proliferation involving S-phase arrest by polyphenol parent compounds including ferulic acid and *p*-coumaric acid [44] and resveratrol [196].

The anti-proliferative activity associated from the Caco-2 cell treatments could be mediated by the induction of apoptosis, which can occur through different molecular pathways including modulation of the activity of the caspase family. Among the caspase family, the proapoptic protein caspase-3 plays an important role towards inducing apoptotic events received from both intrinsic and extrinsic apoptotic pathways [207]. Western blot experiments were undertaken to elucidate the involvement of caspase-3 as an apoptotic pathway in Caco-2 cells treated with CGA, CA and MIX. The presence of cleaved caspase-3 in the cells treated with CGA, CA and MIX demonstrated the capability of those compounds to cleave caspase-3. The findings showed that caspase-3 was activated in a dose-dependent manner by CGA. Increased caspase-3 activation from the combined compounds present in the MIX treatment was also suggested. CGA has been shown to activate caspase-3 through various pathways including production of reactive oxygen species and via activation of caspase-8 [185, 216]. Caspase-3 activation is a confirmatory marker for apoptosis [263]. Previous studies have shown CGA-induced apoptosis in leukemia HL-60 cells [198] and that CA caused apoptosis and inhibited cancer-cell proliferation in HT-1080 human fibrosarcoma cell line [210] and human leukemia cells [211]. Apart from caspase-3, further studies are needed

to provide insight regarding the other signaling pathways that could be involved in the apoptotic effects of CGA and its microbial breakdown compounds. Molecular targets could include apoptotic regulating proteins such as cyclin-dependent-kinases, NF-κB, Bcl-2, caspase-9 and PARP. Previous studies have indicated that polyphenols can affect cell cycles via different molecular pathways depending on the cancer-cell type and the chemical structure of the polyphenol [44, 48].

Apoptosis induction in Caco-2 cells was independent of mtDNA content alterations for CGA, CA and BA in this study, which indicates that mtDNA was not involved in the mechanisms of action for the apoptotic events involving those compounds. Interestingly, maintaining an adequate balance in mtDNA content during the induction of apoptosis has been proposed as a promising avenue for cancer therapy as both decreased or increased levels of mtDNA content have been associated with resistance to anti-cancer drugs [224]. On the other hand, 3-PPA treatment was associated with reduction of mtDNA, which can be a mechanism for the apoptosis seen with this metabolite. Resveratrol-mediated depletion of mtDNA content in breast- and colon-cancer cells has been linked with autophagy as well as induction of caspase activation and apoptosis [226]. Curcumin treatment was also shown to involve mtDNA reduction as an apoptosis trigger in hepatoma HepG2 cells [264].

In summary, the combination of CGA and its microbial metabolites was shown to exert enhanced anti-cancer effects in Caco-2 cells at subtoxic levels that involve inhibition of cell proliferation reflected by a lengthening of the S-phase and apoptotic cell death. The study findings also demonstrate that involvement of mtDNA in apoptosis can vary according to the nature of the microbial metabolite as 3-PPA was the only metabolite associated with alteration of mtDNA, within the power of the tested biological replicates. These results support the contention that the

combination of polyphenol metabolites formed during digestive processes can function together to increase anti-colon cancer effects. As such, these findings provide new avenues for in vitro and in vivo investigations regarding the anti-cancer roles of polyphenol-derived microbial compounds, which could lead to a better understanding of the molecular features involved in anti-cancer activities of dietary polyphenols.

Acknowledgments

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Author Contributions

Shima Sadeghi Ekbatan designed the study, carried out the experiments, analyzed the data and drafted the manuscript. Xiu-Qing Li carried out the PCR analysis. Mohammad Ghorbani performed the Western blot analysis. Behnam Azadi was involved in the flow cytometry analysis and DNA extraction. Stan Kubow initiated the original idea of the study and was involved in the study design, oversaw all the aspects of the study and provided extensive input to the manuscript. All authors read and approved the final manuscript.

Conflict of Interest

The authors declare no conflict of interest.

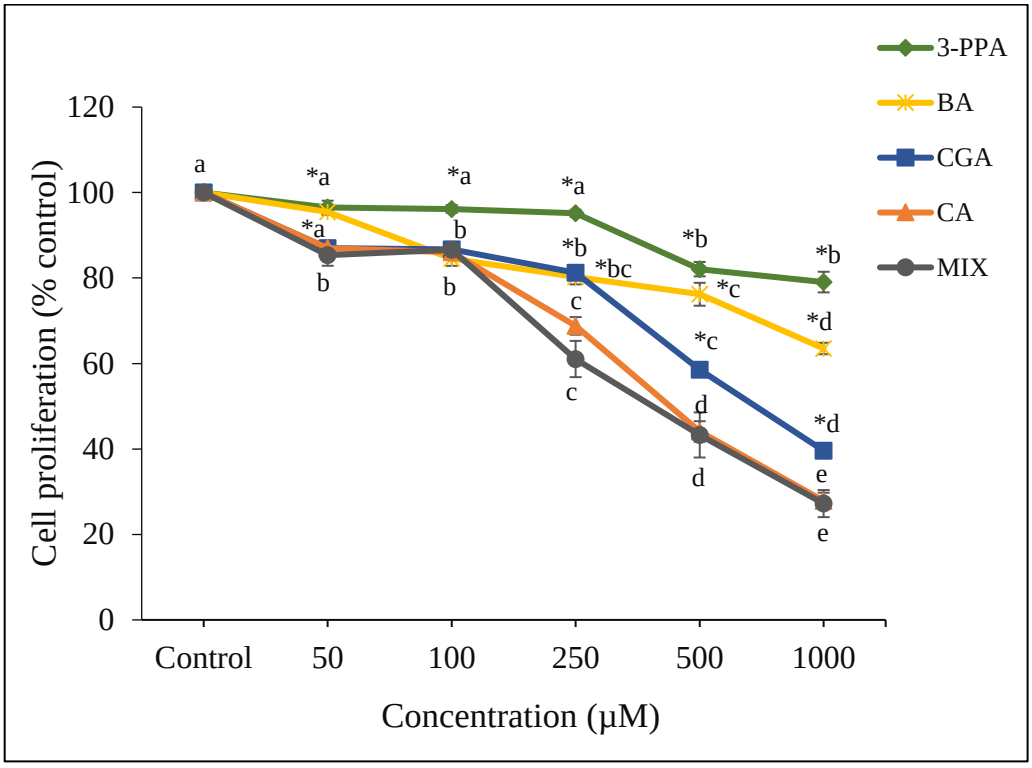


Figure 4.1. Effect of treatment with different doses of CGA, CA, 3-PPA, BA and MIX for 24 h on Caco-2 cell proliferation as measured by the MTT assay. Data are represented as mean ± standard error (SE). Statistical analysis was performed via two-way analysis of variance (ANOVA) using treatment and dose as factors. Doses within the same treatment not sharing common letters are significantly different ($p < 0.05$). The symbol * represents a significant difference ($p < 0.05$) of CA and MIX as compared to CGA, 3-PPA and BA at a specific dose. CGA = chlorogenic acid; CA = caffeic acid; 3-PPA = 3-phenylpropionic acid; BA = benzoic acid; MIX = equimolar mixture of the four tested compounds.

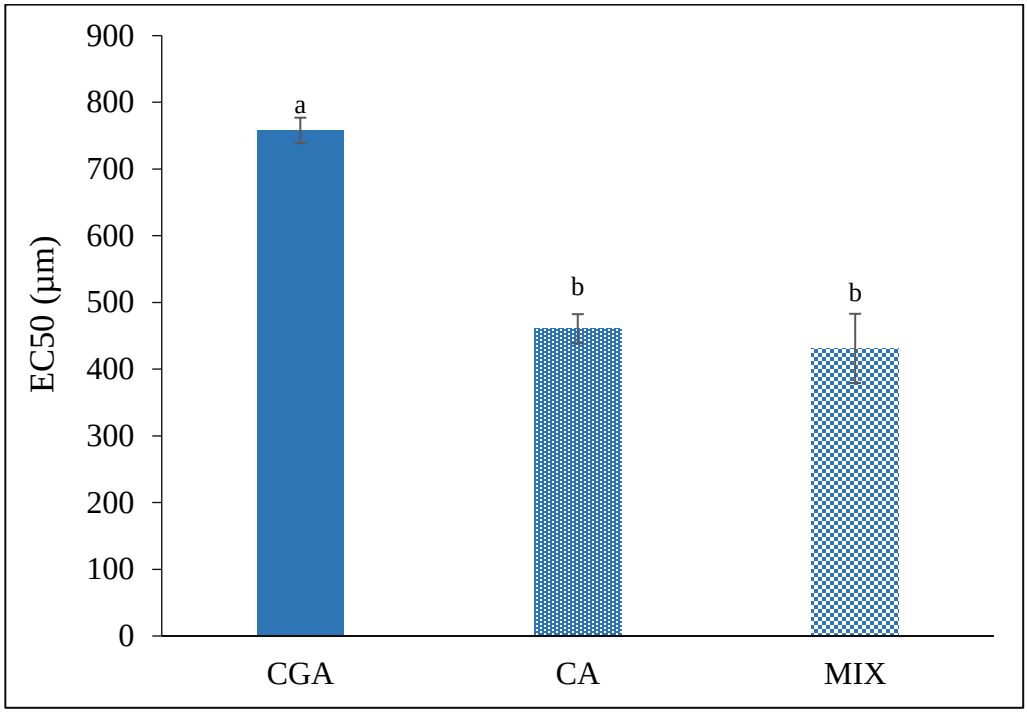


Figure 4.2. The concentrations of CGA, CA and MIX that decrease cell viability by 50% (EC_{50}). Data are represented as mean ± SE. Statistical analysis was performed via one-way ANOVA. Bars not sharing the same letters are significantly different ($p < 0.05$) from each other. CGA = chlorogenic acid; CA = caffeic acid; MIX = equimolar mixture of the four tested compounds.

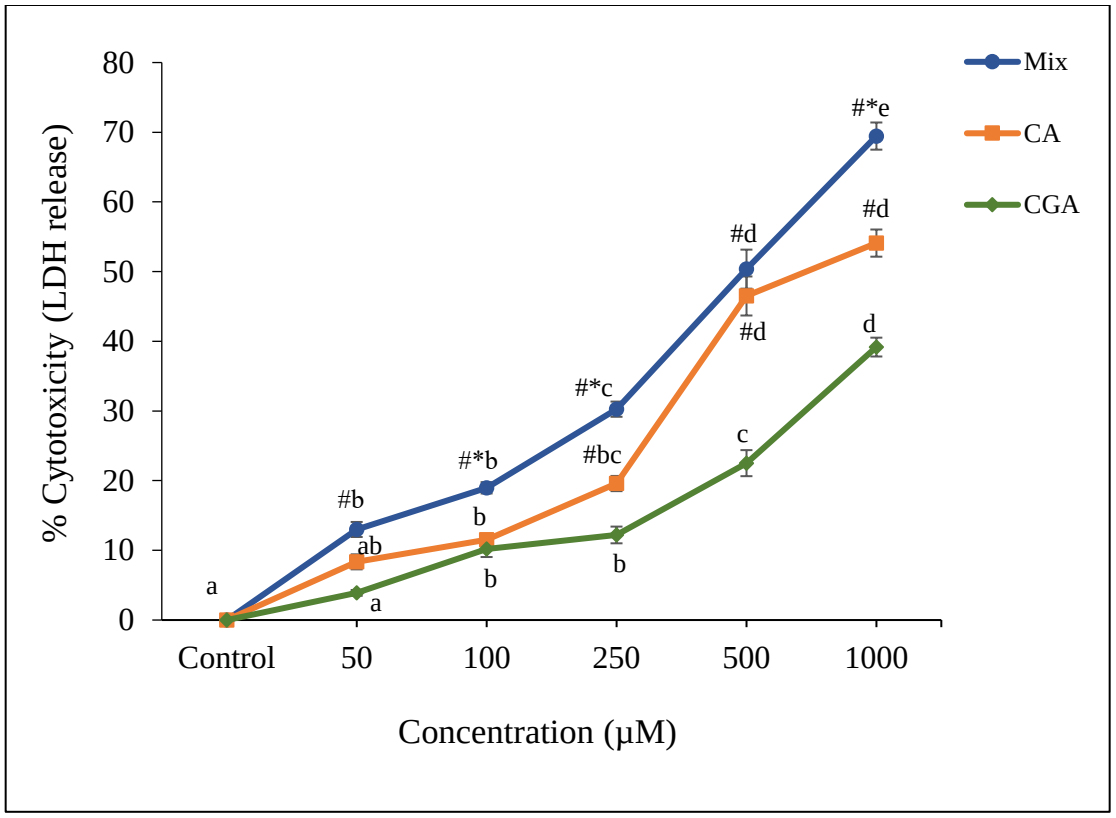
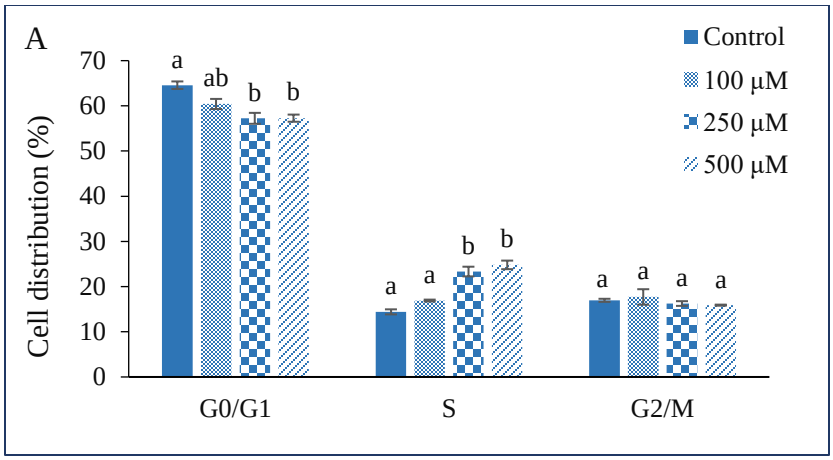


Figure 4.3. Effect of treatment with different doses of CGA, CA and MIX for 24 h on Caco-2 cell cytotoxicity as evaluated by the LDH assay. Data are represented as mean ± SE. Statistical analysis was performed via two-way ANOVA using treatment and dose as factors. Doses within the same treatment not sharing common letters are significantly different ($p < 0.05$). The symbol # represents a significant difference ($p < 0.05$) between CGA and either of the two other treatments at a specific dose. The symbol * represents a significant difference ($p < 0.05$) between MIX and CA at a specific dose. CGA = chlorogenic acid; CA = caffeic acid; MIX = equimolar mixture of the four tested compounds; LDH = lactate dehydrogenase.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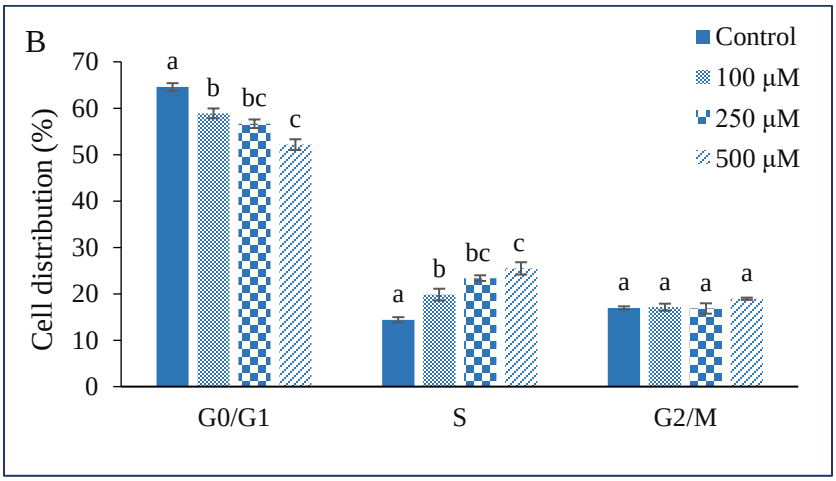
B
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100 µM
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Cell distribution (%)
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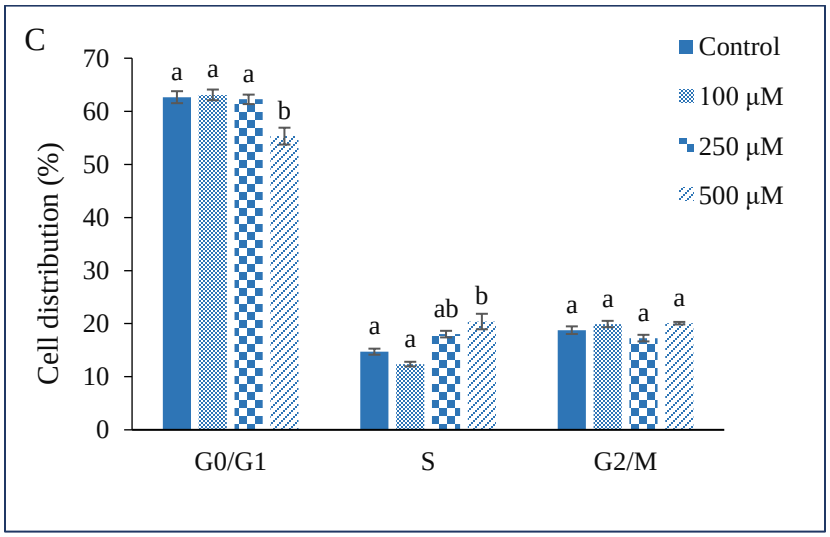
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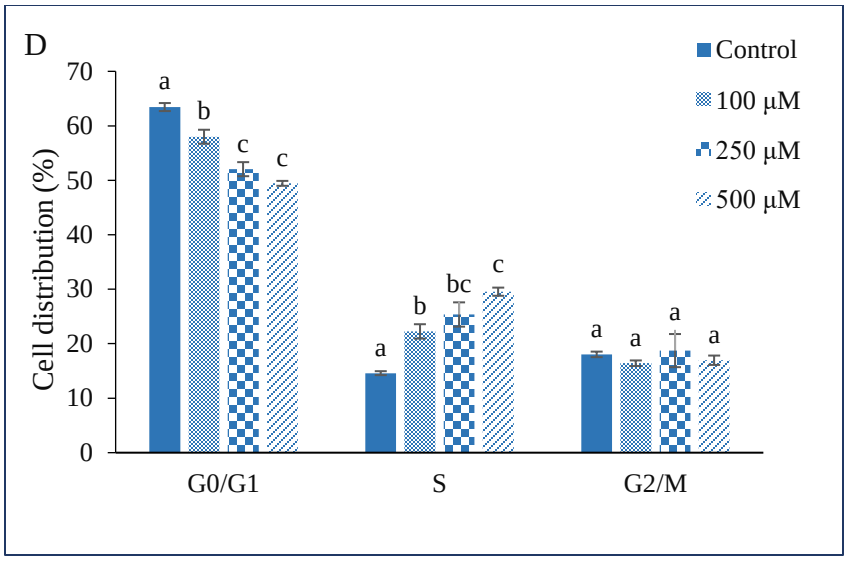

Figure 4.4. Effects of chlorogenic acid (A); caffeic acid (B); 3-phenylpropionic acid (**C**) and MIX (D) treatments for 24 h on Caco-2 cell-cycle distribution as determined by flow cytometry. Data are represented as mean ± SE. Bars not sharing the same letters within each cell cycle are significantly different ($p < 0.05$) from each other.

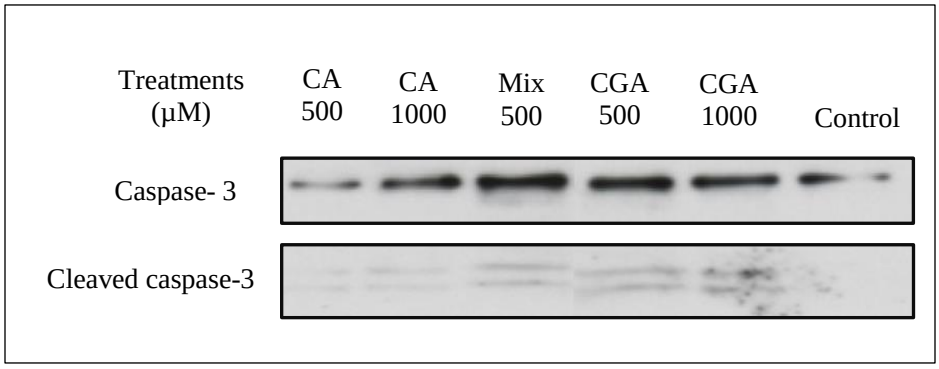

Figure 4.5. Effect of CGA, CA and MIX treatment for 24 h on cleaved and uncleaved caspase-3 levels as evaluated by Western blotting. CGA = chlorogenic acid; CA = caffeic acid; MIX = equimolar mixture of the four tested compounds.

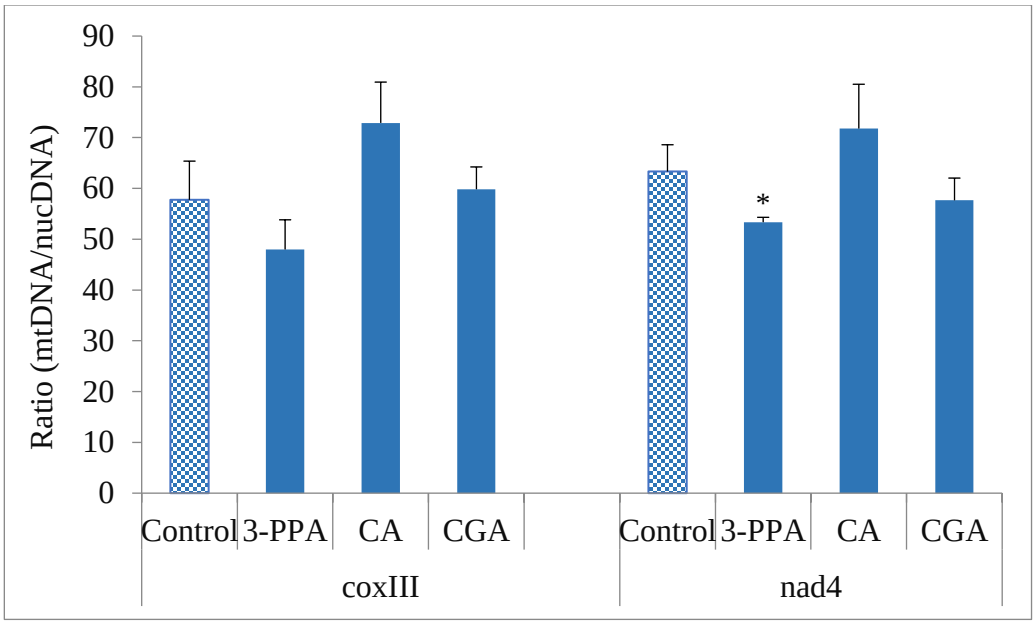

Figure 4.6. Effect of 24 h treatment with 1000 µM CGA and its metabolites on Caco-2 cells on the mtDNA content (ratio of mtDNA/nucDNA) as measured by droplet digital polymerase chain reaction (PCR). Data are represented as mean ± SE. CGA = chlorogenic acid; CA = caffeic acid; 3-PPA = 3-phenylpropionic acid. *: significant from the control ($p < 0.05$).

CONNECTING STATEMENT III

The results from the in vitro digestion study (Study 1) showed extensive biotransformation of the pure polyphenol compounds following enzymatic digestion and gut microbial metabolism. Microbial metabolites including phenylpropionic, benzoic, phenylacetic and cinnamic acids were generated following in vitro digestion. In the second study, the CGA and its major identified post-digest metabolites exhibited anti-colon cancer effects on Caco-2 cells. To exert their systemic health effects, however, polyphenols and their metabolites undergo further absorption and metabolism processes involving intestinal and hepatic cells before becoming available to the target organs [15]. To further elucidate the transport and metabolism of polyphenols and their metabolites, we applied a testing approach that coupled the microbial digests generated from the simulated dynamic GI digestion system with a co-culture of human intestinal Caco-2 and hepatic HepG2 cells. The co-culture system of Caco-2 and hepatocytes has been validated for the prediction of xenobiotic bioavailability in animal models [61] and humans [62]. The combination of the GI model and the co-culture system was used to investigate the bioavailability and biotransformation of a polyphenol-rich potato extract (PRPE), containing CGA, CA, FA and RU as the major polyphenol constituents. The PRPE underwent an in vitro digestion by GI model and the digesta from the colonic compartments were then collected and added to the apical side of the Caco-2/HepG2 cells. The samples were collected at different time points from apical and basolateral sides of the Caco-2/HepG2 system. The presence of the polyphenols and their metabolites in each compartment were measured by LC-MS analysis to assess their transport across Caco-2 cells as well as their metabolism by Caco-2/HepG2 cells.

CHAPTER 5: PAPER 3

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Absorption and metabolism of phenolics from digests of polyphenol-rich potato extracts using the Caco-2/HepG2 co-culture system

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5.1. Abstract

The bioactivity of dietary polyphenols depends upon gastrointestinal and hepatic metabolism of secondary microbial phenolic metabolites generated via colonic microbiota-mediated biotransformation. A polyphenol-rich potato extract (PRPE) containing chlorogenic, caffeic, and ferulic acids and rutin was digested in a dynamic multi-reactor gastrointestinal simulator of the human intestinal microbial ecosystem (GI model). Simulated digestion showed extensive degradation of the parent compounds and the generation of microbial phenolic metabolites. To characterize the transport and metabolism of microbial phenolic metabolites following digestion, a co-culture of intestinal Caco-2 and hepatic HepG2 cells was exposed to the PRPE-derived digests obtained from the colonic vessels. Following a 2 h incubation of the digesta with the Caco-2/HepG2 co-cultures, approximately 10–15% of ferulic, dihydrocaffeic, and dihydroferulic acids and 3–5% of 3-hydroxybenzoic, 3-hydroxyphenylpropionic, and coumaric acids were observed in the basolateral side, whereas 3-hydroxyphenylacetic acid, phenylpropanoic acid, and cinnamic acid were not detected. Subsequent HepG2 cellular metabolism led to major increases in ferulic, dihydrocaffeic, 3-hydroxyphenylpropionic, and coumaric acids ranging from 160–370%. These findings highlight the importance of hepatic metabolism towards the generation of secondary metabolites of polyphenols despite low selective Caco-2 cellular uptake of microbial phenolic metabolites.

Keywords: in vitro digestion; Caco-2/HepG2 co-culture; potato; chlorogenic acid; ferulic acid; caffeic acid; rutin

5.2. Introduction

There is increasing evidence from epidemiological studies and randomized clinical trials showing a strong association between consumption of polyphenols and reduced risk of several chronic diseases such as type 2 diabetes, cardiovascular disease, and some types of cancer [272]. The predominant dietary sources of polyphenols are primarily plant foods that are consumed regularly in large amounts, such as potatoes that are an important food staple in many populations [273]. Potatoes are a good source of common dietary polyphenols including chlorogenic acid (CGA), caffeic acid (CA), ferulic acid (FA), and rutin (RU) which are linked with health-promoting properties [4, 5]. We have shown that a polyphenol-rich potato extract (PRPE) containing CGA, CA, FA, and RU as the primary polyphenols inhibit the development of glucose intolerance and obesity in the diet-induced obesity mouse model [57].

Due to their glycosidic linkages, polyphenols are generally poorly absorbed in the small intestine and so the majority of these compounds reach the colon where glycosides are cleaved by microbiota to generate the aglycon [26]. The aglycons can undergo further hydrolysis, cleavage, and reduction reactions by gut microbiota to produce a variety of small molecular weight end-products that can enter the circulation via transporters or by passive diffusion [16, 17, 23, 102, 274]. To study the effects of digestive processes on polyphenol degradation, in vitro digestion models have been used extensively but the majority of those studies have excluded colonic fermentation [275]. To examine the production of colonic microbial metabolites of polyphenols, we utilized a dynamic multi-reactor gastrointestinal simulator of the human intestinal microbial ecosystem (GI) digestion model, which consists of five interconnected vessels representing the stomach and small intestine, as well as reactors pertaining to the ascending, transverse, and descending colon inoculated with human fecal matter [11, 258, 276]. Our GI model studies have

shown that colonic microbiota metabolism of CGA, CA, FA, and RU generates phenylpropionic acids (3-(3,4-dihydroxyphenyl)propionic acid, 3-(phenyl)propionic acid, 3-(4-hydroxy-3-methoxyphenyl)propionic acid), benzoic acids (protocatechuic acid, benzoic acid, vanillic acid, 3-hydroxybenzoic acid), phenylacetic acid (3-hydroxyphenylacetic acid), and cinnamic acids (caffeic acid, dihydrocaffeic acid, ferulic acid, coumaric acid, cinnamic acid) [258]. Identification of microbial phenolic metabolites is pertinent to the bioactivity of polyphenols as several of these secondary microbial phenolic metabolites have demonstrated antioxidant and anti-inflammatory properties via in vitro and in vivo studies [277]. The presence of such metabolites in human plasma has been suggested to be more relevant for the health benefits of polyphenol-rich plant foods than the less bioavailable parent compounds [43].

There is limited knowledge regarding cellular uptake of microbial phenolic metabolites due to the complexity of the processes affecting the absorptive processes including diffusion and gut transporters, as well as intestinal and hepatic phase II metabolism [25]. First-pass intestinal and hepatic metabolic reactions involving native polyphenols and microbial phenolic metabolites lead to methylated, glucuronidated, or sulfated derivatives that appear in plasma and urine [17, 19]. As processes involved in polyphenol biotransformation and transport have been difficult to fully explore in vivo, recent approaches have coupled in vitro digestion models to double-layered Caco-2 and HepG2 co-cultures [63]. Following treatment of the co-cultures with food digests from GI digestion models, the cell media from the apical and basolateral components were obtained to assess for the biotransformation and transport of metabolites. The human intestinal Caco-2 cell line has been widely used to study the mechanisms of absorption, transport, and metabolism of both drugs and dietary phytochemicals such as polyphenols [118, 122]. Caco-2 cells are differentiated when confluence is reached and after 20 days they form monolayers with well-

established polarity and tight junctions and express transporter and efflux proteins [119, 121]. The human HepG2 cell line is a reliable model that is commonly used to mimic hepatic metabolism of xenobiotics including polyphenols [278]. Combined Caco-2 and liver cell systems have been used for the prediction of oral bioavailability of xenobiotics in animal models [61] and humans [62]. These latter studies have shown very good correlation between the in vitro area under the concentration–time curve and the in vivo bioavailability of the same compound, which indicates that such co-cultures can be used to mimic absorption and first-pass effects.

The aim of the current study was to use the Caco-2 and HepG2 co-culture system to assess intestinal and hepatic-mediated transport and biotransformation of microbial phenolic metabolites generated from the digestion of PRPE in the GI model.

5.3. Materials and Methods

5.3.1. Preparation of the ‘Onaway’ Potato Extract

The potato extract was produced by POS Bio-Sciences (POS Bio-Sciences, Saskatoon, SK, Canada) as previously described [57]. Briefly, *Solanum tuberosum* L. ‘Onaway’ potatoes (20 kg) were washed, chopped, and freeze-dried. The extraction was performed by agitating the freeze-dried potatoes with 200 L of a 90% (*v*/*v*) aqueous ethanol solution for 1 h at room temperature. The ratio of powder to aqueous ethanol was 1:10 (*w*/*v*). The extract was then separated from the solids by centrifugation at 1076× *g* for 10 min and concentrated under vacuum at 40–50 °C until a volume of approximately 15 L was reached. During the evaporation, water was added back to lower the ethanol content to less than 10% as measured by a hydrometer. The extract powder was then generated by freeze-drying the concentrate. The phenolic content of the ‘Onaway’ extract in milligram per gram (mg/g) dry matter basis was 8.9 for CGA, 0.6 for CA, 0.2 for FA, and 1.2 for RU. PRPE was stored at −80 °C until used for simulated GI model digestion studies.

5.3.2. In Vitro Gastrointestinal Digestion of Polyphenol-Rich Potato Extract

PRPE was subjected to in vitro digestion by the GI model as described previously in detail [258]. Briefly, the model was composed of five double-jacketed vessels representing the stomach, the small intestine, the ascending colon, the transverse colon, and the descending colon. The model was fully computer controlled. The pH was automatically controlled by addition of 0.2 M HCl and 0.5 M NaOH solution upon a change in pH in the vessels to simulate the in vivo conditions in the different segments of the human gastrointestinal tract. The temperature was kept at 37 °C and anaerobic conditions were maintained in the fermentation vessels by flushing nitrogen gas for 20 min into the airspace every day. The experiment was started after a 2-week stabilization period in which fecal slurry, obtained from five healthy volunteers with no history of GI disease or antibiotic use in the previous 6 months, were inoculated into the last three vessels. After the 2-week stabilization period, 130 g PRPE was dissolved in the GI food mixture, which was composed of the following ingredients: arabinolactan, pectin, xylan, starch, glucose, yeast extracts, peptone, mucin, and cysteine powders—a composition previously developed by Molly *et al.* (1993) [41]. The GI food mixture was stored at 4 °C during the study. Samples were collected from all three colonic vessels before and 24 h after addition of PRPE to the GI digestion model. Each digestion was carried out in triplicate. Samples were centrifuged at 1000× *g* and stored at −80 °C for later analysis by liquid chromatography–mass spectrometry (LC-MS) and for the cell culture experiments.

5.3.3. Sample Preparation for the Cell Culture Experiments

The samples that were collected from the last three colonic vessels of the gut model after 24 h digestion were pooled after each experiment and prepared according to the method described previously [279]. The samples were centrifuged at 36,000× *g*, 4 °C for 2 h. The supernatant was

collected, and the pH was neutralized to pH 7 before being filter sterilized with a 25 mm syringe filter (0.2 μm, MCE, Fisher Scientific, Ottawa, ON, Canada) and stored at −80 °C until used for the Caco-2/HepG2 cell experiments. Samples collected before the addition of PRPE were considered as the controls.

5.3.4. Cell lines and Culture Conditions

Cells were obtained from the American Type Culture Collection (ATCC, Burlington, ON, Canada) and cultured according to the company's procedures and Li *et al.* (2007) [121], as briefly explained below. The Caco-2 and HepG2 cells were cultured in Eagle's minimum essential medium supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 2 mM L-glutamine, and 0.1% penicillin-streptomycin mixture. Cells were incubated at 37 °C with 5% CO_2 and 90% humidity and were monitored daily. The cells were subcultured at 80% confluence with a 0.25% trypsin-EDTA solution for 5–10 min and were cultured in a new flask or seeded onto an HTS Multiwell™ insert system with polyethylene terephthalate membrane (12 wells, 0.4 μM pore size, 1.12-cm^2 area).

5.3.5. Caco-2/HepG2 Co-Culture System

Caco-2 cells were seeded onto inserts at a density of 60,000 cells/cm^2 and were grown for 21 days under the same incubation conditions mentioned above. During this time, the medium was changed every other day. Before starting the treatments, the integrity of the monolayer was checked by measuring the transepithelial electrical resistance (TEER). A dose-response experiment was carried out in order to determine the optimal dose of the digesta with the minimum effect on the Caco-2 cells' tight junctions as measured by TEER. Caco-2 monolayers having reached a TEER of 250 ohm/cm^2 or greater were used for the transport experiment [123]. As the concentration of 10% (*v*/*v*) digesta in cell culture medium resulted in a minimal decrease in TEER

(data not shown), this concentration was used to investigate the transport and metabolism of phenolic compounds by the Caco-2/HepG2 system. HepG2 cells were added to the basolateral side of the insert system at a concentration of 1 million cells/mL (1.5 mL). Medium (500 µL) containing digesta of PRPE was added to the apical side of the wells and incubated for up to 2 h. After 2 h, the donor plate was removed and the incubation was continued for 3 h with HepG2 cells in the receiver compartment. Samples were taken from the receiver well after 2 and 5 h. After incubation of the 10% digesta of PRPE with Caco-2/HepG2 cells, the supernatant of each compartment (apical and basolateral) was collected at different time points. All samples were centrifuged at 2000× *g* for 15 min and the supernatants were stored at −20 °C for further analysis by LC-MS.

5.3.6. LC-MS Analysis for Identification of Phenolics Using Targeted Metabolite Analysis

After thawing, samples were vortexed and filtered using 25 mm syringe filters (0.45 µm, MCE, Fisher Scientific, Ottawa, ON, Canada) and transferred to HPLC vials for LC-MS analysis. Phenolic compounds were separated using a reverse phase column Gemini-NX (5 µm, 100 mm × 4.6 mm) (Phenomenex, Torrance, CA, USA) with a 4.6 mm × 2.0 mm guard column based on a method developed by Shakya and Navarre (2006) [280] and modified in our previous work [258]. Phenolic compounds and metabolites were eluted using a gradient of solvent A (10 mM formic acid, pH 3.5) and B (5 mM ammonium formate solution in 100% methanol), starting with 5% B, increasing to 30% B in 5 min, 70% B in 7 min, and 100% B at 9 min. This condition was maintained for 3 min. A solvent flow rate of 1.0 mL/min was used and 20 µL of sample was injected into the LC system. Accurate mass data were obtained using an Agilent 1200 series HPLC system equipped with an Agilent 6210 electrospray ionization, time-of-flight (ESI-TOF) mass spectrometer (Agilent, Santa Clara, CA, USA), with internal mass calibration. The analyses were performed in both positive and negative modes and the data was acquired over a mass range of

m/*z* 100–1000. The source parameter settings were: temperature 350 °C, gas flow 12 L/min, nebulizer 50 psi capillary voltage (+/−) 4000 V, fragmentor 100 V, and skimmer voltage 60 V. Reference masses (internal calibration of high resolution spectra) were *m*/*z* 121.050873, 922.009798 for positive mode and *m*/*z* 119.03632, 966.000725 for negative mode. The data were acquired and processed using Agilent Mass Hunter software version B.04.00. Extracted ion chromatograms of accurate masses for deprotonated (MH−) or protonated (MH+) ions were used for confirmation of the presence of parent phenolic compounds as well as metabolites (within 10 ppm). The relative abundance of the compounds was calculated relative to the quantification of benzoic acid as the reference peak, as benzoic acid was present in all the cellular compartments at the same concentration at all time points.

5.4. Results and Discussion

5.4.1. Composition of the Digesta of PRPE Identified by Targeted Metabolite Profiling

The phenolic profiles from the pooled digesta from the three colonic vessels of the PRPE are shown in Table 5.1. The four parent phenolics (CGA, CA, FA, and RU), as well as 12 microbial-derived metabolites, were detected, including: dihydroferulic acid, dihydrocaffeic acid, 3-hydroxyphenylpropionic acid, coumaric acid, 3-hydroxyphenylacetic acid, phenylpropanoic acid, cinnamic acid, 3-hydroxybenzoic acid, and benzoic acid. This result is consistent with previous in vitro digestion studies showing that phenylpropionic, benzoic, phenylacetic, and cinnamic acids were the main metabolites generated after gut microbiota-mediated digestion of the above four parent phenolic compounds [258].

5.4.2. Transport and Metabolism of Phenolic Compounds by Caco-2/HepG2 Cells

The profile of phenolic compounds present in the co-culture system incubated with 10% digesta of PRPE is shown in Table 5.2. A total of 10 microbial-derived metabolites were detected

in the digesta of PRPE when this was diluted to a 10% concentration to maintain TEER above 250 ohms/cm^2 for the transport studies. The presence of some microbial metabolites in control digesta incubated with the cell culture medium before PRPE addition (Table 5.3) may have arisen from the microbial fermentation of proteins and carbohydrates found in the GI nutrient medium [112] or could be explained by the lack of a polyphenol-restricted diet prior to the collection of the fecal samples. The presence of these metabolites has been reported previously in human fecal water [159].

FA was the only parent polyphenol detectable in the 10% digesta (Table 5.2). In agreement with previous Caco-2 cell transport studies, FA exhibited low permeability across the Caco-2 cell monolayer, which involves a concentration-dependent process involving a monocarboxylic acid transporter [123, 281]. After the 2-h Caco-2 cell incubation, a high proportion of the initial FA concentration in the digesta was present in the apical side (90%) while only 11% was located in the basolateral side. Similarly, Koinishi and Shumizu (2003) reported that only a small percentage of FA (3.42%) was transported to the basolateral side of the Caco-2 cell monolayer [123].

Following the 2-h Caco-2 cell incubation, the apical side showed 78% and 67% of initial digesta concentrations of dihydroferulic acid and dihydrocaffeic acid, respectively. Those metabolites were the most predominant in the basolateral compartment showing 15% and 10% of the digesta concentrations for dihydroferulic acid and dihydrocaffeic acid, respectively. The presence of dihydroferulic acid could be partly attributable to Caco-2 cell-mediated biotransformation of FA as dihydroferulic acid has been indicated to be the main metabolite of Caco-2 metabolism of FA [127]. The observed concentration in the basolateral side for dihydrocaffeic acid was higher in comparison to a previous study that reported a permeability of 0.5% for dihydrocaffeic acid across Caco-2 cells involving a 1-h co-culture of Caco-2 and HT29-

MTX cells [127]. The differences between studies might be attributable to the presence of mucus in the latter study, which can affect Caco-2 cell tight junctions and permeability [127]. Alternatively, or in addition, it is conceivable that absorption of dihydrocaffeic acid in the present work may have been enhanced by the concurrent presence of other microbial phenolic metabolites in the fecal digesta [282].

3-Hydroxyphenylpropionic acid was the predominant metabolite in the digesta of PRPE incubated with Caco-2 cells. The apical side showed that 69% of the digesta concentration was present after 2 h but only 4% of the initial concentration was noted in the basolateral side. The transport of 3-hydroxyphenylpropionic acid across the Caco-2 cell monolayer has been previously reported [124]. Likewise, 3-hydroxybenzoic acid and coumaric acid appeared to be poorly transported across Caco-2 cells. 3-Hydroxybenzoic acid and coumaric acid showed respective apical concentrations of 50% and 68% from values observed in the 10% digesta but basolateral concentrations were only 5% and 3% of the initial digesta values, respectively. The transport of *m*-coumaric and *p*-coumaric acids across Caco-2 cells via the monocarboxylic acid transporter has been previously reported with bidirectional transport of the apical to basolateral sides [123, 124]. The relatively low concentration of coumaric acid in the basolateral compartment could be partly due to the basolateral to apical transport reported for coumaric acid in the absence of a proton gradient [123].

A major proportion (65–70%) of 3-hydroxyphenylacetic, phenylpropanoic, and cinnamic acids of their initial digesta concentrations was detectable in the apical side after the 2 h incubation but none of these three compounds were measurable in the basolateral component. Their absence in the basolateral side could be due to either low permeability across Caco-2 cells or further metabolism by Caco-2 cells. In concert with this observation, Konishi (2005) has reported that

99% of apically loaded hydroxyphenylacetic acid isomers remained in the apical side of Caco-2 cells, which was suggested to be due to their restricted transport via tight junctions [283].

Relative to the initial FA values in the basolateral compartment, the incubation with HepG2 cells alone for 3 h at the basolateral side led to a major 166% (1.6-fold) increase in FA concentration (Table 5.2). This increase could be accountable via hepatic-mediated dihydroferulic acid dehydrogenation as reported by Poquet *et al.* (2008) who showed the generation of FA from dihydroferulic acid after 30 min metabolism by rat liver slices [127]. Such metabolism can be partly responsible for circulating free FA in addition to a small percentage of glucuronide and sulfate FA conjugates [127]. The present work thus supports the contention that a significant proportion of plasma FA originates from in situ hepatic generation [161, 284], particularly in view of its poor absorption seen in the current study and previous research [123].

After the 3 h incubation with HepG2 cells, 78% of the initial basolateral dihydroferulic acid concentration was observed. This value could be partly mediated by hepatic hydrogenase enzymes shown to produce dihydroferulic acid from FA [285]. Dihydroferulic acid appears mainly in plasma in an unconjugated form and hydroxycinnamate metabolism has been suggested to be responsible for plasma accumulation of dihydroferulic acid over time [17]. A remarkable three-fold increase in dihydrocaffeic acid concentrations in the basolateral compartment was seen following the HepG2 cell incubation. This increase could be partly mediated via hepatic dehydrogenation and *O*-demethylation reactions shown to generate dihydrocaffeic acid from FA and dihydroferulic acid [285]. Although not measured in the present study, both dihydrocaffeic and dihydroferulic acids could also undergo further intestinal and hepatic metabolism to generate glucuronidated and sulfated metabolites [286].

An increase of 233% (2.3-fold) in the basolateral concentrations of 3-hydroxyphenylpropionic acid was observed after the 3 h incubation with HepG2 cells, which supports hepatic metabolism as a major contributor to circulating concentrations of this metabolite. 3-Hydroxyphenylpropionic acid has been noted to be one of the most abundant microbial phenolic metabolites in plasma, urine, and fecal samples following polyphenol ingestion in human and animal feeding studies [16, 161]. More than a two-fold increase in coumaric acid concentrations was seen in the basolateral compartment after a 3-h incubation with HepG2 cells, which could be partly due to the generation of coumaric acid from 3-hydroxyphenylpropionic acid by hepatic metabolism as previously suggested [284]. The 3-hydroxybenzoic acid concentration was unchanged in the basolateral compartment post-incubation with HepG2 cells, which suggests minimal hepatic degradation or biotransformation. The present results indicate that 3-hydroxybenzoic acid, one of the major products of gut microbiota metabolism, can be present following post-intestinal and hepatic metabolism. This finding coincides with the appearance of 3-hydroxybenzoic acid in the urine and plasma after consumption of polyphenol-rich diets [284].

5.5. Conclusions

In summary, the present results revealed that regardless of the poor Caco-2 cellular transport of FA and the microbial phenolic metabolites of dihydroferulic, dihydrocaffeic, 3-hydroxyphenylpropionic, 3-hydroxybenzoic, and coumaric acids, a notable increase in the basolateral concentrations of those compounds was observed following metabolism with HepG2 cells. The present data thus support the importance of post-prandial hepatic metabolism as a major source of circulating phenolic metabolites. It is noteworthy that in vivo feeding studies involving polyphenols have shown a gradual post-prandial appearance of plasma microbial phenolic metabolites at 0.5–4 h followed by a secondary increase in those metabolites at 4 h, which could

thus be mediated by hepatic metabolism [64, 287]. Future studies involving the combination of in vitro digestion models and Caco-2/HepG2 cell culture studies could also focus upon the production of sulfated, glucuronidated, or methylated metabolites of polyphenols that are predominant in the circulation [10]. Taken together, the results of the present study signify the importance of considering hepatic metabolism as a major contributor towards circulating phenolic metabolites following the intake of polyphenol-rich food products.

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Author Contributions

S.S.E. designed the study, carried out the experiments, analyzed the data, and drafted the manuscript. M.M.I. was involved in developing the co-culture study and helped edit the manuscript. L.S. carried out the LC-MS analysis. K.S. developed the methods for HPLC and LC-MS analysis, performed the HPLC analysis, and was involved in PRPE preparation. J.K.H. was involved in the in vitro digestion study. S.P. oversaw the in vitro digestions. S.K. initiated the original idea of the study and was involved in the study design, supervised all the aspects of the study, and provided extensive input to the manuscript. All authors read and approved the final manuscript.

Conflicts of Interest

S.S.E., M.M.I., L.S., K.S., J.K., S.P. declare no conflict of interest. S.K. holds a US patent (Methods for treating type-2 diabetes and obesity; US. Patent. 9,446,063 B2).

Table 5.1. Polyphenols and their metabolites after human simulated intestinal digestion of polyphenol-rich potato extract (PRPE)[1].

Theoretical Mass (*m*/*z*) [2]	Measured Mass	Mass Accuracy (ppm)	Retention Time (min)	Common Name	Systematic Name	PRPE
609.1461	609.1422	6.4	8.7	Rutin	Quercetin-3-*O*-rutinoside	+
353.0878	353.0863	4.3	7.5	Chlorogenic acid	(1*S*,3*R*,4*R*,5*R*)-3-{[(2*E*)-3-(3,4-Dihydroxyphenyl)prop-2-enoyl]oxy}-1,4,5-trihydroxycyclohexanecarboxylic acid	+
301.0354	301.0395	13.7	8.0	Quercetin	2-(3,4-Dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-chromen-4-one	T
195.0663	195.0642	10.6	8.6	Dihydroferulic acid	3-(4-Hydroxy-3-methoxyphenyl)propionic acid	+
193.0506	193.0507	0.5	8.5	Ferulic acid	(E)-3-(4-Hydroxy-3-methoxy-phenyl)prop-2-enoic acid	+
181.0506	181.0509	1.6	7.0	Dihydrocaffeic acid	3-(3′,4′-Dihydroxyphenyl)propionic acid	+
179.0325	179.341	8.9	8.0	Caffeic acid	3,4-Dihydroxycinnamic acid	+
167.0350	167.0349	0.5	6.6	Vanillic acid	4-Hydroxy-3-methoxybenzoic acid	+
165.0557	165.054	10	8.4	3-Hydroxyphenylpropionic acid	3-(3-Hydroxyphenyl)propionic acid	+
163.0401	163.0409	4.9	8.4	Coumaric acid	The isomer is not specified from our data	+
153.0193	153.0192	0.6	7.2	Protocatechuic acid	3,4-Dihydroxybenzoic acid	+
151.0401	151.0398	1.98	7.7	3-Hydroxyphenylacetic acid	3-Hydroxyphenylacetic acid	+
149.0608	149.0599	6.03	9.4	Phenylpropanoic acid	Phenylpropanoic acid	+
147.0452	147.0453	0.6	8.5	Cinnamic acid	3-Phenylprop-2-enoic acid	+
137.0244	137.0241	2.1	7.2	3-Hydroxybenzoic acid	3-Hydroxybenzoic acid	+
121.0295	121.0297	1.6	9.0	Benzoic acid	Benzoic acid	+

[1] Determined by LC-MS analysis; [2] Identification based on previous literature data [18, 19, 101, 103]; (+) present, (T) trace amount.

Table 5.2. Polyphenols and their metabolites in PRPE digesta (t = 0 h) and amount expressed as a percentage of initial digesta values in the apical (t = 2 h) and basolateral (t = 2 h) chambers in the Caco-2 cell transport bioassay and basolateral (t = 5 h) chamber in the HepG2 cell metabolism bioassay [a].

Theoretical Mass (*m*/*z*)	Measured Mass	Mass Accuracy (ppm)	Retention Time (min)	Common Name	PRPE			
					Digesta [b] (0 h)	Percentage of Digesta Value [c]		Percentage of Basolateral Value [d]
						A (2 h)	B (2 h)	B (5 h)
195.0663	195.0642	10.6	8.6	Dihydroferulic acid	1.22	78	10	78
193.0506	193.0507	0.5	8.5	Ferulic acid	1.07	90	11	166
181.0506	181.0509	1.6	7.0	Dihydrocaffeic acid	3.00	67	15	338
165.0557	165.054	10	8.4	3-Hydroxyphenylpropionic acid	15.11	69	4	233
163.0401	163.0409	4.9	8.4	Coumaric acid	2.44	68	3	212
151.0401	151.0398	1.98	7.7	3-Hydroxyphenylacetic acid	1.02	70	-	-
149.0608	149.0599	6.03	9.4	Phenylpropanoic acid	0.32	65	-	-
147.0452	147.0453	0.6	8.5	Cinnamic acid	0.31	67	-	-
137.0244	137.0241	2.1	7.2	3-Hydroxybenzoic acid	5.12	50	5	100
121.0295	121.0297	1.6	9.0	Benzoic acid	+	+	+	+

[a] Identification based on previous literature data [18, 19, 101, 103]; [b] Quantities of polyphenols and their metabolites in 10% (*v*/*v*) potato extract digesta are calculated relative to the concentration of benzoic acid. [c] Amount expressed as a percentage of initial digesta values in the apical chamber (A) and basolateral chamber (B) after 2 h in the Caco-2 cell transport bioassay. [d] Amount expressed as a percentage of initial digesta values in the basolateral chamber (B) after 3 h in the HepG2 metabolism bioassay. (+) present, (-) absent.

Table 5.3. Polyphenols and their metabolites in control digesta (t = 0 h) and amount expressed as a percentage of initial control values in the apical (t = 2 h) and basolateral (t = 2 h) chambers in the Caco-2 cell transport bioassay and basolateral (t = 5 h) chamber in the HepG2 cell metabolism bioassay [a].

Theoretical Mass (*m*/*z*)	Measured Mass	Mass Accuracy (ppm)	Retention Time (min)	Common Name	Control			
					Control [b] (0 h)	Percentage of Control Value at 0 h [c]		Percentage of Basolateral Value [d]
						A (2 h)	B (2 h)	B (5 h)
181.0506	181.0509	1.6	7.0	Dihydrocaffeic acid	1.1	26	26	123
165.0557	165.054	10	8.4	3-Hydroxyphenylpropionic acid	0.53	90	-	-
151.0401	151.0398	1.98	7.7	3-Hydroxyphenylacetic acid	0.42	100	-	-
149.0608	149.0599	6.03	9.4	Phenylpropanoic acid	0.28	100	-	-
121.0295	121.0297	1.6	9.0	Benzoic acid	+	+	+	+

[a] Identification based on previous literature data [18, 19, 101, 103]; [b] Quantities of phenolic metabolites in controls are calculated relative to the concentration of benzoic acid. [c] Amount expressed as a percentage of initial values of control samples in the apical chamber (A) and basolateral chamber (B) after 2 h in the Caco-2 cell transport bioassay. [d] Amount expressed as a percentage of initial values of controls in the basolateral chamber (B) after 3 h in the HepG2 metabolism bioassay. (+) present, (-) absent

CHAPTER 6: GENERAL SUMMARY AND CONCLUSIONS

6.1. General Discussion

The thesis describes a series of studies involving the combination of a simulated gut digestion with cell culture systems for the investigation of the biotransformation, bioavailability and bioactivity of commonly consumed polyphenols (CGA, CA, FA and RU) and their metabolites. The Computer Controlled Dynamic Human Gastrointestinal Model (GI model) was used in Study 1 to assess the impact of enzymatic digestion and microbial metabolism on polyphenolic structure, antioxidant activity and SCFA generation following intake of pure polyphenol compounds in the form of CGA, CA, FA and RU. The GI model represents an advance over static batch reactor digestion models that have been predominantly used for the study of polyphenol bioaccessibility and biotransformation. In contrast to batch reactors, the GI model considers dynamic digestive responses that follow the introduction of food and food components including increases in pH, the steady transit of food compounds throughout the GI tract and the varying biotransformation processes taking place among the three colonic segments. The findings of Study 1 highlighted the significant role of colonic gut bacteria in the process of digestion and metabolism of the tested polyphenols with minimal biotransformation of the native polyphenols seen in the upper GI vessels and extensive bacterial breakdown occurring in the colonic compartments. These results are in agreement with previous studies reporting the extensive microbial degradation of hydroxycinnamate derivatives as evidenced by the detection of secondary phenolic metabolites in plasma and urine following polyphenol intake [16, 161]. The phenolic profile differences observed among the three colonic vessels in Study 1 could be partly explained by the pH difference in the vessels, which was previously shown to lead to different bacterial communities in the colonic reactors of dynamic gastrointestinal simulators [29]. Such variations

in gut bacterial profiles can lead to their differing capacities for polyphenol biotransformation [239]. In support of the latter concept, considerable inter-individual variability in the excreted levels of polyphenol metabolites was observed following coffee consumption in a human trial, which was attributed to be due to differing gut bacterial profiles among subjects [161]. In an in vitro study, the differing microbial profiles in different segments of the multisector GI model was shown to differentially influence the release of secoisolariciresinol from lignan and its transformation into enterolignans. The hydrolysis of the secoisolariciresinol precursor, in the latter study, occurred in the ascending colon and its bioactivation into enterolignans started from the transverse colon onward [288]. Microbial metabolism of polyphenols consists of various hydrolyzing processes such as ring-fission, dihydroxylation, demethylation and decarboxylation for which bacterial species differ in their capacities [25]. The catabolism of the phenolic compounds was initiated in the first AC vessel, which was followed with further degradation in the last two colonic compartments leading to the generation of microbial metabolites such as phenylpropionic, benzoic, phenylacetic and cinnamic acids. Hence, the metabolic processes leading to the profiles of phenolic metabolites in the TC and DC reactors is partly a consequence to the types of secondary phenolic metabolites that flowed from the preceding colonic vessel (Figure 6.1). Similar urinary metabolites, particularly FA derivatives, have been associated with hydroxycinnamate metabolism following coffee consumption in humans [161]. Previous studies have reported a long residence time of 24 - 48 h in the blood circulation for some of the microbial metabolites identified in Study 1 such as phenylacetic acids, which could be partly due to an enterohepatic circulation of these compounds [25].

The multistage dynamic GI system was also used to measure the antioxidant capacity and SCFA profiles in different colonic segments as those factors can lead to health benefits from

colonic polyphenol metabolism. The stomach, SI and AC showed a significant increase in antioxidant capacity at 8 h, which was not seen in the TC and DC reactors. This result is attributable to the presence of the native polyphenols in the upper intestinal vessels as those polyphenolics have significant antioxidant potential [83, 170, 172]. After 16 h of digestion, however, the TC and DC reactors showed similar antioxidant capacity as the upper intestinal vessels, which signifies that the microbial metabolites can exert antioxidant activity in the gut to a similar extent as their precursors. The latter unique finding contrasts with previous batch reactor studies demonstrating relatively low antioxidant capacity of microbiota-generated polyphenol metabolites [107, 289], which is likely due to the shorter fermentation time of only a few hours in those studies. Polyphenol microbial metabolites have been indicated to play a significant role in increasing the antioxidant capacity of the plasma following consumption of polyphenol-rich foods as the concentrations of some of these metabolites have been shown to be up to four times greater than their precursors [15]. The mixture of common dietary polyphenols used in Study 1 also significantly increased the level of SCFAs including an enhancement of the proportion of propionic to acetic acid which may have implications for weight management [290]. It has been shown that propionate can play a role in appetite regulation and energy intake via inducing the release of pancreatic peptide YY (PYY) and glucagon-like peptide 1 (GLP-1) from intestinal cells. In addition, increased levels of propionate may inhibit the conversion of acetate into lipid in the liver and adipose tissue [290]. An increase in gut production of SCFAs has been associated with several gut health-promoting effects, including enhanced probiotic bacterial growth [253] as well as anti-inflammatory and anti-cancer outcomes [136]. The thesis study also showed differences in SCFA profiles among the colonic reactors, which is in line with the variation in the microbial phenolic metabolites and antioxidant capacity shown among the colonic compartments. The differences in SCFA profiles among the

colonic vessels are likely due to the different microbial communities present in each compartment [112]. Similar to our findings, microbial biotransformation of polyphenol-rich red wine led to generation of differing SCFA profiles in the colonic compartments of a dynamic GI model system. This was characterized by higher level of total SCFAs in the TC and DC reactors [112].

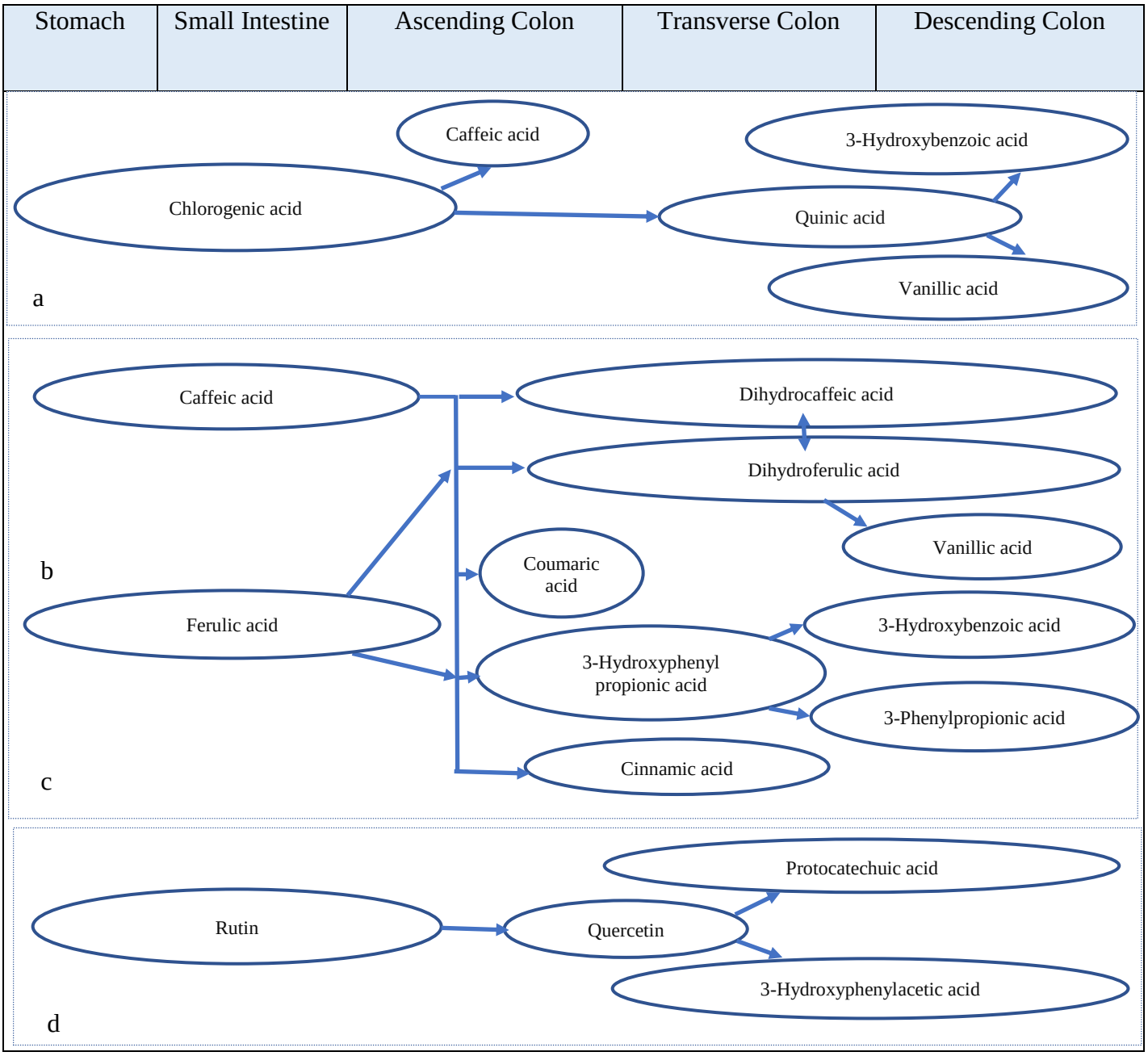

Figure 6.1. Overview of the biotransformation of chlorogenic acid, caffeic acid, ferulic acid and rutin observed during their 24 h digestion in the GI model [10, 25]. All the polyphenols were presented in the stomach, SI and AC vessels. The bacterial biotransformation of the parent compounds and the appearance of the microbial metabolites started in the AC. (a) Chlorogenic acid (m/z 353.0870) was transformed to caffeic acid (m/z 179.032) and quinic acid (m/z 191.056)

found in the TC and DC. Quinic acid was further catabolized to 3-hydroxybenzoic acid (m/z 137.024) and vanillic acid (m/z 167.035) in the TC and DC. (b) Caffeic acid was converted to dihydrocaffeic acid (m/z 181.050), dihydroferulic acid (m/z 195.066), cinnamic acid (m/z 147.045), coumaric acid (m/z 163.040) and 3-hydroxyphenylpropionic acid (m/z 165.055) in the AC. (c) Ferulic acid (m/z 193.050) was transformed to dihydroferulic acid (m/z 195.066) and 3-hydroxyphenylpropionic acid (m/z 165.055) in the AC. 3-Hydroxyphenylpropionic acid (m/z 165.055) was converted to 3-phenylpropionic acid (m/z 149.0608) and 3-hydroxybenzoic acid which were detected in the AC, TC and DC. Dihydroferulic acid was transformed to vanillic acid. (d) Rutin (m/z 609.146) was converted to quercetin (m/z 301.035) in the AC and TC and was further degraded to 3-hydroxyphenylacetic acid (m/z 151.040) and protocatechuic acid (m/z 153.020) which were found in the AC, TC and DC. SI = small intestine; AC = ascending colon; TC = transverse colon; DC = descending colon.

As a large amount of ingested CGA reaches the colon and undergo extensive biotransformation and metabolism by colonic bacteria, the gut cells are likely a major target of the bioactivities pertaining to CGA and its microbial metabolites described in Study 1. In Study 2, the anti-cancer effects of CGA and main CGA-derived microbial metabolites, CA, 3-PPA and BA [16, 51], were tested in the human colonic Caco-2 cancer cell line. To our knowledge, there is no previous evidence linked to the possible synergistic anti-cancer effects of the combination of CGA with its microbial-derived metabolites. Polyphenols can exert anti-cancer activities via multiple diverse molecular pathways involved in carcinogenesis including antioxidant and anti-inflammatory effects, induction of cell cycle arrest and apoptosis [153]. The findings from Study 2 demonstrated that CGA, CA and MIX exerted anti-cancer activities that included inhibition of cell proliferation and the induction of cell cytotoxicity, cell cycle arrest and apoptosis.

Significantly, the MIX exhibited the strongest anti-proliferative and cytotoxic activities followed by CA and CGA. Anti-proliferative and cytotoxic effects of CGA and CA were seen at several-fold lower concentrations within the equimolar mixture than when they were provided singly. The stronger anti-cancer activities of MIX indicates that the combination of CGA and its microbial metabolites generated from colonic microbial metabolism could play more important anti-cancer roles than previously realized. Earlier studies have shown synergistic anti-cancer effects of a combination of parent polyphenols rather than the interaction with their post-digest metabolites [153]. Such studies, however, have questionable physiological relevance since the anti-colon cancer effects of polyphenols are more likely to be due to combinations containing polyphenols and their gut microbial metabolites rather than solely to the native precursor compounds. The observed synergistic interaction in present study could thus better explain the reported in vivo benefits of CGA-rich diet on colon cancer [254, 255]. Figure 6.2 illustrates a summary of possible molecular mechanisms for anti-cancer activity of polyphenols.

The inhibition of cell cycle progression has been shown to be associated with anti-proliferative activities of a variety of anti-cancer compounds including polyphenols [190, 291]. Not all polyphenols can exert cell cycle arrest in cancer cells as this depends on the chemical structure of the polyphenols [291]. Likewise, the mechanisms of cell cycle arrest by polyphenols is dependent upon the cancer cell line [199, 201]. The growth arrest may occur at different phases and through varying molecular pathways including modulation of cyclin proteins or cyclin dependent kinase [184, 199-201]. Previous studies have shown S-phase cell cycle arrest is an anti-cancer mechanism exerted by various types of polyphenols [199, 200]. The present thesis work indicates for the first time that hydroxycinnamic acids such as CGA and their microbiota-generated metabolites can exert anti-proliferative effects via induction of S-phase cell cycle arrest. The

induction of cell cycle arrest could also lead to apoptosis in cancer cells with the involvement of several genes in both processes [292]. Apoptosis induction is one of the most potent mechanisms of anti-cancer agents [293]. Apoptosis induction by polyphenols can occur through activation of intrinsic or extrinsic apoptosis triggers leading to caspase-3 activation [204]. CGA and CA have shown to induce apoptosis in leukemia cells [198] and human cervical cancer cells [218], respectively. In that regard, ROS production, upregulation of p53, cytochrome c release and caspase-3 activation have been indicated to be involved [198, 218]. The p53 molecule is one of the key apoptotic factors that links cell cycle arrest to apoptosis [292]. Combinations of polyphenols with synergistic interactions can exert pleiotropic effects by affecting multiple molecular pathways of carcinogenesis [153]. For the first time, PPA and BA were shown to exert anti-proliferative activities with no cytotoxicity. The anti-proliferative effects of 3-PPA was associated with both S-phase cycle arrest and mtDNA depletion. Among all the tested compounds, only 3-PPA showed alterations of mtDNA content in association with anti-proliferative effects. This is interesting finding considering that 3-PPA is the end microbial metabolite of many phenolic compounds [245] and one of the most predominant metabolites detected in human fecal water [232]. The slight anti-proliferative effects observed for BA and 3-PPA in the concentration range tested in the present work (50-1000 μM) would worthy further investigation using the higher concentrations as concentrations of 3-PPA and BA in human fecal water have been reported to reach up to 2136 μM [160] and 4625 μM [159], respectively. The above findings are in line with previous studies involving ellagitannins showing similar molecular anti-cancer mechanisms for both parent polyphenols (ellagitannins) and their microbial-derived metabolites urolithin A [277]. The present work extends the results of the latter studies with respect to chlorogenic acid-derived microbial metabolites.

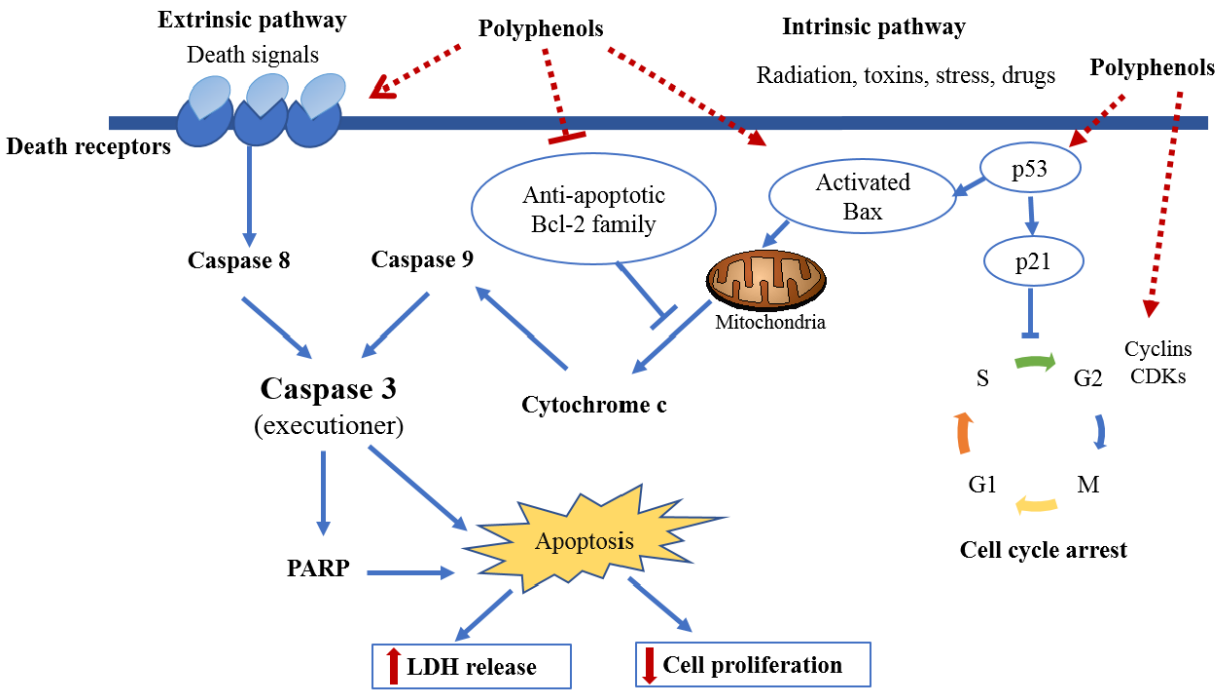

Figure 6.2. Summary of the possible molecular pathways that lead to cell cycle arrest and apoptosis and can be affected by polyphenols and their microbial metabolites (dashed lines). They may exert their anti-proliferative activities through extrinsic or intrinsic pathways, which both lead to activation of caspase-3 and apoptosis. Caspase-3 can also induce apoptosis via activation of poly (ADP-ribose) polymerase (PARP). Polyphenols may also induce apoptosis via activation of p53, which is involved in the induction of cell cycle arrest through activation of p21. Polyphenols may induce cell cycle arrest through other molecular pathways such as modulating the activity of cyclin proteins, cyclin dependent kinases (CDKs) and CDKs inhibitors. p53: tumor suppressor p53; p21: CDK-interacting protein 1; Bax: apoptosis regulator (Bcl-2-associated X protein); Bcl-2: apoptosis regulator (B-cell lymphoma 2); M: mitotic phase; S: synthesis phase; G1:gap 1; G2: gap 2; LDH: lactate dehydrogenase assay. Adapted from: Mrakovcic and Fröhlich 2018; D'Archivio *et al*., 2008; Rodríguez *et al*., 2013 [223, 294, 295].

Prior to their entry into systemic circulation, post-digest metabolites of polyphenols undergo sequential transport and metabolism by intestinal and hepatic cells [1]. There is relatively little information pertaining to the intestinal and hepatic transport and biotransformation that is attributable to difficulties in examining the complex nature of polyphenol metabolism, which is further complicated by their considerable structural diversity. One of the limitations of the GI digestion simulators is that the absorption processes are not simulated in such models. In order to more comprehensively study the absorption and metabolism of post-digest phenolic metabolites, we applied a testing system that coupled the colonic digests obtained from the dynamic continuous GI system with a co-culture of intestinal Caco-2 cells and hepatic HepG2 cells. Previous studies have used the Caco-2 cells to study the transport and metabolism of pure synthetic polyphenols or polyphenol-rich plant extracts [122, 123, 296]. In those studies, however, the effects of enzymatic and microbial transformation of polyphenols that take place during gut digestion before their uptake by the intestinal cells were not taken into account. We utilized the combined dynamic digestion model and Caco-2/HepG2 co-culture to study the bioavailability and metabolism of microbiota-derived metabolites generated from digestion of a polyphenol-rich potato extract (PRPE). FA and microbial-derived metabolites including dihydroferulic, dihydrocaffeic, 3-hydroxyphenylpropionic, coumaric, 3-hydroxyphenylacetic, phenylpropanoic, cinnamic, 3-hydroxybenzoic and benzoic acids were identified in the digesta collected from the colonic vessels of the GI model before addition to the Caco-2/HepG2 cells (Table 5.1). Our findings demonstrated poor transport of FA and all the metabolites across the Caco-2 cells as the tested compounds were predominantly detected in the apical compartment after 2 h incubation with the Caco-2 cells. A permeability of 10-15% was observed for FA, dihydroferulic and dihydrocaffeic acids and the lower permeability rate of 3-5% was observed for 3-hydroxyphenylpropionic, 3-hydroxybenzoic

and coumaric acids. These results are in agreement with previous studies reporting poor transport of FA and dihydrocaffeic acid across Caco-2 cells when these pure compounds were tested individually [123, 127]. The bidirectional transport of the apical to basolateral sides could also explain the low recovery of some of the metabolites from the basolateral compartment [123], which could be investigated in future studies. It is conceivable that the interaction between the phenolic metabolites in the digesta used in the present study could have altered the permeability rate of the identified compounds [282]; however, the mixture of post-digest phenolic metabolites is physiologically more relevant to in vivo conditions than testing single synthetic phenolic compounds as per previous studies [123, 124, 127].

Following further incubation of the metabolites with HepG2 cells for 3 h, a marked increase in concentrations of FA, dihydrocaffeic, 3-hydroxyphenylpropionic and coumaric acids was observed. The above data has demonstrated that liver cells can contribute significantly to the generation of these phenolic metabolites that have been previously characterized in the systemic circulation following intake of hydroxycinnamic compounds [17]. Despite the high dietary intake of phenolic acids little is known about their hepatic metabolism. There have been few studies reporting the hepatic generation of some of the tested phenolic compounds including FA, dihydrocaffeic and coumaric acids using rat hepatocytes [127, 284, 285]. In an ex vivo study, hepatic production of FA was reported following incubation of dihydrocaffeic and dihydroferulic acids with rat liver slices [127]. CA, FA, dihydrocaffeic and dihydroferulic acids has been shown to metabolize to each other by isolated rat hepatocytes [285]. Coumaric acid was shown to form from hydrogenation of 4-hydroxyphenylpropionic acid by rat liver homogenate [284]. Although there are no direct evidence regarding the hepatic generation of 3-hydroxybenzoic acid, its urinary excretion in rats fed procyanidins has been suggested to either originate from microbial

biotransformation of protocatechuic acid or from biotransformation of 3-hydroxyphenylpropionic acid in the liver [284]. The present findings further elucidate the important contribution of human hepatic metabolism in generation of the above circulating phenolic metabolites, which has not been investigated previously. To our knowledge, the present results provide the first evidence describing generation of 3-hydroxyphenylpropionic acid by human hepatic cells.

This is the first time that the transport and metabolism of a mixture of secondary phenolic metabolites of CGA, FA, CA and RU has been studied using a co-culture of human intestinal and hepatic cells. The measurement of the polyphenol metabolites in the plasma and urine in human and animal studies do not allow the capacity to distinguish the role of colonic and post-colonic intestinal and hepatic metabolism in the biotransformation processes involved for the excreted metabolites. By utilizing the combined in vitro digestion and cell culture systems we were able to derive new information regarding the relative contribution of each stage in the intestinal and hepatic biotransformation processes of the tested polyphenols. Figure 6.3 represents a summary of transport and biotransformation pattern of PRPE post-digest metabolites by Caco-2/HepG2 cells.

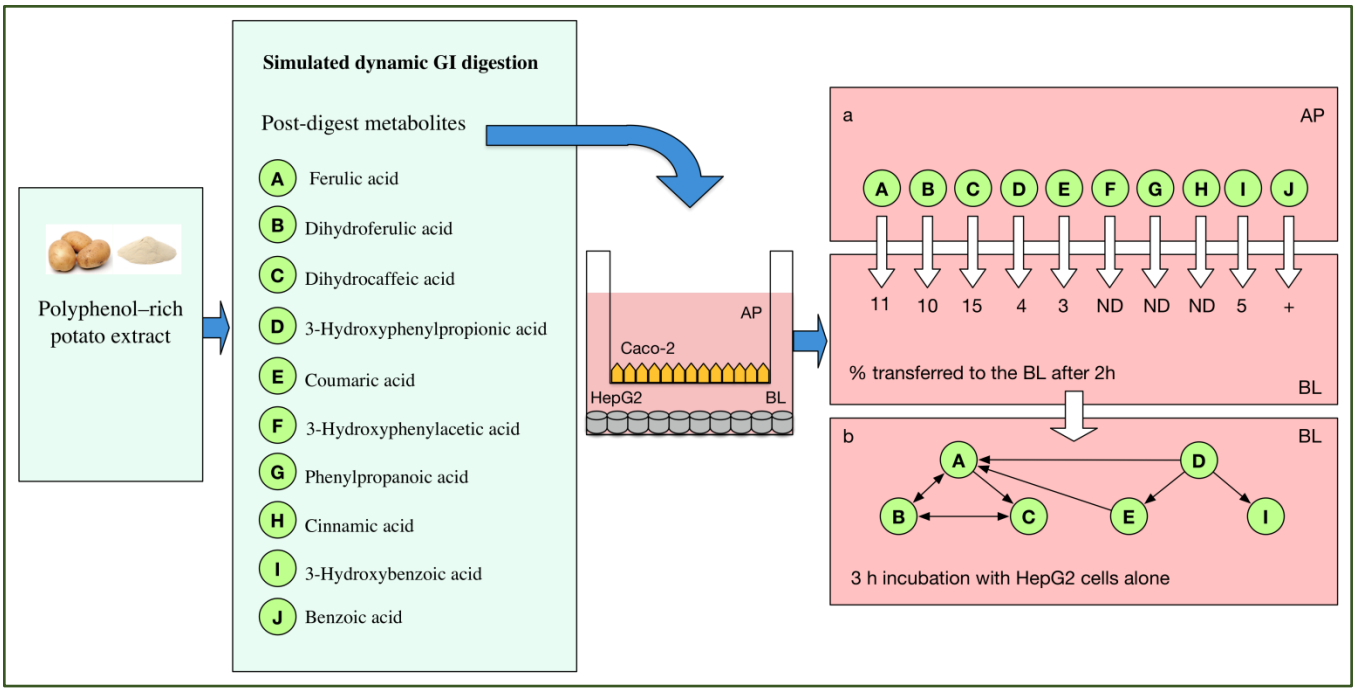

Figure 6.3. Summary of transport and metabolic pathways of post-digest phenolic compounds by Caco-2/HepG2 cells. (a) The percent of initial concentration of each compound that was detected in the BL compartment after 2 h incubation with Caco-2 cells. About 3-15% of the initial concentrations of the phenolic compounds was transported to the basolateral side. (b) Possible biotransformation pathways of transported compounds by hepatocytes after 3 h incubation with HepG2 cells only. Ferulic acid can be converted to dihydroferulic acid via the reduction of its aliphatic double bond [127, 285]. Dihydroferulic acid oxidation would produce ferulic acid by the action of dehydrogenase enzyme [127, 285]. Ferulic acid can undergo *O*-demethylation and further hydrogenation to form dihydrocaffeic acid [127]. 3-Hydroxybenzoic acids can be formed from β-oxidation of 3-hydroxyphenylpropionic acid [284]. 3-Hydroxyphenylpropionic acid can biotransform to ferulic acid via methylation and hydroxylation [297]. 3-Hydroxyphenylpropionic acid hydrogenation produces coumaric acid, which can undergo hydroxylation and methylation to produce ferulic acid [284]. AP = apical, BL = basolateral

6.2. Strengths and Limitations

As compared with previous in vitro studies using batch fermentation models to assess microbial biotransformation of polyphenols, the use of the continuous multi-stage GI model in Study 1 enabled us to perform more physiologically relevant evaluation of the biotransformation patterns of polyphenols in the different colonic segments. This data was accompanied by measurements of the changes in antioxidant capacities and SCFA profiles produced by the microbial metabolites in different colonic segments. Such information might have implications for prevention or treatment of colonic diseases by designing the dietary interventions that target specific colonic regions. In addition, the use of a mixture of pure polyphenols in the absence of other food components in the present work, provided more accurate information regarding the effects of biotransformation of pure supplemental polyphenols and the interaction between phenolic compounds on their bioactivity. The identification of polyphenols and their metabolites in Study 1 and 3 was performed by LC-MS analysis, which is a powerful and accurate method for identification of phenolic compounds. Previous Caco-2 studies involving phenolic transport have relied primarily upon HPLC analysis, which have been noted to be less sensitive to detect minute changes in phenolic transport and absorption [123, 124]. As pure standards of the phenolic microbial metabolites were unavailable, the use of relative abundance in the present thesis work provided quantitation based on the changes in relative amounts of each targeted compound following their metabolism involving the simulated GI model and the Caco-2/HepG2 system. Once internal standards of the phenolic metabolites become available, quantification using such standards will provide a more accurate quantitation of such compounds.

Another strength of this thesis is that for the first time the anti-cancer effects of CGA were compared with its microbial metabolites (Study 2). In concert with findings obtained from Study 1, Study 2 also demonstrated bioactivities of the parent polyphenols and their secondary microbial

phenolic metabolites. In addition, important molecular features involved in cancer development were assessed as potential anti-cancer mechanisms of action of CGA and its microbial metabolites including measurement of the mtDNA content that has been sparsely studied in relation to polyphenols. This investigation led to the novel finding regarding the involvement of mtDNA depletion in anti-proliferative effects of 3-PPA. This finding is supported by previous studies indicating that the effects of polyphenols on mtDNA content in cancer cells depends on the dose and nature of the polyphenol [226, 264]. One of the limitations of Study 2 is that the molecular pathways underlying the cell cycle arrest and apoptosis were not investigated.

Part of the novelty of Study 3 was the testing of the bioavailability and biotransformation of microbial phenolic degradation by-products via the combined GI model and Caco-2/HepG2 co-culture system, which has not been done previously. An earlier study combined upper GI in vitro digestion with Caco-2/HepG2 co-culture to assesses the bioavailability of by-products of olive oil and thyme extract [63] but the effects of gut microbial biotransformation processes were not considered. Another strength of this thesis study was the use of dietary achievable concentrations of the tested polyphenols pertaining to an average daily intake of 1 g, which provided more dietarily and physiologically relevant information regarding bioaccessibility and bioavailability of polyphenols and their secondary metabolites following their digestion [15]. In addition, polyphenol digesta used for the above first pass metabolism experiment provides a better simulation of in vivo absorption, as opposed to simply dissolving individual phenolic metabolites into the co-culture medium. The use of human liver cells in the co-culture model also more closely simulates human hepatic metabolism of the tested phenolics as compared to previous studies using the rat liver cells or tissue [127, 285]. One of the limitations of Study 3 was the lack of measurement of the sulfated, glucuronidated or methylated metabolites that could be generated by

the Caco2/HepG2 system as these are primary forms of the circulating metabolites [10]. It is also important to note that the epithelium in vivo is considerably denser than the cellular layers of Caco-2 cells cultured in an insert system, which may lead to a lower in vitro permeability than in vivo [35]. In addition, the Caco-2 cells lack the intestinal mucus layer which could affect the polyphenol bioavailability [120]. Another limitation to the Caco-2 cell culture model is that the apical digest solutions were in a static state as opposed to the movement of chyme that occurs along the intestinal tract lumen. Recently, the HIEC cell model has been described as a useful non-tumorigenic model to assess post-digest intestinal metabolite transport [298]. Nevertheless, previous Caco-2 studies have shown good correlation with human in vivo studies regarding xenobiotic and nutrient bioavailability [62, 121, 132].

6.3. Considerations for Future Research

The findings from the present work provide novel information regarding the biotransformation of polyphenols in the GI tract, their transport and metabolism by intestinal and hepatic cells and their anti-cancer activity. There are, however, still many unanswered questions that could be further investigated considering the following insights from the thesis studies.

Study 1: The identification of the microbial metabolites in the present work was performed using the targeted approach by searching for the known microbial metabolites. A metabolomic approach may provide more information about the unknown/non-predicted metabolites derived from the biotransformation of the tested polyphenols. Identification of a wider range of polyphenol metabolites provides a platform for further investigation of their biological activities. This approach has recently been used to characterize the phenolic profiles of urine and fecal water and investigate the inter-individual variations in the metabolism of polyphenols [299-302]. Additionally, the assessment of gut microbial catabolism of the tested polyphenols when fortified

in a food matrix context would be informative including information regarding the effects of such fortification on the antioxidant status and SCFA profiles of the digesta as well as on the gut microbiome.

The above findings are also in concert with the concept that the composition of gut microbiota affects the biotransformation of polyphenols and their subsequent biological activities. In that regard, inter-individual alterations in bacterial composition due to geographic location, intestinal disease states or medication use has been associated with altered patterns of microbial polyphenol metabolism [25]. Future gut model studies could use fecal samples from individuals with GI diseases such as irritable bowel syndrome (IBS) or ulcerative colitis to study their impact on polyphenol biotransformation and how alterations of gut microbiota composition by polyphenol treatment could affect their biotransformation and subsequent health effects. The testing of the metabolism of polyphenols derived from potatoes using gut microbiota of individuals from communities who consume polyphenol-rich potatoes as an important food staple could be studied to investigate as to whether their microbiota may have evolved to more efficiently catabolize potato-based polyphenols as compared to other populations. Additionally, the protective effects of polyphenols on gut microbiota against antibiotic and environmental toxicants could be investigated using the GI system. Finally, in order to improve the utility of in vitro digestion systems towards possible clinical applications, there is a need for more in vitro-in vivo correlation studies.

Study 2: The use of the physiologically relevant concentrations of the tested phenolics in Study 2 results indicate their potential applicability for further investigations in human population cancer studies as future directions could focus on the inter-relationship of diet, plasma concentrations of the tested microbial compounds and colorectal cancer risk. Phenolic compounds may also act in synergy with chemotherapy drugs to increase their efficacy and to reduce their side

effects [14]. In that regard, an interesting future direction would be to test CGA and its metabolites in combination with chemotherapy drugs on cancer cells. Additionally, future investigations could perform more in-depth mechanistic assessments on anti-cancer effects of the tested compounds, particularly with regards to identification of key molecular components involved in cell cycle arrest and apoptosis induction. Such measurements could include cyclin proteins, the CDKs, PARP, anti- and pro-apoptotic proteins such as p53, Bcl-2, Bax and cytochrome c. In addition to anti-proliferative activities of the test compounds in Caco-2 cells, it is unclear how these compounds may act upon non cancer-derived intestinal cell lines. Therefore, testing the effects of these compounds in control intestinal cells such as the recently established human small intestinal epithelial cell (HIEC) line [298] could provide additional information regarding their effects on non-tumorigenic cells.

Study 3: Future studies could involve co-culturing Caco-2/HepG2 cells with mucus-secreting cells such as HT29-MTX cells [120] as bioavailability of some polyphenols may be affected by the intestinal mucus layer [120]. In that regard, FA transport has been shown to be reduced and dihydroferulic acid transported more efficiently than the dihydrocaffeic acid in the presence of mucus [303]. In addition, the use of non-tumorigenic intestinal cell lines such as the newly available HIEC cell line [298] or the co-culture system could provide further information about polyphenol bioavailability. Likewise, the human HepaRG cells could be utilized in the co-culture model as this cell line has been described to provide functional protein expression for transporters and phase I and II metabolizing enzymes similar to primary human hepatocytes [304, 305]. Additionally, identification of the metabolites in the intracellular fraction in the co-cultured cells will provide further understanding of the involvement of intestinal and hepatic polyphenol metabolism. Future in vitro and in vivo studies could also be directed to the study of the biological

effects of polyphenol metabolites in different cell lines, tissue and organ systems, which are exposed to much lower concentrations of the microbial phenolic metabolites than observed in the GI tract. In that respect, the conjugated form of post-digest microbial phenolic metabolites can be considered due to their conjugation by intestinal and hepatic cells before exerting their effects in target cells [98]. Considering the poor bioavailability of polyphenols, future work could explore polyphenol delivery systems to enhance their in vivo bioavailability for chemopreventative and therapeutic purposes. There has been growing interest in the development of various delivery systems to improve the bioavailability of polyphenols and their consequent health outcomes [306]. The delivery systems can be designed to release the compound at a specific tissue location or to control and prolong its release during digestion [307]. A wide range of technologies and formulations have been applied to develop a novel delivery systems including polymeric nanoparticles, nanocapsules, liposomes, phytosomes, nanoemulsions, microspheres, transferosomes, and ethosomes [307]. In addition, identification of specific synergistic interactions among polyphenols has been suggested as an approach to improve polyphenol bioavailability via multiple mechanisms such as modulating the enzymatic activities of gut bacteria, gut epithelial cell transporters or phase I and II enzymes [308]. This is a newly emerging research area and so many issues need to be resolved including the improvement of the carrier materials [307]. The use of the combined in vitro digestion and cell culture model described in this thesis could also be used as a screening tool to assess various delivery systems developed to improve polyphenol bioavailability.

6.4. Conclusions

In summary, this dissertation has utilized a human simulated gut digestion model together with intestinal and hepatic cell cultures to study the gastrointestinal biotransformation of

polyphenols commonly present in potato, an important component of the human diet (CGA, CA, FA and RU), and the bioavailability and bioactivities of the generated microbial metabolites. The results obtained from this work have demonstrated the extensive biotransformation of the above polyphenols following their microbial metabolism in the GI model, which has led to generation of varying profiles of secondary metabolites with differing bioactivities in different colonic segments. The results from the present thesis also demonstrated the key potential role of the secondary phenolic metabolites in gut health, particularly via their additive or synergistic interactions in terms of anti-proliferative effects on colonic tumor cells. This data coincides with recommendations to increase fruit and vegetable consumption that is associated with higher polyphenol intake and decreased risk of colorectal cancer [50]. The results from the co-culture study also highlighted the role of hepatic metabolism in the generation of some of the circulating microbial phenolic metabolites despite their relatively poor bioavailability from the GI tract. The use of in vitro systems described in this thesis provides a feasible and cost-effective platform for screening approaches and understanding of the complex processes involved in polyphenol bioaccessibility, bioavailability and bioactivity towards improved human health.

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