The role of Nrf2 in the antigenotoxic potential of raspberry metabolites.

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CONTENTS

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SUMMARY

Colorectal cancer (CRC) is the third most prevalent cancer worldwide; as it has an increasing incidence rate in westernised countries. This correlation has been attributed in part to poor diet. In contrast, individuals with high consumption of (poly)phenol rich foods such as fruit and vegetable have a reduced risk of CRC. Berries are a particularly rich source of (poly)phenols and in recent years have been associated with a range of anticancer activities. When consumed, these berries undergo a range of structural transformations as they are digested, which could alter their potential bioactivity. The aim of this thesis was to develop an *ex-vivo* model with which to produce physiologically relevant samples that represent the colonic contents post-raspberry consumption. Eleven post-berry ileal samples were collected following a human feeding study and subsequently *in vitro* fermented. The potential antigenotoxic activity of these samples was assessed in a model of normal colonic epithelium (CCD 841 CoN) and a cell model of colonic adenocarcinoma (HT29). The anticancer activity, previously demonstrated by crude berry extracts and isolated (poly)phenols, was retained following digestion. Both post-berry ileal samples and post-berry ileal fermentate samples decreased H_2O_2 induced DNA damage. Following this discovery, it was important to attempt to identify the mechanism by which this antigenotoxic activity occurs. There was sufficient previous evidence to suggest that (poly)phenolic compounds have the potential to induce the nuclear factor-erythroid 2-related factor 2 (Nrf2)/ antioxidant response element (ARE) pathway and this warranted further investigation. The post-berry IFF samples showed the ability to significantly induce the mRNA expression of Nrf2 and its downstream targets heme-oxygenase 1 (HO-1), N-acetyltransferase (NAT), NADPH quinine oxidoreductase 1 (NQO-1). In addition to these findings a novel raspberry seed derived triterpenoid was identified within the post-berry ileal fluid samples. This compound was present in the original

raspberry seeds and survived *in vivo* digestion, it therefore was pertinent to assess its antigenotoxic capacity. As with the other raspberry metabolites investigated in this study, the triterpenoid rich fraction (TRF) significantly decreased H_2O_2 induced damage in CCD 841 CoN and HT29 cells, in conjunction with the up-regulation of HO-1 and NQO1.

In conclusion, this thesis has determined that the berry metabolites produced following *in vivo* digestion and *in vitro* fermentation retain the antigenotoxic activity previously demonstrated by crude berry extracts. This activity in maybe part due to the activation of the Nrf2/ARE pathway. These findings further expand our understanding of the role of berry metabolites as potential dietary anticarcinogenic agents.

ABBREVIATIONS

4HBA	4-Hydroxybenzoic Acid
3PPA	3-Phenylpropionic Acid
3HPPA	3-(3-Hydroxyphenyl)propionic Acid
ACF	Aberrant Crypt Foci
AOM	Azoxymethane
ANOVA	Analysis of Variance
APC	Adenomatous polyposis coli
Apc$^{min/+}$	Adenomatous polyposis coli (Multiple Intestinal Neoplasia)
ARE	Antioxidant Response Element
BA	Benzoic Acid
CARE	Colon-Available Raspberry Extract
COX-2	Cyclooxgenase-2
CRC	Colorectal Cancer
DCA	Deoxycholic acid
DCC	Deleted in Colorectal Carcinoma
DMEM	Dulbecco‟s Modified Eagle Medium
DNA	Deoxyribonucleic Acid
ECACC	European Collection of Cell Cultures
F344	Fischer 344 rat
FAP	Familial Adenomatous Polyposis
FBS	Foetal Bovine Serum
GAE	Gallic Acid Equivalents
GC	Gas Chromatography
GCL	Glutamate-cysteine ligase
GST	Glutathione S-transferase
HO-1	Heme oxygenase 1

H_2O_2	Hydrogen Peroxide
HBA	Hydroxybenzoic Acid
HPRT	hypoxanthine phosphoribosyltransferase
LC	Liquid Chromatography
MS	Mass Spectrometry
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
NQO1	NAD(P)H:quinone oxidoreductase 1
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
SPE	Solid Phase Extraction

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The subsequent compositional LC-MS analysis, metabolomic investigation and GC-MS analysis of fermented berry extract was carried out under the supervision of Dr. Gordon McDougall.

Chapter 1: General Introduction

Colorectal Cancer:

Colorectal cancer (CRC) is one of the leading causes of mortality and is responsible for over 500,000 deaths a year globally. It accounts for 9% of all cancer incidence, the third most common cancer for men and second in females worldwide (1-3). Incidence rates of CRC within the USA, Australia, New Zealand and Western Europe have stabilised in recent years. However, countries in Eastern Europe, Asia and South America have seen rapid increases CRC incidence and mortality (4, 5). CRC remains an increasing burden upon the global community that necessitates continued research.

Anatomy and Morphology of the Colon:

The colon is the last section of the digestive tract, extending a total of 1.5m from the caecum to the rectum and is comprised of 6 distinct regions; the caecum, ascending colon, transverse colon, descending colon, sigmoid colon and rectum. Known as the right side of the colon, the caecum, ascending colon and transverse colon are responsible for the absorption of water, nutrients and the products of bacterial fermentation. (6) The primary function of the left side which consists of the descending colon, sigmoid colon and rectum, is the formation, storage and excretion of faecal matter (7). The wall of the colon consists of several layers including; epithelium, lamina propria, muscularis mucosa, submucosa and muscular layer. There are four main cell types present within the colon; the epithelial colonocytes, mucus-secreting goblet cells, peptide hormone-secreting endocrine cells and Paneth cells. The simple columnar epithelial cells form a single highly polarised layer with intricate cell-to-cell junctions. These are responsible for the absorption of nutrients and water from within the lumen, a process facilitated by a dense capillary network. Unlike the small intestine the colon does not contain villi but rather is characterised by colonic crypts. The crypts are

approximately 50 cells deep with 1-10 multipotent stem cells located at the base (8). These stem cells generate clonal daughter cells which reside in the lower third of each crypt, ensuring they are removed from any potentially toxic colonic contents, therefore reducing the exposure to mutagenic substances. Rapid replication and division by the stem cells drive migration and differentiation of the daughter cells as they travel up the crypts and join the epithelial lining, until they under go apoptosis and are shed from the lumen. This cycle from replication to apoptosis last approximately 5 days, this rapid turn over ensures limited contact with any mutagenic substances with the colon (7).

Tumorigenesis:

Colorectal cancer (CRC) is the result of a complex multistep process, described by Fearon and Vogelstein, that follow the aberrant crypt foci-polyp- adenoma-carcinoma sequence of events that develops due to the accumulation of genetic mutations (Figure 4) (9). With cases of sporadic cancer these mutations develop over time and can be a result of aging or exposure to exogenous substances. The disease originates in the epithelial cells of the colon and is the result of the alteration in a range of genes. Mutation of the APC (Adenomatous polyposis coli) gene has been identified in more than 75% of all sporadic CRC and causes abnormal cellular adhesion, proliferation and migration (10). Disruption of the gene coding for this tumour suppressor can result reduced cellular migration within the colonic crypt causing exposing already mutated cells to come in sustained contact with toxic substances, enable further genetic alterations and enable the formation of a polyp (11).

Secondary mutations which include; K-*ras*, DCC (deleted in colon cancer), BRAF (v-Raf murine sarcoma viral oncogene) and the loss of chromosome 18q promote the progression from a polyp to adenoma (12-16). A final late occurring event is the mutation in p53, a

tumour suppressor gene found on chromosome 17p (17). In normal conditions p53 is a transcriptional activator for several genes which regulate the cell cycle in response to DNA damage (18). The inactivation of this gene can therefore result a loss of cycle control and unchecked DNA damage, this is associated with the progression from adenoma to carcinoma (19). It is not necessarily the order in which these mutations occur but rather the accumulation over time that results in the development and progression of CRC (20, 21).

Risk factors for colorectal cancer

CRC is a global disease but there is considerable variation between incidence rates and mortality statistics worldwide (23). The highest rates are seen within "westernised" countries with the developed world accounting for over 66% of all CRC cases (24). These statistics combined with the knowledge that only 5-10% of CRC are due to inherited conditions, such as hereditary nonpolyposis colorectal cancer (HNPCC) or familial adenomatous polyposis (FAP), suggests that environmental factors play a key role in the development of CRC (25). The correlation between environmental risk and CRC can be seen first-hand in studies which assess the incidence rate in migrants and their children. Those individuals who move from low-risk to high risk countries see the incidence of the first-generation trend towards to the higher rates of the host country (26). Therefore, a portion of CRC cases are considered theoretically preventable as they are induced by a variety of modifiable causes (27). As with other major cancers a range of lifestyle choices including; poor diet, obesity, alcohol consumption and exercise are contributing factors to CRC development. Diet has the ability to strongly influence the risk of CRC with poor food habits resulting in increased cancer burden. Those diets high in fat and meat consumption have been linked to an increased rate of CRC (28, 29). There a few underlying mechanisms by which meat could initiate

carcinogenesis, but the presence of heme iron and production of heterocyclic amines and polycyclic aromatic carbons following cooking at high temperatures are most likely to induce DNA damage (30-32). Conversely diets with a high fibre content are associated with a decreased risk of colorectal cancer. Fibre can act in several ways to reduce CRC which includes increasing the stool bulk, therefore diluting faecal carcinogens and limiting contact with the epithelial cells. It can also bind to the potentially carcinogenic bile acid or alternatively is broken down into short-chain fatty acid such as butyrate which have the potential to inhibit carcinogenesis (33, 34). Diet rich in fruit and vegetables have also been linked to a reduction in CRC incidence. These plant-based foods are a source of fibre, vitamins and a range of non-nutrients. There had been a considerable interest in the anticancer potential of these compounds in particular phytochemicals and (poly)phenols.

Anticancer effects of berries:

Berries are rich source of (poly)phenols which can contain up to 1000mg polyphenols per 100g fresh weight of fruit (FW) (37, 38). There has been considerable research into the potential health benefits of these fruits with much focussed on the anticancer properties their polyphenolic constituents (39, 40). Recent evidence suggests that a large portion of theses phenolic metabolites have poor bioactivity, therefore pass through the small intestine unabsorbed and come into contact with the colonic epithelium where they can exert anticancer effect (42). A variety of *in vitro* and *in vivo* studies have shown the anticancer potential of berry (poly)phenols and their metabolites upon models of CRC and as a consequence these compounds have been considered as potential putative dietary agents against carcinogenesis (43).

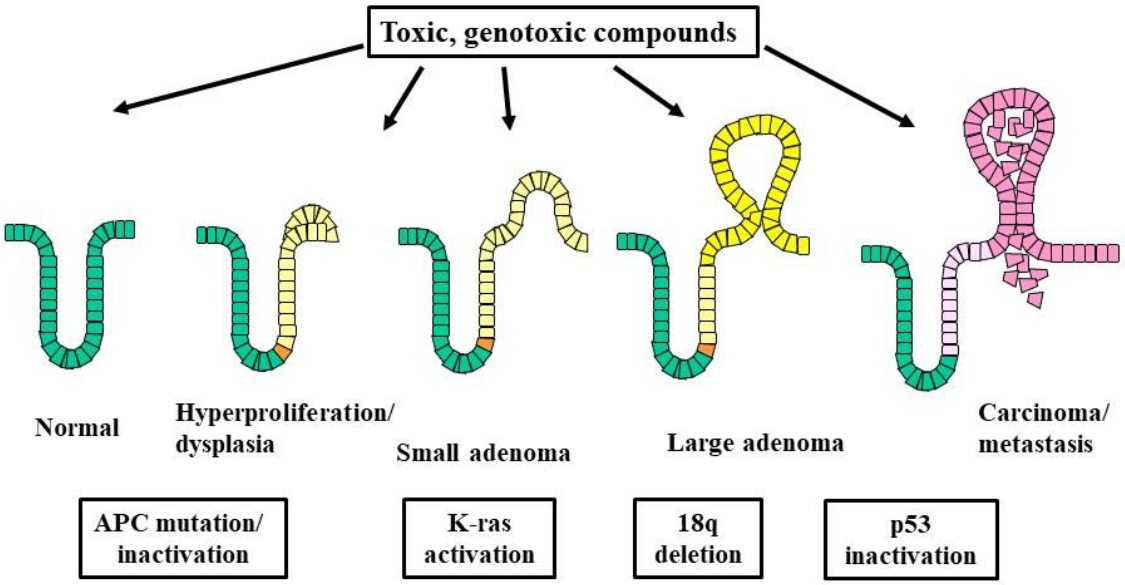

Figure 1: Multistep pathway of colorectal cancer development. * adapted from Gill *et al.* [(22)]

Rational of thesis:

There is a significant quantity of *in vitro* and *in vivo* evidence which describes the anticancer effects of berries. However, these investigations have used crude berries, berry extracts or berry-derived compounds and have not taken into consideration the compositional changes which occur due to digestion and colonic fermentation and how this may impact upon bioactivity. The rational of this thesis was to develop physiologically relevant samples that accurately represented colon-available berry metabolites, to subsequently assess the antigenotoxicity of compound and further elucidating a potential molecular mechanism of action within *in vitro* models of colon carcinogenesis.

Chapter two examines the current literature to evaluate the role of berries and berry metabolites within colorectal cancer in both *in vitro* models and human intervention studies. This review of recent studies showed that berry metabolites produced via *in vitro* digestion maintain the antigenotoxic potential seen with crude extracts, as well as identifying a potential molecular mechanism which induces this anticancer activity.

Chapter three describes the human feeding study and method of *in vitro* fermentation used to produce the biologically relevant samples for this study. The compositional changes which occurred during digestion and bath culture where analysed using HPLC LC-MS and GC-MS.

Chapter four investigated the antigenotoxic potential of post-berry ileal fluids, post-berry ileal fluid fermentate and 4 individual phenolic compounds upon normal colonic (CCD 841 CoN) and adenocarcinoma cell lines (HT29). The comet assay was used to assess the ability of these sampled to decrease H_2O_2 induced damage.

Chapter five focuses on the role of the Nrf2/ARE pathway in regulating antigenotoxic activity of berry metabolites. The effect of IFF samples and individual phenolics upon the expression Nrf2 and two of its downstream targets was analysed using qPCR.

Chapter 6 investigates the anticancer potential of a raspberry-derived triterpenoid-rich fraction. Using the comet assay and qPCR analysis the bioactivity of this novel extract was assessed.

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Chapter 2: Literature Review: "Antigenotoxic potential of berry and berry metabolites: *in vitro* and *in vivo* models of Colorectal Cancer"

Introduction:

Diets containing a plentiful supply of fruit and vegetables contribute to a range of health benefits and can play a key role in reducing the risk of CRC (1-4). These plant-based foods contain a selection of compounds including; fibre, vitamins, micronutrients and phytochemicals which have demonstrated protective and preventative effects upon the human body (5-7). They have been linked with the prevention of various disease states including cardiovascular disease, neurodegeneration, diabetes and cancer (8, 9). (Poly)phenols are a diverse subgroup of non-nutritive phytochemicals which consist of over 8000 individual compounds (10, 11). These contain an aromatic ring to which one or more hydroxyl groups are attached but are classified by the number and orientation of their carbon atoms and are initially categorised as either flavonoids or non-flavonoid (12). Flavonoids can be identified by the presence of 15 carbons with two phenolic rings (Ring A and B) attached with a 3-carbon bridge forming a C6-C3-C6 backbone. Flavonoids, the most abundant (poly)phenols in our diet, can be divided into several groups; flavonols, flavones, flavanones, isoflavones and anthocyanidins (13). Non-flavonoid (poly)phenols include phenolic acids, hydroxycinnamates and stilbenes (14). These compounds are found at high concentrations (100-100mg/100g) in a range of beverages; including tea, coffee and red wine, in fruit; such as apples, pomegranates and berries; in vegetables particularly; carrots, legumes, onions and tomatoes, as well as present in a variety of herbs (15).

Berry (Poly)phenols:

Berries are a commonly consumed source of (poly)phenols these include; blackberries (*Rubus* sp.), blackcurrants (*Ribes rugrum*), cranberries (*Vaccinium macrocarpon*), blueberries (*Vaccinium corymbosum),* raspberries *(Rubus ideaus*) and strawberres *(Fragaria ananassa*) which can contain up to 1000mg polyphenols per 100g fresh weight of fruit (FW) (16, 17) whose phenolic composition differs from species to species but are comprised of anthocyanins, ellagitannins, flavanols and cinnamic acids (18). Blueberries for example contain anthocyanins (54%) including; delphindin, petunidin, malvidin derivatives; flavonols (11%) in the form of quercetin derivatives, and cinniamic acids (30%) such as chlorogenic acid, producing total (poly)phenol content between 500-800mg/100g FW (19). In contrast raspberries are primarily comprised of cyanidin-based anthocyanins (40%), ellagic acids and ellagitannins (44%) including sanguiin and lambertianin with a total (poly)phenol content of 200mg/100g FW (20). However the phytochemical composition of these berries can be influenced by a range of environmental factors and will differ from growing season to growing season (21, 22). Different culitvars will also produce compositional variants, for example within raspberries the total anthocyanin content of 10 raspberry cultivars ranged from 37.5-325mg/100g FW, with total phenolic content from 118-208mg/100g FW (23). Irrespective of the precise (poly)phenolic content, berries have been associated with a range of beneficial properties including; antioxidant, anti-inflammatory, immunodulatory and chemopreventive potential (24). Previous studies have illustrated the putative anticancer activity berries have upon models of CRC, with (poly)phenols inducing free radical scavenging, activating the Nrf2 pathway and modulating the signalling pathways involved in DNA repair, cell proliferation, apoptosis and invasion (25-28). This review aims to assess the *in vitro* and *in vivo* evidence for anticancer activities of berry polyphenols in relation to DNA damage and Nrf2 activity.

DNA damage:

Exposure to carcinogenic compounds can cause DNA damage and initiate the process of carcinogenesis. In CRC if this damage is permanently incorporated into the genome it can begin the multistep process of cancer development resulting in the activation of oncogenes e.g. KRAS, PIK3CA and loss of tumour suppressors e.g. SMAD4, APC, TP53 (29, 30). These mutations can disrupt cell signalling, cell proliferation, cell cycles resulting in increased cellular invasion and eventual metastasis (31, 32). Reactive oxygen species (ROS) which are generated under normal cellular conditions and produced during the process of metabolism in the form of superoxide anions (O_2^-) or hydroxyl radicals (OH^-), activate a range of cellular receptors and signalling pathways responsible for proliferation, differentiation and cell survival. Alternatively ROS can place cells under oxidative stress and result in the oxidation DNA bases, DNA strand breaks, formation of DNA lesions, disruption of the cell membrane and lipid peroxidation (33). This activity contributes to the development and progression of CRC and as a consequence greater emphasis has been placed on detection and reduction of DNA damage (34, 35).

As can be seen in Table 2 a variety of *in vitro* laboratory assays are utilised to assess the extent of cellular DNA damage. The comet assay, or single-cell gel electrophoresis is a rapid and relatively sensitive technique to identify DNA strand breaks within individual cells. Named due to the distinctive structure which forms as a spherical head of undamaged DNA and an elongated tail of damage DNA, several variations of this protocol exist (36). Either a genotoxic agent, such as hydrogen peroxide is used to induce oxidative damage or alternatively a lesion-specific enzyme such as endonuclease III (EndoII) or formamidopyrimidine DNA glycosylase (FPG) can excise DNA at the site of oxidised pyrmindines or oxidised purines respectively (37-39). Alternatively rather than measure DNA damage the dichlorofluorescein (DCF) assay can be used to detect the levels of oxidative

stress within cells. Cells are treated with 2’7’-dichlorofluorescin-diacetate (DCFH-DA) which readily crosses the cellular membrane, in the presence of intracellular oxidative challenge this is converted to the highly fluorescent DCF (40). These *in vitro* studies can utilise a range of colon cell lines, at different disease states see Table 1.

Table 1: Commonly used colonic cell lines

Name	Cell Type	Disease Status	Clinical Origin	Doubling time	Mutations critical to CRC development [100-101]
Caco-2	Epithelial	colorectal adenocarcinoma	72yr old Caucasian male	62h	TP53
CCD 841 CoN	Epithelial	Normal	21week old female fetus	96h	–
CCD-18Co	Fibroblast	Normal	2.5 month old Black Female	72h	–
DLD-1	Epithelial	Dukes' type C, colorectal adenocarcinoma	Adult male	48h	TP53, KRAS, PIK3CA, MSI, CIMP+
HCT 116	Epithelial	Dukes' type D, colorectal carcinoma	48yr old male	18h	KRAS, PIK3CA, MSI, CIMP+
HCT-15	Epithelial	Dukes' type C, colorectal adenocarcinoma	Male	20h	TP53, KRAS, PIK3CA, MSI, CIMP+
HT29	Epithelial	Dukes' type C, colorectal adenocarcinoma	44yr old Caucasian female	24h	TP53, BRAF, CIMP+
NCM460	Epithelial	Normal	68yr old male	26h	–
SW480	Epithelial	Dukes' type B, colorectal adenocarcinoma	50yr old Caucasian male	38h	TP53, KRAS

Anticancer Activity of Berry (Poly)phenols

Previous *in vitro* studies have demonstrated the anticancer properties of a variety of berry extracts and berry derived (poly)phenolics, including the modulation of pathways involved in cell proliferation, apoptosis and cell signalling (41). However within the context of this review will concentrate on the role the phenolic compounds have upon oxidative stress and DNA damage (Table 1). Most of these investigations have used the comet assay to show assess how berry components reduce DNA damage, for example Olejnik *et al*. have shown the potential of both freeze dried elderberry (EB), *Sambucus nigra* L, and blackcurrant (BC), *Ribes nigrum* L, to induce antioxidant and antigenotoxic activity (42, 43). Both extracts decreased H_2O_2 induced DNA damage in a dose dependent manner within their respective cell lines. When at 0.01mg/ml, 01mg/ml and 1mg/ml EB extract decreased % tail DNA by 12, 20 and 30% respectively within NCM460 cells challenged with 100μM H_2O_2. BC extract followed a similar pattern in Caco-2 cells, a maximum reduction in oxidative damage was observed (27%) with 1mg/ml concentration. This antigenotoxic action corresponded with a reduction of intracellular ROS levels, which was detected using DCF assay. Treatment with concentrations of 0.01, 0.1 and 1mg/ml reduced DCF fluorescence intensity; EB decreased this oxidative stress by 13, 20 and 40% while BC resulted in 29, 43, 48% reductions. The results from these papers indicate that both EB and BC extracts have anticancer potential. Although this activity has been linked to the (poly)phenol rich nature of the original berries, however HPLC-DAD-ESI-MS analysis has highlighted the compositional differences between both samples. EB extract contained a total of 9 distinct anthocyanins which accounted for 93.4% of total (poly)phenol content and were predominantly glycosylated cyanidin and pelargonidin compounds. Anthocyanins contributed to 85% of all (poly)phenols within the BC extract, a total of 14 compounds primarily consisting of delphinidin and cyanidin extracts, with the remaining content comprised of hydroxycinnamic

acids, flavan-3-ols and flavonols. This evidence suggests that a variety of phenolic compounds can activate this pathway of antigenotoxicity and the presence of multiple (poly)phenols may be of greater benefit (42, 43). This combined effect was further explored with the actions of bilberry extract (BE) (*Vaccinium myrtillus* L) and bilberry derived fractions upon Caco-2 cells. The original BE extract reduced intercellular ROS levels at 500μg/ml and decreased Md-induced DNA damage at 5μg/ml concentration. The phenyl carbonic acid rich fraction (PCF) was the only bilberry derived fraction which produced a reduction in DNA damage at 1.5μg/ml. The anthocyanin fraction and polymeric fraction had no significant effect upon intercellular ROS or md-induced DNA damage (44). In contrast to the protective effect of blackcurrant, elderberry and bilberry upon colon cancer cell lines, several studies have illustrated the reverse effect with greater concentrations of (poly)phenols inducing DNA damage. Several of these studies have shown that raspberry derived extracts in particular ellagitannins result in the fragmentation on DNA (45, 46). However, the studies described in Table 1 are not of physiological relevance as they do not address the effect of digestion and colonic fermentation upon (poly)phenols (44-52). Recent studies have placed a greater emphasis upon role of the metabolic changes which occur to (poly)phenols as they pass through the small intestine, enter the colon and interact with the microbiota found within this region (53-54).

After ingestion of berries the (poly)phenolic components found with the fruit undergo a variety of structural modifications. Human feeding studies have been used to determine the bioavailability of berry (poly)phenols. Analysis of urine and plasma samples collected after ingestion of berries has demonstrated that the parent compounds present in the fruit have low bioavailability. A raspberry intervention completed by Ludwig *et al.,* identified 8 anthocyanins, 2 ellagic, 3 ellagitannins, 1 hydroxycinnamate and 1 phenolic acid, with a total concentration of 553μM polyphenols within their Glen Magma raspberries (55). UHPLC-

MS-SRM analysis of urine samples, collected post-digestion, detected 27 compounds which were metabolites of parent anthocyanins (benzoic acid derivates, phenylacetic acids, phenylpropanoid derivatives) and ellagitannins (urolithins and ellagic acid derivates) which only accounted for 15% of the original (43.9±8μM) of the anthocyanin intake and 7% (17.9±4μM) of the ellagitannin intake. Combined with evidence from McDougall *et al.* whom identified a range of phenolic compounds present in the ileostomy fluid collected following consumption of 300g of raspberry puree, indicates that the majority (40% of phenolic intake) of raspberry polyphenols reach the large intestine (20). These compounds are subjected to further degradation as they are acted upon by colonic microflora which breakdown the flavonoid C-ring structures of the anthocyanins and ellagitannins are converted to ellagic acid and urolithins (14, 56, 57). To accurately assess the bioactivity of berry derived (poly)phenols upon colonocytes, it is important to utilise biologically relevant samples, which contain phenolic compounds that are readily available within the colon.

The complexity of human digestion has been modelled in vitro using a number of approaches, these can be either be static where the physical movements of digestion (mixing and hydration) are not simulated, or dynamic where this mechanical processes are mimicked (58). Static models of *in vitro* digestion use the addition of enzymes (pepsin) and bile salts in conjunction with acidic pH (HCl) to simulate the processes in the stomach and small intestine (58). Generally based on the methodology developed by Miller *et al.* many adaptations can be made to customise this assay for a specific purpose for example to examine the bioavailability of a variety of phytochemicals including; phytosterols, (poly)phenols and carotenoids. (59-62). These comparatively simple models allow for high throughput with multiple samples run at the same time. Dynamic models of *in vitro* digestion can simulate the changes in enzyme concentration, pH and introduce the peristaltic movements that occur within the GI tract. Many of these systems involve multiple compartments as with the TNO

gastrointestinal model (TIM-1) which has separate vessels to represent the stomach, duodenum, jejunum and ileum (63). With TIM-1 the changes in digestive conditions are controlled by computer software, allowing protocols to be developed for a range of purposes i.e. to differentiation between infant, adult and elderly digestion as well as between humans and animals (64, 65). This simulation has been expanded in TIM-2 which also mimics the physiological conditions within the large intestine, therefore providing a combined model of *in vitro* digestion and *in vitro* fermentation (66). SHIME or (Simulator of the Human Intestinal Microbial Ecosystem), is another example of a multi-chamber model of *in vitro* digestion and fermentation, comprised of individual vessels to represent the stomach, small intestine, ascending colon, transverse colon and descending colon (67). Both models require inoculation with faecal microbiota to introduce colonic microflora into the systems (66, 67). Although these dynamic GI models are more expensive, with more complex set-up and limited throughput they are considered a more accurate representation of the *in vivo* digestion (68). Both types of model of *in vitro* digestion produce samples whose composition will differ greatly from the original crude extracts and more closely mimic the contents found in the colon.

A variety of the methods described above have been utilised to produce physiological relevant samples which mimic the digestion of berries, the antigenotoxic potential of which are described in Table 2. The studies investigating elderberry and blackcurrant bioactivity which were conducted by Olejnik *et al.* and previously described above also examined the potential of *in vitro* digested berries (42, 43). In both instances the protective effects demonstrated by the original berry extract persisted following simulated digestion. With the elderberry digest (EBD) only 0.1 and 1mg/ml showed this effect, decreasing H_2O_2 DNA damage in NCM460 cells by 25 and 46% respectively, which improved upon the reduction

seen with pure extract (20% and 37%). A similar pattern was seen with the blackcurrant digest (BCD) at 1mg/ml decreasing DNA damage in Caco-2 cells by 54%. In contrast the capacity of berry sample to reduced intercellular ROS was reduced following digestion; 1mg/ml EBD resulting in 22% decrease compared to 37% in the original extracts, while 0.1mg/ml BCD reduced ROS levels by 28% compared to the 43% decrease with the original. These differences are probably due to the differences in (poly)phenolic composition. Following digestion of both berries there was a significant reduction in the concentration of anthocyanins present in each sample, this is a result of (poly)phenols being broken down into much simpler compounds. Chen *et al.* have used alternative assays to demonstrate the persistence anticancer and antigenotoxic activity of raspberry and blackberry extracts following digestion (69-71). Hoescht nuclear staining illustrated the ability of raspberry digest (RD) and blackberry digest (BD) to decrease ethyl carbamate (EC) induced DNA fragmentation within Caco-2 cells. RD and BD samples also decreased the level of intracellular ROS, reductive oxidative damage to the mitochondrial membrane and moderated EC depletion of cellular glutathione (70, 71). *Brown* et al. used *in vitro* digestion in conjunction with *in vitro* fermentation (IVF) to produce lingonberry fermentate samples which retained the ability to reduce (50% decrease) H_2O_2 induced DNA strands breaks following fermentation. However, this was a decrease from the *in vitro* digested (IVD) lingonberry which resulted in a 90% following pre-treatment of HT29 cells with 50μg/ml GAE concentration (72). This variation can be attributed to the changes in (poly)phenolic content with total phenol content of IVD sample equal to 670±39μg/ml while the phenol content for IVF samples was 158±18μg/ml. However, the IVD samples was primarily comprised of cyanidin and quercetin derivates while IVF contained simple phenolic compounds including 4-Hydroxybenzoic acid, phenylacetic acid and 3-(Phenyl)propionic acid. Based on the results described within Table 2 (42, 43, 69-75), it is possible to conclude

that although the (poly)phenolic content within berries underwent significant compositional changes due to digestion and fermentation these colon available extracts still maintain anticancer activity.

Human intervention studies or human feeding studies have been utilised in two different ways to determine the protective effects. Most studies use systemic markers that are relevant to cancer studies, for example peripheral blood mononuclear cells (PBMCs). These consist of a range of lymphocytes (T-cells, B-cells and Natural-killer cells) as well as monocytes, cells of the innate and adaptive immune systems which have a single round nucleus (76). Several studies have both demonstrated the ability of blueberries to offer antioxidant protection against endogenous and oxidative induced DNA damage (78, 79). Ten volunteers on the study designed by Del Bo *et al.* consumed either 50g of blueberry puree or 50g of a control jelly, blood samples were collected before consumption then 1, 2 and 24hr following consumption (78). PBMCs were then separated from the blood challenged with H_2O_2 and a comet assay performed. Blood collected 1hr after ingestion of the blueberry puree decreased DNA damage from 52% to 43%, when compared to the control jelly. However, this effect was transient and blood PBMCs from later time points showed no protective activity, nor did the treated group demonstrate any reduction in FPG-sensitive sites (78). Alternatively Ferk *et al.* assessed the antigenotoxic potential following the consumption of an individual phenolic, gallic acid (GA). Three days of intervention with 12mg GA dissolved in water, resulted in a significant decrease in the Endo III-sensitive sites and FPG-sensitive sites in the lymphocytes collected post study. H_2O_2 induced DNA damage and intracellular ROS levels were both significantly reduced following GA consumption (80). Although these investigations effectively demonstrate the antigenotoxic potential in the compounds, they are not specifically relevant to CRC cancer (77-81). An alternative to the *in vitro* models of digestion is collecting samples from ileostomists following the consumption of berries. These

human feeding studies generally collected 2 ileal samples, 1 pre-consumption and 1 post-consumption of the foodstuff under investigation to allow for compositional analysis these samples can then be used like the original berry extracts to treat colonocytes (55, 76). Brown *et al.* were able to use this method to compare *in vitro* digested (IVD), *in vitro* fermented (IVF) and *in vivo* lingonberry samples (73). One male ileostomist, provided a pre-berry or 0h ileal fluid sample prior to consuming 150g of pureed lingonberries, a secondary post-berry sample was collected 7hr after feeding. Only 3% of the original anthocyanin content of the berries was contained with the 7hr ileal fluid sample. Although greatly reduced in concentration (6.6±0.6μM in comparison to 210±6μM) a total of 7 of the original 10 anthocyanins where still present within the ileal fluid. Total (poly)phenol content followed a similar pattern with a reduction from 943±115μM to 446±65μM. The post-berry ileal fluid at 200μg/ml GAE significantly reduced H_2O_2 induced DNA damage from 50% to 36%. This was a greater concentration than was required for IVD and IVF lingonberry samples to reduced DNA damage, 50μg/ml GAE and 16μg/ml respectively, however the ileal fluid is a true representation of the compounds which would enter the colon and therefore is a more accurate depiction of potential anticancer (82). These studies which use ileal fluid to treat *in vitro* models accurately represent the contents which pass from the ileum into the large intestine, but they do not consider the effect colonic fermentation has upon the (poly)phenols. An *ex-vivo* model which incorporates collection of ileal fluid post-berry consumption and subsequently *in vitro* ferments these samples would produce the most physiologically relevant samples.

Table 2: Effect of berry extracts on models of DNA damage.

Berry or berry constituent	Colonocyte cell line	Dose and Duration	Assay	Effect	Observation	Reference
Delphinidin	HT29	1, 10, 50, 100µM; 30min, 1h	Antigenotoxic activity (Comet Assay) Intracellular ROS (DCF assay)	+* +*	↓ ATX-II induced DNA damage by ~75% after 100µM and 30min. ↓ ROS levels after 1hr incubation with concentrations of 1, 10 and 100µM.	(50)
Elderberry (*Sambucus nigra*)	NCM460 (non-transformed, non-tumorigenic colon cell line)	0.01, 0.1, 1, 10mg/ml; 1hr	Antigenotoxic activity (Comet Assay) Intracellular ROS (DCF assay)	+ * +*	↓ in H_2O_2 induced DNA damage after co-incubation with non-digested extract at 0.01 (↓12%), 0.1 (↓20%) and 1mg/ml (↓37%). ↓ in ROS after 0.01, 0.1 and 1mg/ml by 13, 20, 40%.	(42)
Blackcurrant (*Ribes nigrum*)	Caco-2	0.01, 0.1, 1 and 10mg/ml; 30min	Antigenotoxic activity (Comet Assay) Intracellular ROS (DCF assay)	+* +*	↓ in H_2O_2 induced DNA damage in a dose dependent manner, 1mg/ml = 27% reduction in DNA damage ↓ROS levels after 0.01, 0.1 and 1mg/ml by 29, 43 and 48%.	(43)
Bilberry (*Vaccinium myrtillus*.) extracts- Bilberry Extract (BE), Phenyl carbonic acid rich fraction (PCF), Anthocyanin fraction (AF), Polymeric fraction (PF)	Caco-2, HT29	1.5-500µg/ml; 1hr, 24hr	Antigenotoxic activity (Comet Assay) Intracellular ROS (DCF assay)	+* +* +* +*	↓ Md-induced DNA damage (~55%) after 5µg/ml and 24hr. ↓ Md-induced DNA damage (~40%) after 1.5µg/ml PCF and 1hr. ↓ ROS levels in Caco-2 after 1hr or 24hr with 500µg/ml BE. ↓ ROS levels in HT29 cells at 1hr with 500µg/ml BE.	(44)

Bilberry (*Vaccinium myrtillus.)* and Blueberry (*Vaccinium corymbosum*.) anthocyanin-rich extracts.	Caco-2	0.5-50µg/L; 1hr incubation	Intracellular ROS levels (CAA assay)	+*	↓ intracellular ROS after incubation with 0.5µg/L	(45)
Bilberry (*Vaccinium myrtillus*) anthocyanin-rich extract	Caco-2, HT29	10-500µg/ml; 1hr and 24hr incubation	Antigenotoxic activity (Comet Assay) Intracellular ROS levels (CAA assay)	+* +*	↓ in H_2O_2 induced DNA damage by ~50% after 50µg/ml and 24hr. ↓ in ROS levels by ~30-40% in Caco-2 and HT29 after 250µg/ml and 24hr	(46)
Quercetin	Caco-2	1µM or 100µM; 1hr and 4hr	Antigenotoxic Activity (Comet Assay) DNA repair (hoGG1)	+* +*	↓ in H_2O_2 induced DNA damage by 42% at 1µM and 57% at 57% at 100µM and 1hr. ↑ in mRNA expression of hOGG1 at 1hr and 4rh after 100µM and H_2O_2	(47)
Anthocyanin fraction from Blackberry (*Rubus fruticosus)*	Caco-2	3.15 – 50 µg/ml; 3hr	Intracellular oxidation Oxidative cytotoxicity (CellTiter-Glo assay)	+* +*	↓ in intracellular oxidation in a dose-dependent manner after 3hr ↓in APPH induced cytotoxicity at 1.6 – 25 µg/ml and 3hr	(48)
Cyanidin Cyanidin-3-glucoside Quercetin	Human colon epithelial cells (HCEC)	50 µM; 4hr	Antigenotoxic Activity (Comet assay)	+* +* +*	↓ H_2O_2 induced DNA damage by ~ 39 % at 50µM cyanidin and 4hr ↓ H_2O_2 induced DNA damage by ~ 35 % at 50µM cyanidin-3-glucoside and 4h r ↓ H_2O_2 induced DNA damage by ~ 30 % at 50µM quercetin and 4hr	(49)

Raspberry (*Rubus idaeus* L.) extracts Raspberry Ellagitannin preparation (REP), sanguiin H-6 (SH-6), lambertianin C (LC)	Caco-2	2.5-160µg/ml REP 9.3-378µM LC 12.6-256µM SH-6	Comet Assay	-*	1hr incubation with REP ↑ DNA damage in a dose dependent manner from 2.5-80µg/ml (7-56% tail DNA) 1h incubation with LC ↑ DNA damage at 18.9µM (12% tail DNA) 1 h incubation SH-6 ↑ DNA damage at 26.7µM (20% tail DNA)	(51)
Ellagic Acid (EA)	HCT-15	60µM EA	DNA Fragmentation ROS Annexin V-PI assay	-* -* -*	↑ in internucleosomal fragmentation of DNA. ↑ in the production of ROS ↑ in annexin V staining, ↑ in apoptotic cells	(52)

Table 3: Effect of *in vitro* digested berries on models of DNA damage.

Berry or berry constituent	Colonocyte cell line	Dose and Duration	Assay	Effect	Observation	Reference
in vitro digested Elderberry (*Sambucus nigra*.)	NCM460 (non-transformed, non-tumorigenic colon cell line)	0.01, 0.1 and 1mg/ml digested extract; 30min, 1hr.	Antigenotoxic activity (Comet Assay) Intracellular ROS (DCF assay)	+* +*	↓ in H_2O_2 induced DNA damage after with 0.1 (↓25%), 1mg/ml (↓46%) and 1hr. ↓ in ROS after 0.1 (↓9%), 1mg/ml (↓22%) and 30min.	(44)
in vitro digested *Blackcurrant (Ribes nigrum.)*	Caco-2	0.01, 0.1 and 1mg/ml digested extract; 1hr	Antigenotoxic activity (Comet Assay) Intracellular ROS (DCF assay)	+* +*	↓ in H_2O_2 induced DNA damage in a dose-dependent manner, 1mg/ml = 54%. ↓ in ROS after incubation with colon digested extract at 0.01 (↓14%) and 0.1mg/ml (↓28%).	(45)
in vitro digested Wild Raspberry *(Rubus hirsuts Thunb.)*	Caco-2	2mg/ml Raspberry Digest; 2hr.	Hoescht 33258 Nuclear Staining Mitochondrial Membrane (MM) Lipid Peroxidation Cellular glutathione	+*	↓ in acrylamide (AC) induced DNA damage following pre-treatment with raspberry digest. ↓ in AC induced lipid peroxidation of the mitochondrial membrane, demonstrated by ↑ RH123 fluorescence (21% to 66%) ↓ in AC mediated GSH depletion, demonstrated by ↑ in NAD (~23% to 73%)	(69)
in vitro digested Wild Raspberry *(Rubus hirsuts Thunb.)*	Caco-2	2mg/ml raspberry digest (RD); 2hr	Hoescht 33258 Nuclear Staining Mitochondrial Membrane Potential (MMP) Intracellular ROS (DCF assay)	+*	↓ in ethyl carbamate (EC) induced DNA damage following pre-treatment with RD. ↓ in EC induced oxidative damage to MMP, demonstrated by ↑ in NAO fluorescence (35% to 79%) ↓ in ROS production from 392.76% to 120%	(70)

			Cellular glutathione		↓ in EC mediated GSH depletion, demonstrated by ↑ in NAD fluorescence (~30% to 70%)	
in vitro digested Blackberry	Caco-2	0.5mg/ml blackberry digest (BD); 2hr	Hoescht 33258 Nuclear Staining Mitochondrial Membrane Potential (MMP) Intracellular ROS (DCF assay) Cellular glutathione	+* 	↓ in ethyl carbamate (EC) induced DNA damage following pre-treatment with BD. ↓ in EC induced oxidative damage to MMP, demonstrated by ↑ in NAO fluorescence (20% to 91%) ↓ in ROS production from 241% to 111% ↓ in EC mediated GSH depletion, demonstrated by ↑ in NAD (~45% to 87%)	(71)
in vitro digested and *in vitro* fermented Lingonberry (*Vaccinium vitis-idaea*.)	HT29	IVD: 3.125-50µg/ml GAE IVF: 16µg/ml GAE 24hr	Antigenotoxic activity (Comet Assay) Mutation Frequency Assay	+* +*	↓ in H_2O_2 induced DNA damage at all concentrations of IVD (50µg/ml= ↓90%) ↓ in H_2O_2 induced DNA damage by ~50% (16µg/ml) after 24hr 16µg/ml IVF. ↓ in relative mutation frequency following incubation with IVD and IVF	(72)
in vitro digested (IVD) and *in vitro* fermented (IVF) raspberry (*Rubus idaeus*), strawberry (*Fragaria x ananassa*), and blackcurrant (*Ribes nigrum*)	HT29	IVD: 3.125-50µg/ml GAE IVF: raspberry-15.5µg/ml strawberry- 13.9µg/ml black currant-12.4µg/ml	Antigenotoxic activity (Comet Assay) Mutation Frequency Assay	+ * + *	↓ in H_2O_2 induced DNA damage by ~40% following 24hr incubation with all IVD at 6.25-50µg/ml ↓ in H_2O_2 induced DNA damage by ~30% following 24hr incubation with all fermentate ↓ relative mutation frequency >50% following 24hr with IVD. ↓ relative mutation frequency ~40% following 24hr with fermentate Significant anti-cancer activity persists following *in vitro* digestion and *in vitro* fermentation	(73)

in vitro digested Blackcurrant (*Ribes nigrum* L.)	HT29, NCM460	0.01-20mg/ml of digested extract	BrdU-incorporation Tunel Assay Comet Assay	-* -* -*	↓ in DNA synthesis in both HT29 and NCM460 after 24hr treatment with 10mg/ml ↑ in DNA degradation in a dose dependent manner ↑ in DNA strand breaks following treatment with 10mg/ml (9%) and 20mg/ml (19%)	(74)
Raspberry *(Rubus idaeus)* in vitro digested extract	HT29	0-50µg/ml; 24hr	Antiogentoxic Activity (Comet Assay)	+*	↓ in H_2O_2 induced DNA damage in a dose-dependent manner with 50µg/ml=↓ 50%	(75)

Table 4: Effects of Supplementation of Berry Extracts/Components on Markers Of Anti-Genotoxicty in Human Intervention Studies

Berry or berry constituent	Subjects	Dose and exposure	End point	Effect	Observation	Reference
Lingonberries (*Vaccinium vitis-idaea*.)	1 male ileostomist (43yrs old0	150g of pureed lingonberries	Antigenotoxic activity (Comet Assay)	+*	↓in H_2O_2 induced DNA in HT29 cells following 24hr incubation with post-berry ileal fluid (↓25%)	(72)
Strawberry (*Fragaria x ananassa*)	18 volunteers-8 males, 10 females (35±10years)	500g strawberries daily for 14days Blood samples pre and post treatment	Antigenotoxic activity (Comet Assay)	+*	↓ in H_2O_2 induced damage in PBMCs following strawberry intervention.	(77)
Bilberry (*Vaccinium myrtillus)*	5 female ileostomy volunteers 5 healthy female volunteers	10g BE in 150g yoghurt	Antigenotoxic activity (Comet Assay) qPCR analysis for Nrf2 pathway	+* +*	↓ DNA damage in PBMCs 2h post-consumption healthy control group ↓DNA damage in PBMCs NQO1 ↑ in PBMCs from 1h post-digestion, at 8hr post-digestion relative transcription 142%	(80)
Blueberry puree	10 volunteers (average age= 20.8 ±1.6 years)	Randomised Crossover 1x 300g blueberry or control jelly. Blood Samples collected 0hr, 1hr, 2hr, 24hr	Antigenotoxic activity (Comet Assay)	+*	↓ H_2O_2 induced DNA damage (18%) in MNBCs 1hr post blueberry consumption. No effect on H_2O_2 induced damage at 0hr, 2hr, 24hr. No effect on basal levels of DNA damage, 0hr, 1hr, 2hr, 24hr.	(78)

Wild blueberry drink	18 Male volunteers (average age= 47.8 ±9.7 years)	1x25g of blueberry powder daily for 6 weeks, 6 weeks washout, 6 weeks placebo. Blood samples collected pre- and post-treatment	Antigenotoxic activity (Comet Assay)	+*	↓ in FPG-sensitive sites in PBMC cells following Blueberry treatment. (~12.5 to 9.6%) ↓ in H_2O_2 induced DNA damage following blueberry treatment. (~45 to 37%).	(79)
Gallic Acid (GA)	8 male volunteers (34±5years) 8 female volunteers (32±5years)	0.2mg/kg Gallic Acid dissolved in 500ml H_2O daily for 3 days. Blood samples collected pre- and post-treatment	Antigenotoxic activity (Comet Assay) Intracellular ROS (DCF assay)	+* +*	↓ in FPG-sensitive sites (~13%) and ENDO-III sensitive sites (~11%) in lymphocytes after consumption. ↓ in H_2O_2 induced DNA damage (~11%) in lymphocytes after consumption ↓ in ROS levels after consumption.	(80)
Red mixed berry juice (red grape, blackberry, sour cherry, black currant and elderberry)	21 Haemodialysis patients	Parallel study 200ml red berry juice daily for 4 weeks (n=9) Control juice for 4 weeks (n=9) 3 week washout Blood collected at the end of each week on treatment	Antigenotoxic activity (Comet Assay)	+*	↓ in H_2O_2 induced DNA damage (40%) in lymphocytes following treatment.	(81)

Nrf2/ARE pathway:

Although the tables above have demonstrated the anticancer potential of berry (poly)phenols there has been limited research to identify the mechanism by which this occurs. The activation antioxidant response element (ARE) / nuclear factor-erythroid 2-related factor 2 (Nrf2) pathway may contribute to the antigenotoxic activity of dietary (poly)phenols (83). The interaction of Nrf2 and ARE up-regulates the transcription of phase II detoxifying enzymes and antioxidant enzymes, critical cellular response proteins which provides cytoprotection against carcinogens (84, 85). The Nrf2/ARE pathway may therefore present a novel approach for the prevention and treatment of CRC (86, 87). ARE is the regulatory element found in the promoter region of a variety of genes encoding for phase II detoxifying and antioxidant enzymes. These enzymes include, λ-glutamylcysteine synthase (GCL), glutathione peroxidase (GPX), gluthathione S-transeferase (GST), heme-oxygenase 1 (HO-1), N-acetyltransferase (NAT), NADPH quinine oxidoreductase 1 (NQO-1), sulfiredoxin (SRXN), sulfotransferase (SULT), thioredoxin reductase (TrxR), UDP-glucuronosyltransferase (UGT) (88). These cytoprotective enzymes decrease the levels of intracellular ROS, therefore decreasing the risk of DNA damage. The actions of these enzymes are discussed in greater detail in Chapter 5 but as a brief description, when up-regulated NQO1 and HO-1 act a superoxide scavenger and reduce the production of free radicals (89,90). These two have previously been connected to CRC; polymorphisms within NQO1 are linked with increased risk of cancer and HO-1 has been identified as a mediator of inflammation within the colonic mucosa (91, 92). The transcriptional activation of these ARE related genes is mediated by Nrf2.

Nrf2 a member of the CNC subfamily of transcriptional activators is a ubiquitously expressed protein which is sequestered within the cytoplasm under normal homeostatic condition. Interaction with its cytosolic repressor protein, Keap1 creates a complex with

Cul3-type E3 (Cul3) ligase which ubiquitinates Nrf2 and results in degradation by the 26s proteasome (93, 94). However, in conditions of oxidative stress or in the presence of electrophilic xenobiotics the bond between Nrf2 and Keap1 is disrupted, releasing the transcriptional activator. Once Nrf2 translocates and accumulates in the nucleus it can bind to the ARE promoter region found in many genes coding for a variety of antioxidant and detoxifying enzymes (95,96). Activation of these proteins reduces the amount of cellular ROS (reactive oxygen species), decreases genomic instability and reduces the risk of CRC progression (96). A negative feedback loop can be observed within this action, as oxidative stress results in the release of Nrf2 protein from the Keap1-Cul3 complex which in turn produces an up-regulation of antioxidant and phase II detoxifying enzymes and reduces the amount of cellular ROS.

This Nrf2-ARE system acts as a critical sensor for stress, protecting cells from damage induced by multiple sources. It has therefore emerged as an important target for chemotherapeutics in cancer and a range of other chronic diseases. The significance of Nrf2 in the progression of CRC has been demonstrated with knock-out mice models. $APC^{min/+}$ mice crossed with $Nrf2^{-/-}$ mice produced Nrf2 knockout model (Nrf2KO) with enhanced tumorigenesis. The loss of Nrf2 produced a significant increase in the number and size of polyps formed in the intestine, resulting in a lower expression of NQO1 protein and an increased expression of inflammatory makers when compared to $APC^{min/+}$ mice (94). A loss of Nrf2 resulted in a reduction of the antioxidative stress pathway, which in turn lead to inflammation, increased cellular proliferation in aberrant crypts and ultimately enhanced carcinogenesis. If the loss of this pathway can increase the risk of cancer, can an increase the amount of available Nrf2 result in greater chemoprevention?

A variety of dietary (poly)phenols have shown the potential to stimulate the Nrf2/ARE pathway, the details of which are described within Chapter 5 but for completeness a few

examples will be discussed below. Vari *et al.* utilised a range of *in vitro* techniques to illustrate the activation of Nrf2 and upregulation of target genes following treatment with protocatechuic acid (PCA), an anthocyanin metabolite. Murine macrophage cells, J744 A.1, incubated with 25μM PCA showed increased expression of GPX mRNA, after 1h of treatment which resulted in increased protein expression after 6hr incubation and corresponded to upregulation of Nrf2 mRNA and protein levels. These results were not replicated within Nrf2 silenced cells, therefore confirming the correlation between Nrf2 and GPX (97). Similar result have been seen with models of colorectal cancer, the commonly consumed dietary flavonoids, quercetin and kaempferol, have demonstrated the ability to induce Nrf2 activity within the Caco-2 cell line. When incubated with these (poly)phenols for 48h mRNA levels of Nrf2 and two of in target genes (NQO1 and GST) were significantly up-regulated (98). The activation of the Nrf2/ARE pathway can also be seen within *in vivo* models of CRC. Azoxymethane (AOM)-induced Balb/C mice treated daily with 1.2mg/kg of the bioflavoind luteolin elevated the protein levels of Nrf2 and increased the expression of the phase II enzyme GST (99). The combined evidence from these studies strongly suggests that the Nrf2/ARE pathway is a potential target for chemotherapeutic intervention within CRC.

Conclusion

In conclusion the evidence from Tables 2+3 indicate that berry (poly)phenols have anticancer properties, in this instance the ability to reduce intracellular ROS levels and decrease DNA damage. Studies have used a variety of approaches to assess this activity, Table 2 demonstrates the antigenotoxicity induced by crude berry extracts and individual poly(phenols) upon a range of colonic cell lines. These results establish the chemotherapeutic potential of berry derived (poly)phenols but do not represent the actual content and compounds which are available within the colon (42-52). In recent years a greater significance has been place upon the compositional changes that occur to as (poly)phenolics pass through the human body and how these will affect the bioavailability and bioactivity of these compounds (53, 54).

The investigations in Table 2 attempt to improve upon the relevance of these berry samples. To more accurately design an *in vitro* study several conditions must first be met to produce appropriate samples. This includes taking into consideration metabolic transformations, the presence of multiple phenolic compounds and ensuring these are used at physiological concentrations. A variety of *in vitro* systems have been developed to mimic the process of digestion, with the dynamic models simulating the peristaltic movements, pH and enzyme changes within the GI tract considered the most accurate. Alternatively, human feeding studies which collect ileal fluid post-berry consumption provide samples which truly represent the colonic contents (72). Regardless of the sample time the results above demonstrate that although the berry original (poly)phenols undergo considerable structural changes during digestion, the metabolites produced retain antigenotoxic activity. However, with greater research further improvements could be made to these models of digestion.

Although extensive research has established the anticancer properties of berry (poly)phenols and their metabolites upon CRC, the cellular mechanism behind this activity has yet to be fully understood. The Nrf2/ARE pathway has shown potential as a target of interest. Activation of this pathway up-regulates a series of phase II detoxifying enzymes (e.g. NQO1, HO-1, GST) which have the capacity to reduce intracellular oxidative stress, reduce DNA damage and in turn decrease the risk of CRC. Recent research suggests that an interaction between the Nrf2/Keap1/Cul3 complex and (poly)phenols, or their metabolites, results in thiol modifications which allows the Nrf2 protein to dissociate from Keap1 and translocate to the nucleus. Once in the nucleus, Nrf2 acts as a transcriptional activator for ARE-related genes. Such evidence implies that Nrf2 is a promising target from chemotherapeutic intervention.

Based on the literature reviewed above it is possible to conclude that berry (poly)phenols and their metabolites demonstrate antigenotoxoicity, and could be considered as a dietary agent of anticancer activity. However, further research is required to entirely comprehend the metabolic transformation which occur during digestion and how this alters the (polyphenol concentration and composition within the colon.

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Chapter 3: Bioavailability of Raspberry (Poly)phenols

Introduction:

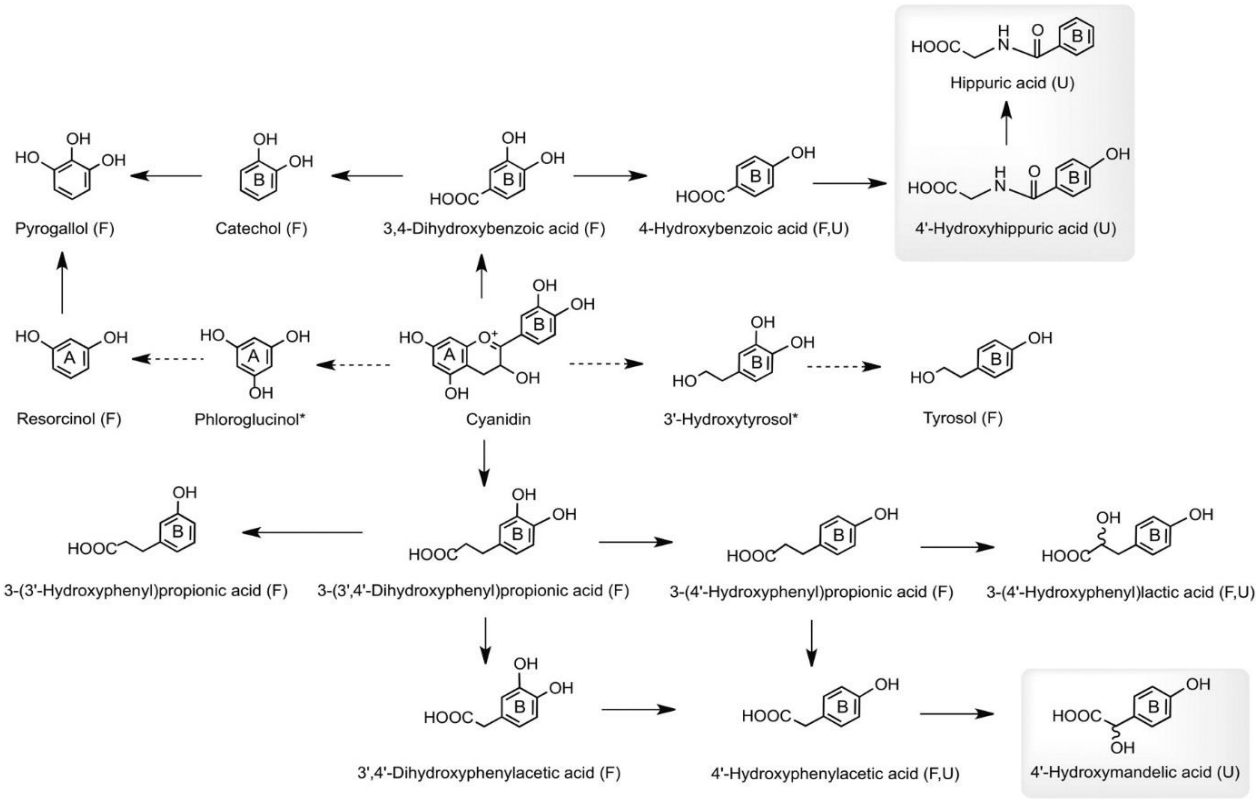
Berries are a particularly rich source of phytochemicals which have demonstrated a range of chemopreventive properties, with the potential to reduce the risk of a variety of cancers, including colorectal cancer (CRC) (1, 2). This anti-cancer activity is widely attributed to the abundance of (poly)phenols found within these fruits. Raspberries, for instance, are primarily comprised of a range of anthocyanins (cyanidin-3-*O*-sophoroside, cyanidin-3-(2″-*O*-glucosyl)rutinoside, cyanidin-3-*O*-glucoside) and ellagitannins (sanguiin H-10, sanguiin H-6, lambertianin C) with other phenolic compounds such as flavonols, hydroxycinnamic acids and hydroxybenzoic acids found at much lower concentrations (3-5). The total (poly)phenolic content and composition of the fruit varies dependant on the variety of raspberry, the environmental conditions, storage and processing of the berries (6-8). As raspberries are consumed and digested the phytochemical content changes as the fruit is degraded to its constituent parts. Therefore, these values do not represent the composition or concentration of the bioavailable and bioactive compounds within the body (3, 4). As with the majority of (poly)phenolics, both anthocyanins and ellagitannins have poor bioavailability and are not readily absorbed in the small intestine (9, 10). These two groups of compounds undergo considerable molecular changes as a consequence of digestion, but due to their diverse chemical structures the pathways of degradation are different for each (Figure 1A and B).

Anthocyanins are subgroup of flavonoids, which contribute on average 42% of the total (poly)phenols within raspberries, they are comprised of 2 aromatic rings (A and B) linked by a 3-carbon bridge to a heterocyclic ring (C), bound to sugar moiety (6, 11). There are a wide range of anthocyanins with six of these highly pigmented compounds; Pelargonidin (Pg), Peonidin (Pn), Cyanidin (Cy), Malvidin (Mv), Petunidin (Pt) and Delphinidin (Dp) commonly found in fruit and vegetables (12). These primarily occur as glycosides of

aglycones bonded to sugar moieties such as glucose, galactose, rhamnose and arbainsose as mono-, di or trisaccharides (13). Anthocyanins are highly reactive molecules that are sensitive to degradation and as such are susceptible structural changes with passage through the gastrointestinal tract. Unlike other flavonoids anthocyanins are sensitive to pH and as a consequence of this are found as a stable red flavylium form in the acidic conditions of the stomach, while in the small intestine at a basic pH a colourless carbinol is formed. Food digestion begins in the oral cavity (pH 5.6-7.9) where biotransformation of anthocyanins commences, but it is the intestinal tract (pH 6.7-7.4) where the anthocyanins are fully degraded into low molecular weight phenolics (14). Due to limited absorption in the small intestine, anthocyanins are not readily metabolised to glucuronide, sulfate or methylated derivates (15). Raspberry feeding studies have illustrated that 40% of the total anthocyanin intake remains in the ileal fluid and therefore has the potential to enter the large intestine (16). Under the anaerobic conditions of the colon the microflora act upon the cyanidin aglycone resulting in C-ring fission and the release of phenolic acids from both A and B rings (See Figure 1).

Ellagitannins are a group of nonflavonoids which make up an average of 57% of the raspberry phenolic content; and consist of gallic acid and hexahydroxyphenol units linked to glucose moieties which form dimers or oligomers (6, 17). This gives rise to a range of structurally diverse compounds found in a variety of food sources, for example, sanguiin H6, sanguiin H10 and lambertianin C are most commonly found in berries, while punicalagins and punicalins have been identified in pomegranates (10,18). Due to their complex chemical structure these compounds are relatively unstable and are susceptible to hydrolysis, which results in the production of ellagic acid. Both ellagitannins and ellagic acid have poor bioavailability, passing through the gastrointestinal tract un-absorbed and are subject to extensive metabolism by gut microbiota (19).

A)



B)

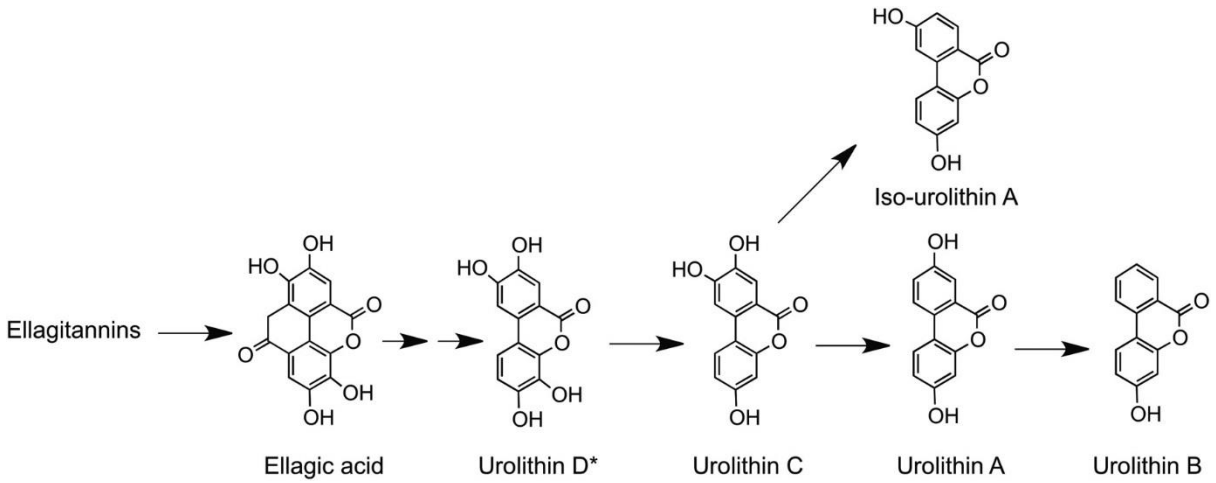


Figure 1: Proposed pathway for the conversion of **A**) red raspberry anthocyanins to phenolic acids. **B)** Proposed pathway for the conversion of red raspberry ellagitannins to ellagic acid and urolithins. *Adapted for González-Barrio *et al*.[16]

Ellagic acid is degraded through decarboxylase and dehydroxylase activity resulting in the loss of one lactone and successive removals of hydroxyl groups, producing dibenzopyran-6-one derivates which are more commonly known as urolithins (20). This group of benzo-coumarins are more readily absorbed than their parent compounds and therefore may contribute significantly to the health benefits previously ascribed to ellagitannins and ellagic acid (19, 21). The exact mechanism of urolithin production is not fully understood but the reaction is known to be catalysed by presence of gut microbiota. Tannases (tannin acyl hydrolase), produced by lactic acid bacteria e.g *L. plantarum, L. parplantarum* and *L.pentosus*, hydrolyse the ester bonds present in ellagitannins. This reaction produces hexahydroxy-diphenic acid, which is spontaneously converted to ellagic acid. Further bacterial enzymes cleave the lactone-ring and promote decarboxylation and dehydroxylation creating ellagic acid metabolites known as nasutins (10). Alternatively, breaking one of the lactone rings within ellagic acid releases the luetic acid which is immediately decarboxylated to create Urolithin M-5 a pentahydroxy-urolithin (Uro-M5), a key intermediary metabolite which is required to produce further urolithins. The removal of one hydroxyl group from distinct positions on the lactone of Uro-M5 creates one of three tetrahydroxy-urolithins, Uro-D, Uro-M6 or Uro-E (22). Loss of additional hydroxyl groups results in formation of the trihydroxy-urolithins (Uro-C and Uro-M7), which in turn are converted to dihydroxy-urolithins (Uro-A and IsoUrolithin-A) and the monohydroxy-urolithin, Uro-B (23). This pathway of metabolism is catalysed by gut microbiota, the specific bacteria involved in this process were recently identified as two strains, *G. urolithinfaciens* and *G. pamelaea,* both urolithin-producing species of *Gordonibacter* (24, 25).

The anti-cancer properties of native, parent berry (poly)phenols has been well established, with the compounds demonstrating the ability to modulate cellular functions and signalling pathways associated with antioxidant, antiangiogenic, anti-metastatic, and anti-inflammatory

mechanisms (1). However, these studies have not considered the effect of digestion or colonic fermentation upon the raspberry phytochemicals (20, 26). As described above the (poly)phenols under-go a variety of structural changes as they pass through GI tract. The cells within the colon are exposed to a mixture of (poly)phenols predominantly consisting of conjugated metabolites produced after phase II metabolism, as well as the simple and aromatic phenolics produced by colonic catabolism. It has become necessary to reassess the anti-genotoxic properties attributed to (poly)phenolic compounds using physiologically relevant samples. To produce such samples several groups have utilised various models of *in vitro* digestion to mimic the (poly)phenolic metabolites that pass through gastrointestinal tract (27-29). Simulated gastrointestinal digestion is generally more rapid, more cost effective and does not have the same ethical restrictions associated human intervention or feeding studies. These protocols utilise digestive enzymes, pH, salt concentration and digestion time to mimic *in vivo* conditions and can be either simple static models or complex dynamic models (30). Often these models only represent the metabolism that occurs in the upper GIT and small intestine and are therefore supplemented with *in vitro* fermentation to mimic the processes which occur within the large intestine (31, 32).

In recent years there has been much investigation into the health benefits of berry (poly)phenolics with a focus on the prevention of colorectal cancer. However, many of these studies have negated to consider which compounds are present within colon and are available to act upon the epithelial cells. The overall aim of this chapter is to produce and characterise a sample which is representative of this complex mixture of native (poly)phenols, phase II metabolites and colonic catabolites. In an attempt to improve upon previous models, our lab has developed an *ex vivo* model, which is a combination of a ileostomate human feeding study to produce raspberry enriched ileal fluid followed by 24hr batch culture model was utilised to mimic the changes which occur during digestion and fermentation.

Methods:

Ileostomy Feeding Study

The protocol for the raspberry feeding study (11/NI/0012) has previously been described in detail by McDougall *et al.* (4). In brief, following a low-(poly)phenol diet for 48hr and an overnight fast, baseline ileal fluid (T0hr) was taken from 11 ileostomists, The participants then consumed 300g of raspberry purée (*R.ideaus*, Glen Ample variety) and subsequently provided a second post-raspberry ileal sample (T8hr). Ileal fluid samples were collected, processed, aliquoted into falcon tubes (45g) and stored at -80°C

In vitro fermentation

To produce biologically relevant samples, i.e. representative of the substrate within the colon, ileal fluid samples were subjected to *in vitro* fermentation in the presence of faecal inoculum. All reagents used were purchased from Sigma-Aldrich (Poole, Dorset, UK).

Prior to performing 24hr batch culture basal nutrient media was prepared by dissolving; Petone water (2g), yeast extract (2g) NaCl (0.1g), K_2HPO_4 (0.04g), KH_2PO_4 (0.04g), $MgSO_4.7H_2O$ (0.01g), $CaCl_2.6H_2O$ (0.01g), $NaHCO_3$ (2g), Tween 80 (2ml), Hemin (0.05g), Vitamin K (10µl) and L-cysteine HCl (0.5g) in 2000ml of distilled water. The resulting media was aliquoted in glass bottles and autoclaved. This sterile medium was added to pre-sterilised fermentation vessels (see Figure 2 for glassware set-up) connected to a water-bath and a continuous nitrogen gas supply. The pH of each solution was monitored using an electrode and maintained at pH 6.6 with drop-wise addition of acid (0.5M HCl) or base (0.5M NaOH). This set-up left overnight to achieve 37°C temperature and anaerobic conditions.

The fermentation experiment was performed, in accordance with method used by Koutsos *et al.*, supplemented with faecal inoculum from a healthy volunteer, with no history of gastrointestinal disorders, provided a weekly stool sample (33). Stool samples were diluted 1:10 with sterile PBS and placed in a stomacher (Seward, Norfolk, UK) for 2min to homogenise. 50ml of ileal fluid sample and 15ml of faecal slurry was added to the basal media in each fermentation vessel to produce a final volume of 150ml and allowed to mix thoroughly before the pH was adjusted. Cultures were maintained for 24hr with a 10ml aliquot taken at 0h and 50ml collected at 24hr stored in sterile 50ml falcon tube at -80°C until required.

Targeted Analysis of Raspberry Phenolics in Ileal Fluids

Anthocyanins, ellagic acids and ellagitannins in the ileal fluid samples were purified and analysed as previously described by McDougall *et al*. (4). LC-MS^n analysis was performed using HPLC system comprised of Acella PDA detector coupled to an LTQ Orbital XL mass spectrometer and operated with Xcalibur software. Anthocyanins, ellagic acids and ellagitannins were identified by comparing exact mass and retention time to available standards. Anthocyanins were quantified from their chromatographic peak areas recorded at 520 nm and expressed as cyanidin 3-O-glucoside equivalents, ellagitannins detected at 260 nm were expressed as punicalagin equivalents, and ellagic acid and ellagic acid conjugates monitored at 365 nm were quantified in ellagic acid equivalents.

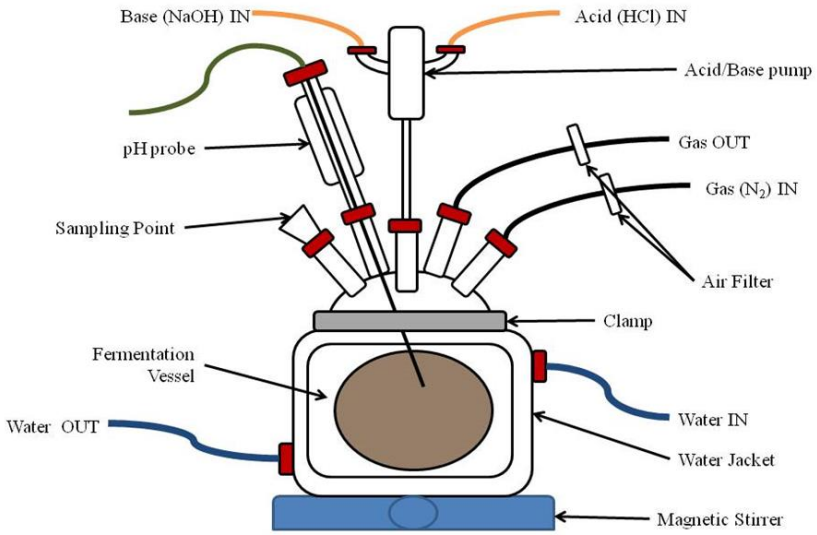


Figure 2: Glassware setup for *in vitro* fermentation

Non-targeted LC-MS^n analysis

The nontargeted LC–MS was performed using the same HPLC system as described above with data from the Orbitrap analysis deconvolved using the SIEVE software program to produce a list of retention time (RT) m/z pairs along with an associated extracted ion chromatogram based on peak area for each sample. The data was analysed statistically and a subset was defined of components whose patterns of abundance were increased after supplementation in all 11 subjects

GC-MS Analysis of phenolic acid catabolites in ileal fluid fermentate samples

The method for extraction and analysis of phenolic compounds from fermented samples was carried out by G.Pereira-Caro (Glasgow University) and performed using an established method (34). In summary, samples were purified using Styrene divinyl benzene solid phase extraction (SPE) cartridges (Strata SDB-L 200 mg), (Phenomenex, Macclesfield, U.K.). Prior to use the SPE cartridges were activated with 3ml of ethyl acetate, conditioned with 3ml methanol followed by 3ml 0.1M HCl. The ileal fluid fermentates (IFF) were acidified with 3ml of 0.2M hydrochloric acid (HCl) and spiked with an internal standard of 60µg 2', 4', 5'-trimethoxycinnamic acid (TCMA). Each sample was then added to the SPE cartridge, washed with a further 3ml 0.1M HCl and dried with nitrogen gas for 20min prior to the elution of phenolic acids with 3ml ethyl acetate. The eluate was reduced to dryness under nitrogen gas at 35°C, with the dried extract silyated with 300µl of pyridine and N-methyl-N-(trimethylsily) trifluoroacetamide (MSTF) (1:4, v:v) on an 80°C heat block for 20min. Samples were then cooled in a desiccator prior to Gas Chromatography- Mass Spectroscopy (GC-MS). A 6890 gas chromatograph fitted with a 5973 mass spectrometer (Agilent Technologies, Berkshire, U.K.), a 7683S autosampler and equipped with ZB-5MS Zebron

30m x0.25mm x 0.25µM (i.d.) capillary column (Phenomenex, Cheshire, U.K.) and a helium carrier gas (1.2ml/min) was used to identify phenolic catabolites from the purified ileal fluid fermentates. The following conditions were used for GC-MS; injection volume injection volume (1µL), split ratio (1:25), initial temperature 40 ºC raised to 160 ºC at 20 ºC/min, to 200 ºC at 1.5º C/min, to 250 ºC at 10º C/min and a final temperature of 300 ºC at 40º C/min, held for 5 min. Injector temperature was maintained at 220 ºC, the MS transfer line was 150 ºC and the ion source was 230 º C. Mass spectra were scanned at *m/z* 50-470 with an ionization energy of 70 ev. Phenolic compounds were identified according to their retention time and mass spectra of authentic standards. When standards were not commercially available, identification was achieved though the integrated NIST mass spectral library, with confidence of 90% or above. Quantification was based on calibration curves of the ratio between the target ion (*m/z*) of the standard of interest and the target ion of the TCMA internal standard.

Results:

Overview of raspberry and ileal compositional analysis

The results of from this part of the investigation can be seen in greater detail in the previously published paper by McDougall *et al.* but are briefly explained below (4). A total of 11 ileostomists, (with a mean age of 44 ± 12yrs) participated in the study, each providing two ileal samples; one baseline sample (T0hr) and one post-raspberry consumption (T8hr). The group mean ileal fluid sample mass at (T0hr) was 187.8 ± 73g (range of 107.4 - 318.0g), while the group mean mass of (T8hr) samples showed a significant increase at 268.0 ± 108g (range of 62.0 – 470.0g). This pattern was repeated with the group mean pH of both sample sets; (T0hr) had a group mean pH of 5.6 ± 0.4 (range 5.0 - 6.3) and (T8hr) with a significantly increased group mean pH of 6.6 ± 0.6 (range 5.3 - 7.5).

LC-MS analysis of the original raspberry extract identified a total of 8 anthocyanins, 2 ellagic acids and 3 ellagitannins (Table 1), which where all absent from baseline T0h ileal fluid samples. In contrast, a range of these (poly)phenolic compounds were detected in the post-raspberry ileal fluid collected from the 11 subjects (See Table 2). A total of 5 major anthocyanins were consistently identified within these samples; cyanidin 3-*O*-sophoroside, cyanidin 3-O-(2″-O-glucosyl)rutinoside, pelargonidin 3-O-sophoroside, cyanidin 3-O-glucoside and cyanidin 3-O-rutinoside. Total anthocyanin concentration varied across the post-berry samples and ranged from 0.55 ± 0.09μM in S05 to 10.2 ± 1.5μM in S01 with an average of 6.30±1.22μM. Cyanidin 3-*O*-sophoroside was the most abundant anthocyanin within both the original raspberry sample and the (T8hr) ileal fluid samples.

Ellagitannins, consisting of sanguiin H-10, sanguiin H-6 and lambertianin C, contributed the most to the total (poly)phenolic content of both the raspberry extract and post-raspberry (T8hr) ileal samples. The total amount of ellagitannin varied greatly from subject to subject, ranging from 1.0 ± 0.1μM in S05 to 53.0 ± 9.0μM in S09. Sanguiin H-6 was the most

abundant of the ellagitannins, found at 61.0±3.0µM in the raspberry extract with an average recovery of 30.5% from the ileal samples.

Ellagic acids, comprised of ellagic acid and ellagic acid-*O*-pentoside, were the only (poly)phenol group present at a higher concentration in the (T8hr) ileal samples than in the original raspberries, an average of 8.8±1.6µM compared to 6.0±0.2µM. Concentration found in ileal samples ranged from 0.8 ± 0.1µM (S05) to 14.6 ± 0.1µM (S03) which corresponded to percentage recoveries of 17-304% and an average of 175.7. There was considerable variation in the concentration and composition of the post-raspberry ileal fluid samples, emphasising the inter-individual variation which exists between the participants.

Non-targeted LC-MS analysis revealed that the concentration of two unknown compounds was increased in all 11 post-raspberry ileal fluid samples, compound 1 (*m/z* 355) and compound 2 (*m/z* 679) (Figure 3). Further investigation as described by McDougall *et al.* determined these compounds are structurally related, with compound 2 most likely as dimer of compound 1 which was putatively identified as an ursolic acid-based triterpenoid (35).

Compositional analysis of ileal fluid fermentate

GC-MS analysis of the ileal fluid fermentate (IFF) illustrated the degradation of (poly)phenolic compounds to simple and aromatic phenolics. A total of sixteen simple phenolics where detected consistently throughout pre and post raspberry IFF samples with benzoic acid and catechol the most abundant compounds within all sample types (Table 3). Compositional analysis of post-berry 24hr IFF samples revealed that all the subjects showed an increase in 2 or more simple phenolics when compared to 0hr samples. Seven of the individual phenolics were increased in over half the 24h fermentate samples with, 4-(hydroxybenzoic acid, 3-phenyllactic acid and 3-(3-hydroxyphenyl) propionic acid each

increased in 8 of the 11 subjects. Benzoic acid and 4-hydroxyphenyllatic acid were detected at greater concentrations in 7 of the 11 subjects, and catechol and 3-(4-hydroxyphenyl) propionic acid increased in 6 subjects. Only (S05) showed an increase in all 7 of these aromatic compounds after fermentation, as well as greater concentrations of 3-phenylpropionic acid, 4-hydroxyphenylacetic acid, 3,4-hydroxybenzoic acid, dihydroferulic acid and 4-coumaric acid. This was a total of 12 individual phenolics which increased in concentration from (S05) 0hr-24hr, a pattern also seen with subject 4 but with increased 3-hydroxyphenylacetic acid rather than benzoic acid. When the average concentration for each individual phenolic was calculated 6 compounds in found in the IFF samples increased from 0hr to 24hr, these included benzoic acid, catechol, 3-phenylactic acid, 3-phenylpropionic acid, 3-(3-hydroxyphenyl) propionic acid and 4-hydroxyphenylactic acid. Catechol demonstrated the largest increase concentration from an average of 385µM at 0h to 594µM at 24hr IFF (a total of 209µM), followed by benzoic acid with a 64µM increase from (0h (76µM) to 24hr (140µM). The remaining 4 phenolics had increases of 20µM or less.

As was demonstrated by the ileal fluid samples, the consumption of raspberry purée increased the total (poly)phenolic content (TPC) in the post-raspberry IFF samples of several subjects. (S01, 02, 04, 10 and 11) all demonstrated increased TPC in post-berry 0hr IFF samples when compared to pre-raspberry 0h IFF. In contrast S01, S02, S06, S09 and S12 increased in TPC following 24hr fermentation. The total phenolic content in both post raspberry 24hr IFF samples was increased in 8 out of the 11 subjects (S01, 03, 04, 05, 06, 08, 09, 10 & 12). There was considerable inter-individual variation within each sample set; the phenolic content of pre-raspberry 0hr IFF ranged from 155 ± 46µM (S10) to 848±109µM (S02) with an average of 513 µM. With pre-berry 24hr fermentation this discrepancy was further extended with total phenolic content differing from 95 ± 17µM (S01) to 1622 ± 178µM (S05) (average of 789µM). Post-raspberry samples also demonstrate this variation

with the phenolic content of post-berry IFF 0hr samples ranging from 55 ± 21µM (S08) to 1618 ± 187µM (S02) with an average of 555µM. The overall concentration of phenolics with this group increased after 24hr fermentation but the variation between samples remains; with phenolic content from 94 ± 9µM (S03) to 1893 ± 227µM (S06).

Table 1: Identification of phenolic compounds detected within raspberries*

Compound	Concentration (µmol/300g)
cyanidin 3,5-O-diglucoside	0.6 ± 0.1
cyanidin 3-O-sophoroside	22 ± 1.0
cyanidin 3-O-(2″-O-glucosyl)rutinoside	4.2 ± 0.3
pelargonidin 3-O-sophoroside	0.25 ± 0.01
cyanidin 3-O-glucoside	9.0 ± 1.0
cyanidin 3-O-(2″-O-xylosyl)rutinoside	0.3 ± 0.1
cyanidin 3-O-rutinoside	2.2 ± 0.2
pelargonidin 3-O-glucoside	0.1 ± 0.1
Total anthocyanins	**38.2 ± 3.2**
ellagic acid O-pentoside	1.2 ± 0.1
ellagic acid	4.8 ± 0.1
Total ellagic acid	**6.0 ± 0.2**
sanguiin H-10	43.0 ± 3.0
sanguiin H-6	61.0 ± 3.0
lambertianin C	20.0 ± 2.0
Total ellagitannins	**124.0 ± 8.0**

*Adapted from McDougall [4]

Data in column 2 is expressed as mean values ± SD (n=2)

Table 2: Recovery of Anthocyanins, Ellagic Acid, Ellagitannins in Ileal fluid collected 8hr post raspberry consumption. *

Compound	S01	%	S02	%	S03	%	S04	%	S05	%	S06	%
Cy-3-*O*-sophoroside	8.0	*36*	4.2	*19*	7	*33*	6.6	*30*	0.41	*2*	6	*27*
Cyanidin-3-*O*-(2"-*O*-glucosyl)rutinoside	1.7	*40*	1.6	*38*	1.7	*39*	1.7	*40*	0.11	*3*	1.6	*38*
Pelargonidin-3-*O*-sophoroside	0.1	*40*	0.1	*40*	0.1	*40*	0.08	*32*	nd	*0*	0.08	*32*
Cyanidin-3-*O*-glucoside	0.1	*1*	0.4	*4*	0.06	*1*	0.12	*1*	nd	*0*	0.09	*1*
Cyanidin-3-*O*-rutinoside	0.3	*14*	0.1	*4*	0.04	*2*	0.03	*1*	0.03	*1*	0.2	*9*
Total anthocyanins	**10.2±1.5**	***27***	**6.4±0.6**	***17***	**8.9±2.7**	***23***	**8.5±1.1**	***22***	**0.55±0.09**	***1***	**7.9±2.5**	***21***
Ellagic acid-*O*-pentoside	0.3	*25*	0.5	*42*	0.50	*42*	0.6	*50*	0.05	*42*	0.5	*42*
Ellagic acid	7.2	*150*	10	*208*	14.6	*304*	12	*250*	0.8	*17*	12	*250*
Total Ellagic acid	**7.5±0.7**	***125***	**10.5±3.1**	***175***	**15.1±0.1**	***252***	**12.6±6.3**	***210***	**0.85±0.11**	***14***	**12.5±3.2**	***208***
Sanguiin H-10	13.5	*31*	6	*14*	8	*19*	6.5	*15*	0.6	*1*	8	*19*
Sanguiin H-6	8.5	*14*	17	*28*	32	*52*	29	*47*	0.4	*1*	19	*31*
Lambertianin	Nd	*0*	0.7	*4*	2.4	*12*	4	*20*	nd	*0*	nd	*0*
Total Ellagitannins	**22.0±2.3**	***18***	**23.7±8.6**	***19***	**42.4±8.7**	***34***	**39.5±21.2**	***32***	**1.0±0.1**	***0.8***	**27±5**	***22***

Compound	S08	%	S09	%	S10	%	S11	%	S12	%
Cy-3-*O*-sophoroside	4	*18*	6.8	*31*	6.3	*29*	3.5	*16*	4.03	*19*
Cyanidin-3-*O*-(2"-*O*-glucosyl)rutinoside	1.0	*24*	1.66	*39*	1.51	*36*	0.9	*21*	1	*24*
Pelargonidin-3-*O*-sophoroside	0.05	*20*	0.10	*40*	0.07	*28*	0.04	*16*	0.02	*8*
Cyanidin-3-*O*-glucoside	0.3	*3*	0.07	*1*	0.06	*1*	0.11	*1*	0.09	*0.3*
Cyanidin-3-*O*-rutinoside	0.4	*18*	0.11	*5*	0.9	*41*	0.02	*1*	0.57	*23*
Total anthocyanins	**5.7±1.4**	***15***	**8.7±0.2**	***23***	**8.8±0.6**	***23***	**4.6±1.3**	***12***	**5.7±1.7**	***15***
Ellagic acid-*O*-pentoside	0.5	*40*	0.4	*33*	0.5	*40*	0.13	*11*	0.8	*33*
Ellagic acid	8.6	*179*	13	*271*	9	*187*	7.3	*152*	7	*83*
Total Ellagic acid	**9.1±0.6**	***152***	**13.4±1.1**	***223***	**9.5±1.1**	***158***	**7.4±0.7**	***124***	**8±2.3**	***134***
Sanguiin H-10	4	*9*	7	*16*	7.4	*17*	6	*14*	5	*13*
Sanguiin H-6	23	*38*	39	*64*	19	*31*	26	*43*	12	*20*
Lambertianin	0.4	*2*	7	*35*	2.3	*12*	1	*5*	1.1	*6*
Total Ellagitannins	**27.4**±6.2	***22***	**53±9**	***43***	**28.7±7.9**	***23***	**33±4**	***27***	**27±5**	***15***

***Adapted from McDougall [4]

The data for each subject presented in column one are expressed as mean values in µM, while the column two represents the amount recovered as percentage of the quantity ingested.

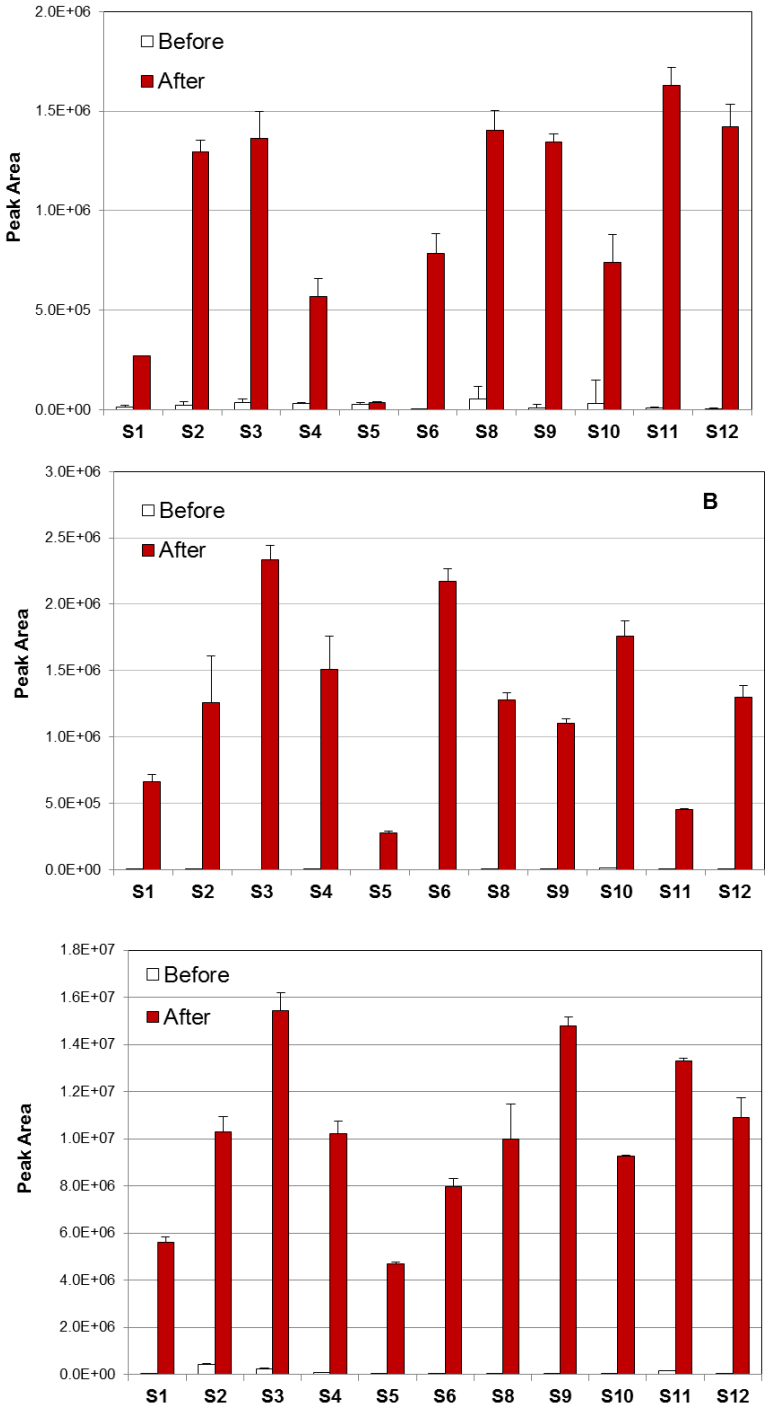


Figure 3: Comparison of abundance of selected MS signals in ileal fluids before and after raspberry intake. Panel A = *m/z* 934 = sanguiin H-6 at RT 17.9 min; B = *m/z* 679 at RT 23.5; C = *m/z* 355 at RT 12.7. Peak areas are in arbitrary MS units.

* Adapted from McDougall *et al.* [35]

Compound µM	S01		S01		S02		S02		S03		S03	
	Pre-Berry		Post-Berry		Pre-Berry		Post-Berry		Pre-Berry		Post-Berry	
	0hr	24hr	0hr	24hr	0hr	24hr	0hr	24hr	0hr	24hr	0hr	24hr
Benzoic acid	141.0	155.7	118.9	208.2*	90.2	53.3	49.2	229.5*	50.8	61.5	123.0	70.5
Catechol	618.2		1009.1	1527.3*	554.5	11.8	1563.6	9.1	233.6	3.6	172.7	
3-phenylpropionic acid			6.0	9.3*	10.0	4.7	6.0	144.0*	4.7	108.0	6.0	4.7
Pyrogallol	9.1		7.3	7.3	5.5	2.7	4.5	3.6	3.6	2.7	4.5	3.6
3-phenyllactic acid	5.4		6.6	136.7*	5.4	42.8	14.5	3.0	14.5	30.1	1.2	11.4*
3-hydroxyphenylacetic acid	21.1		85.5		52.6	3.3	3.3	2.6	7.2		1.3	
4-hydroxybenzoic acid	74.6	58.0	50.7	19.6	17.4	46.4	9.4	43.5*	44.9	5.1	3.6	39.1*
4-hydroxyphenylacetic acid	54.6	37.5	190.8		121.1	10.5	11.2	5.9	65.8	5.9	3.9	5.3*
3-(2-hydroxyphenyl)propionic acid												
3-(3-hydroxyphenyl)propionic acid				9.0*		4.2		12.0*	2.4	33.1	9.6	4.8
3(4-hydroxyphenyl)propionic acid			4.2		3.0		1.8			16.3		2.4*
3,4-dihydroxybenzoic acid			32.5		18.8		3.9					6.5*
Dihydroferulic acid			17.3		10.7	6.6				30.6		1.5
4-hydroxyphenyllactic acid				39.5*		23.0			6.6	2.0		12.5*
3(3,4-dihydroxyphenyl)propionic acid												
4-coumaric acid			14.6		9.8	4.9			4.3		4.9	2.4
Caffeic acid						8.9			13.3			
Isoferulic acid				5.2*		9.3			2.6			
Dihydrocaffeic acid			51.1		39.6	42.3			4.9	4.9		
Total	**783±41**	**95±17**	**1475±406**	**1753±33**	**848±109**	**221±26**	**1618±187**	**223±28**	**408±21**	**242±11**	**207±10**	**94±9**

Table 3B: Recovery of simple phenolics in 0hr and 24hr ileal fluid fermentates pre-raspberry and post-raspberry consumption

Concentration of each phenolic measure in µM

*significant increase in concentration between Post-Berry 0hr and 24hr

Compound µM	S04		S04		S05		S05		S06		S06	
	Pre-Berry		Post-Berry		Pre-Berry		Post-Berry		Pre-Berry		Post-Berry	
	0hr	24hr	0hr	24hr	0hr	24hr	0hr	24hr	0hr	24hr	0hr	24hr
Benzoic acid	97.5	123.0	76.2	71.3	32.0	131.1	32.8	172.1*	65.6	18.9	24.6	123.0*
Catechol	327.3	1272.7	481.8	909.1*	572.7	1245.5	154.5	327.3*	227.3	1154.5	181.8	1600.0*
3-phenylpropionic acid	2.0	24.7	2.7	10.0*	4.0	4.7	4.0	11.3*	4.0	4.7	4.0	20.7*
Pyrogallol	3.6		4.5	0.9	10.9	8.2	10.0	8.2		3.6		4.5*
3-phenyllactic acid	7.8	26.5		16.3*		54.2		26.5*	18.1	15.7	24.1	18.7
3-hydroxyphenylacetic acid				3.3*								
4-hydroxybenzoic acid	50.7	95.7	12.3	26.1*				5.8*	36.2	21.7	29.0	50.7*
4-hydroxyphenylacetic acid	5.3	2.6		9.9*	111.8	105.3	9.2	11.8	13.2		26.3	15.8
3-(2-hydroxyphenyl)propionic acid	0.0		3.6									
3-(3-hydroxyphenyl)propionic acid	3.6	13.3	5.4	6.0*	19.3	30.1	12.7	84.3*		15.1		22.98*
3(4-hydroxyphenyl)propionic acid		4.8	3.6	8.4*		8.4		9.0*				6.0*
3,4-dihydroxybenzoic acid			1.9	2.6				2.6*			19.5	1.9
Dihydroferulic acid		21.4		5.1*		2.6		13.8*	30.6	11.2	7.7	18.4*
4-hydroxyphenyllactic acid	3.3	10.5		9.2*	11.2	32.9		17.8*	6.6	17.8		10.5*
3(3,4-dihydroxyphenyl)propionic acid	3.6								17.9	8.3		
4-coumaric acid				3.7*				11.6*		11.0		
Caffeic acid												
Isoferulic acid												
Dihydrocaffeic acid												
Total	**504±55**	**1595±77**	**592±24**	**1081±209**	**761±178**	**1622±178**	**223±51**	**702±98**	**419±109**	**1282±306**	**316±48**	**1893±227**

Table 3B: Recovery of simple phenolics in 0hr and 24hr ileal fluid fermentates pre-raspberry and post-raspberry consumption

Concentration of each phenolic measure in µM

*significant increase in concentration between Post-Berry 0hr and 24hr

Compound µM	S08		S08		S09		S09		S10		S10	
	Pre-Berry		Post-Berry		Pre-Berry		Post-Berry		Pre-Berry		Post-Berry	
	0hr	24hr	0hr	24hr	0hr	24hr	0hr	24hr	0hr	24hr	0hr	24hr
Benzoic acid	196.7	196.7	32.8	82.0*	73.8	90.2	13.9	131.1*	65.6	82.0	82.0	357.4*
Catechol	45.5	763.6	3.6	54.5*	363.6		163.6	142.7	63.6	1263.6	300.0	
3-phenylpropionic acid	1.3	3.3	1.3	0.7	2.0		2.0		4.7		2.0	
Pyrogallol	3.6	3.6	4.5	2.7	2.7	6.4	0.9	1.8	1.8		0.9	0.9
3-phenyllactic acid	9.0	26.5			6.6			7.8*	1.8	41.6		41.6*
3-hydroxyphenylacetic acid		2.0									30.9	
4-hydroxybenzoic acid				3.6*			13.0	4.3	1.4	6.5	1.4	13.8*
4-hydroxyphenylacetic acid	5.3	6.6		3.3*		46.7			13.2		70.4	
3-(2-hydroxyphenyl)propionic acid												
3-(3-hydroxyphenyl)propionic acid	6.0	10.8	7.8	10.8*		14.5			3.0	61.4		72.9*
3(4-hydroxyphenyl)propionic acid								2.4		11.4	0.6	25.9*
3,4-dihydroxybenzoic acid			5.2	3.9			3.2				17.5	1.3
Dihydroferulic acid		2.6								26.5	2.6	12.8*
4-hydroxyphenyllactic acid	4.6	11.2								9.2		5.9*
3(3,4-dihydroxyphenyl)propionic acid	5.4	6.0					4.2				3.6	
4-coumaric acid	7.9											
Caffeic acid											3.9	
Isoferulic acid												
Dihydrocaffeic acid												
Total	**285±504**	**1032±179**	**55±21**	**161±47**	**448±16**	**157±21**	**200±16**	**290±45**	**155±46**	**1502±42**	**515±72**	**532±19**

Compound µM	S11		S11		S12		S12	
	Pre-Berry		Post-Berry		Pre-Berry		Post-Berry	
	0hr	24hr	0hr	24hr	0hr	24hr	0hr	24hr
Benzoic acid	41.0	196.7	245.9	65.6	52.5	8.2	35.2	30.3
Catechol	409.1	200.0	190.9	109.1	172.7		18.2	672.7*
3-phenylpropionic acid	2.7	29.3	33.3	18.7	24.0		0.7	
Pyrogallol	1.8	5.5	2.7		1.8		0.9	0.9
3-phenyllactic acid	15.1	37.3	16.3	22.9*	12.0			2.4*
3-hydroxyphenylacetic acid								
4-hydroxybenzoic acid		5.8	87.0	20.3	65.9	92.8		5.8*
4-hydroxyphenylacetic acid	131.6	150.0	48.0	43.4	37.5		3.3	
3-(2-hydroxyphenyl)propionic acid						44.0		
3-(3-hydroxyphenyl)propionic acid		77.7	15.7			7.8		3.6*
3(4-hydroxyphenyl)propionic acid		4.2	51.2	12.7		6.0		
3,4-dihydroxybenzoic acid			33.1				13.6	
Dihydroferulic acid			38.3		25.0	40.8		
4-hydroxyphenyllactic acid	12.5	27.6		19.7*	5.3			
3(3,4-dihydroxyphenyl)propionic acid	20.8		27.4				5.4	
4-coumaric acid			37.8					
Caffeic acid					26.7			
Isoferulic acid								
Dihydrocaffeic acid						2.2		
Total	**635±43**	**734±30**	**827±90**	**312±127**	**423±39**	**201±18**	**77±35**	**715±41**

Table 3C: Recovery of simple phenolics in 0hr and 24hr ileal fluid fermentates pre-raspberry and post-raspberry consumption

Concentration of each phenolic measure in µM

*significant increase in concentration between Post-Berry 0hr and 24hr

Discussion:

There has been much use of *in vitro* models to research the bioactivity and anticancer properties of berry derived phytochemicals, investigations which have primarily used whole berry, berry extracts or purified (poly)phenols (1, 2, 36). These studies have highlighted the potential health benefits of such compounds; however, it is important to consider the biological relevance of such interactions. The colonic epithelia will encounter a complex mixture of compounds, consisting primarily of simple and aromatic phenolics (produced from colonic fermentation) combined with much lower concentrations of native berry (poly)phenols and secondary metabolites (produced during digestion). To create a more physiologically relevant material, ileal fluid was collected from a raspberry feeding trial, this *in vivo* digested material was fermented in a gut model to simulate the interaction with colonic microflora. Thus, creating an *ex-vivo* model of greater physiological value than *in vitro* modelling alone.

The initial analysis of the raspberry purée used within the feeding study identified multiple (poly)phenolic compounds, the composition of which was comparable to that detected in previous research which utilised the Glen Ample variety of the fruit (3, 6, 37). The 8 anthocyanins (cyanidin 3,5-*O*-diglucoside, cyanidin 3-*O*-sophoroside, cyanidin 3-*O*-(2-O-glucosyl) rutinoside, pelargonidin 3-*O*-glucoside, cyanidin 3-*O*-(2”-*O*-xylosyl)runtinoside, cyanidin 3-*O*-rutinoside and pelargonigin 3-*O*-glucoside) 2 ellagic acid (ellagic acid *O*-pentoside and ellagic acid) and 3 ellagitannins (sanguiin H-10, sanguiin H-6 and lambertianin C) where present in all the prior studies. However, the total amount of ellagic acids and anthocyanins were significantly reduced within the raspberries used for the study. Glen Ample raspberries analysed by González-Barrio *et al*. presented with greater than 5 times the anthocyanin content as in this study (3). This pattern was also seen with ellagic acid, which was double the concentration detected in our samples at 17µM. Although the

total ellagitannin concentrations in both studies were very similar the profiles differed with the results from this investigation showing much lower levels of sanguiin H-6 but higher levels of sanguiin H-10 and lambertianin C. These are a reflection of the environmental conditions in which the fruit was grown and as such anthocyanin levels suggest that the berries where at differing stages of ripeness when harvested (37-39). The starting (poly)phenolic composition and concentration of these berries ultimately dictated the compounds which are available to act upon the colonic epithelium.

The raspberry feeding study and subsequent collection of ileal fluid highlighted compositional changes which occur due to digestion and identified those (poly)phenolic compounds which reach the large intestine. Although the total number of anthocyanins present was reduced from 8 to 5, all three ellagitannins where detected in the post-raspberry ileal fluid and ellagic acid content showed a substantial increase. This abundance of ellagic corresponds to the breakdown of ellagitannins, whose complex structures are readily hydrolysed during digestion. A similar pattern of results was observed in the study conducted by González-Barrio *et al.*, which examined (poly)phenolic composition of 10 post-raspberry ileal fluid samples (3). They detected a total of 8 anthocyanins with and overall concentration of 81±4μM and 2 ellagic acids, ellagic acid (19 ±3μM) and sanguiin H-6 (23±1μM). These concentrations were higher than those detected within our study, however this can be partially attributed to the greater initial (poly)phenolic content of the raspberry fruit.

The results from this study combined with previous publications indicate that a considerable proportion of the anthocyanins pass through the upper gastrointestinal tract unabsorbed and enter the colon. The lack of anthocyanins present in both the plasma (<1nM) and urine (21.4nM) of 9 participants after consumption of raspberries (containing 292μM total anthocyanins) indicates that these compounds have poor bioavailability (18). In fact, the only parent compound detected within the urine sample was cyanidin O-glucuronide, while the

other constituents were analogues such as peonidin 3-O-glucosides. As most anthocyanins are found as di and tri-saccharides sugar moieties, they are unlikely to be cleaved by the enzymes found in the brush epithelia of the small intestine. The ellagitannin and ellagic acid profiles also give some indication as to the changes which occur *in vivo*. The poor bioavailability of the ellagitannins is due to their large molecular structure and highly polar nature, preventing them from entering the circulation. However, as the compositional analysis above indicates, these compounds reduce in concentration as they pass through the GI tract, with only 21.2% reaching the large intestine and much lower concentration found in the faeces (40). This is in direct contrast to ellagic acid which increases in concentration. The phenolic profile of the ellagitannins present also changes from the original raspberry sample to the ileal fluid, with a decrease in lambertianin C and sanguiin H-6, while sanguiin H-10 increases. These patterns are due to the catabolism of ellagitannins; hydrolysis of the large molecule lambertianin C produces sanguiin H-6, which in turn can be broken down to sanguiin H-10 and ultimately these compounds are all degraded to ellagic acid (17). The ileal fluid samples collected are representative of the changes which occur to (poly)phenolic compounds due to digestion and passage through the small intestine. To mimic the action of colonic microbiota these samples were subjected to *in vitro* fermentation for 24hr.

The colonic catabolism of (poly)phenolic compounds is not yet fully comprehended with many of the pathways involved in this procedure yet to be elucidated. The phenolic metabolites produced during bacterial fermentation will depend upon the initial (poly)phenol. With anthocyanins the end-products are phenolic acids, while ellagitannins produce ellagic acid and urolithins. *In vitro* fermentation models are utilised to predict these breakdown pathways is an attempt to further our understanding. The data produced from this investigation illustrates how the more complex anthocyanin structures are degraded to produce simple and aromatic compounds. As many of the (poly)phenolic compounds reach

the colon, microbiota play a key role in their degradation and the production of phenolic metabolites. Raspberry anthocyanins incubated anaerobically in faecal suspensions for 48hr resulted in the production of a range of phenolic acids which were also present in the post-raspberry IFF samples described above. These included compounds such as catechol, 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, tyrosol and 3-(3'-hydroxyphenyl) propionic acid. The presence of such phenolics suggests that they follow the breakdown pathway described by Gonzalez-Barrio *et al*. (16). During fermentation, the anthocyanins are degraded through the cleavage of sugar moiety, cleavage of the C-ring and breakdown of A and B rings to produce simple and aromatic phenolics (Figure 1). The phenolic compounds produced as the result of 24hr fermentation reached concentrations between 94-1893µM with over half the subjects producing total phenolic concentrations greater than 500µM. Concentrations of benzoic acid were greater than 50µM in 11 of out the 12 post-berry IFF samples. These values are consistent with the concentrations of those few studies which have utilised biologically relevant phenolics to assess potential health benefits (41, 42). The specific bioactivity of the samples produced within this investigation are discussed in greater detail in Chapters 4 and 5.

The findings of this experiment somewhat mirror those of Correa-Betanzo *et al.* whose study simulated the digestion of wild blueberries (*Vaccinium angustifolium*) using a three-step *in vitro* model (31). They demonstrated a reduction in recovery of (poly)phenols and anthocyanins from the 94% and 97% (2.0 and 1.4mg GAE/g fresh weight equivalent) after gastric digestion to a final recovery of 42% and 1.5% (0.91 and 0.023mg GAE/g fresh weigh equivalent) following simulated intestinal digestion and colonic fermentation. As with our study this overall loss of (poly)phenol content corresponded to changes in sample composition with the more complex (poly)phenolics (chlorogenic acid, quercetin arabinoside and syringetin-3-galactoside) and anthocyanins (including delphinidins, cyanidins,

petunidins) within the crude extract and present after gastric and intestinal digestion, while the fermentate samples were primarily composed of simple and aromatic phenolics (syringic acid, hippuric acid, protocatechuic acid).

The colonic degradation of ellagitannins, as described above and illustrated in Figure 2, is dependent upon metabolic phenotype, dictated by the microflora present within the large intestine. This metabolic pathway is further complicated with inter-individual and intra-individual variation, which is dependent upon the differing compositions of gut microbiota within the human population (43). Individuals can be grouped based upon the level of urolithins present within urine samples and classified as either producers or non-producers (44, 45). However prolonged exposure to a diet containing high concentrations of ellagitannins or ellagic acid can activate non-producers, i.e. they start to produce urolithins; these are then classified as responders and those who are not activated are non-responders (47, 48). Individuals can also be grouped into 3 by phenotypes or metabotypes, which are differentiated based upon their end-point of ellagitannin metabolism. The most common group or "phenotype A", excrete only Uro-A metabolites, while the second group or "phenotype B" produced Uro-A, IsoUro-A and Uro-B. The final group are referred to as "phenotype 0" and do not excrete any detectable levels of Uro-A, IsoUro-A or Uro-B. These individuals may be activated with long-term exposure to ellagitannins and ellagic acid, i.e. are responders or may simply be non-producers (48). This inter-individual variation had been attributed to differences in gut microbiota, a theory which is corroborated with correlation between *Gordonibacter* and Uro-A production (49) Romo-Vaquero *et al.* suggested that higher levels of *Gordonibacter* were present in individual with "phenotype 1" (50).

Previous studies have readily demonstrated the conversion of ellagic to urolithins following anaerobic incubation with faecal slurry, with approximately 80% of the total ellagic acid converted to a range of urolithins, dependent upon the phenotype of the faecal donor (16).

In order to minimise variation as a result of metabotype, a single faecal donor was used to provided samples (weekly), to inoculuate the batches of *in vitro* fermentation models. The absence of ellagic acid metabolites, specifically urolithins suggests an absence of *Gordonibacter*, the species of gut microbiota which catalyses this pathway (24, 25, 26). The results from this chapter show a lack of detectable urolithins in the IFF samples, suggesting the individual faecal donor was "phenotype 0". If this study was repeated it would be beneficial to screen the donors prior to producing faecal slurry, use of different donors with other phenotypes could create a greater variety of ellagitannin breakdown products, allowing the inclusion of urolithins A, B and iso-urolithins. In isolation or in combination with ellagic acid *in vitro* assays with these compounds have demonstrated antioxidant, antiproliferative and antimutagenic potential (51-54).

The overall aim of this chapter was to produce substrates representative of the metabolic breakdown of raspberry (poly)phenolics as they travel through the digestion system. This was a two-step process, the initial collection of 0hr and 8hr ileal fluid from 11 participants of a raspberry feeding study. These samples were subjected to 24hr *in vitro* fermentation producing ileal fluid fermentate, to mimic the compounds present within the large intestine after degradation by colonic microbiota. The HPLC and GC-MS analysis of the subsequent samples provided us with a greater understanding of the metabolic degradation which occurs as (poly)phenols pass through the digestive system. Both the ileal fluid and ileal fluid fermentate samples have provided us with biologically relevant substrates with which to reassess the bioactivity and potential health benefits of berry (poly)phenols.

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Chapter 4: Bioactivity of in vivo digested and in vitro fermented raspberries

Introduction:

Epidemiological studies have attributed a wide range of health benefits to a diet rich in fruit and vegetables, including, decreased risk of cancer, cognitive disorders and type-2 diabetes (1-4). These plant based foods contain a variety of bioactive compounds, including fibre, vitamins, minerals and numerous phytochemicals (5). Phytochemicals or (poly)phenols are a diverse group of secondary plant compounds which many health benefits have been attributed (6). The richest sources of dietary (poly)phenols are found in highly coloured fruit, such as berries, which are comprised of a range of (poly)phenolic compounds including, phenolic acids, tannins, stilbenes, flavonoids and ligands (7).

Many studies have demonstrated the anti-cancer properties which these berry (poly)phenols possess including; inhibition of tumour cell proliferation, induction of cell cycle arrest, apoptosis, anti-angiogenesis and anti-genotoxic activity (8). Such research has used a variety of *in vitro* and *in vivo* models to determine the mechanisms by which berry (poly)phenols induce these chemopreventive and chemoprotective effects. These investigations have primarily focussed on the anticancer properties of berry extracts or purified compounds. For example, black raspberry derived anthocyanins used at 0.5 and 5µg/ml to inhibit the aberrant tumorigenic activity of DNA methyltransferases in Caco-2 and HCT116 cells (9). *In vivo* models have also demonstrated the protective effects of berry extracts, e.g. an AIN-76A diet supplemented with 5% strawberry extract reduced tumour incidence in AOM/DSS treated mice by 66% (10). However, few studies have taken into consideration the effect of digestion and colonic fermentation upon the parent (poly)phenolic compounds.

It is important to consider the bioavailability of these berry (poly)phenols; if bound within the food structure they are of little nutritional value. Although average daily (poly)phenol intake is ~1g, only a small percentage (5-15%) of these compounds are excreted in urine (11). This combined with low levels of parent compounds found within the blood, contributes to the understanding that many dietary phytochemicals have poor bioavailability. Recent studies have developed a greater understanding of the absorption, disposition, metabolism and excretion pathways which play a key role in bioavailability (11, 12).

These compounds are subjected to a series of metabolic events as they pass through the gastro-intestinal tract, which results in the production of secondary metabolites. Although these metabolites are more bioavailable, a substantial portion (~40%) still pass through the small intestine unabsorbed (13). Once in the large intestine these compounds are further modified through the action of a variety of enzymes produced by colonic microbiota. This fermentation process generates a range of new (poly)phenol metabolites which may not have been present in the original berry (14). Colonic epithelial cells are more likely to be exposed to a complex mixture of berry (poly)phenols and secondary metabolites. It is therefore essential to utilise physiological relevant samples when investigating the effect of dietary phytochemicals within *in vitro* models.

To produce physiologically relevant samples, berry enriched ileal fluid was collected from 11 ileostomates following a raspberry feeding study (see Chapter 3 for details), the subsequent samples were representative of raspberries post-gastric digestion. To simulate lower GI digestion, colonic fermentation and further the breakdown of aromatic phenolics to simple phenolics, ileal samples were placed in a 24hr-batch culture model under anaerobic conditions with a controlled pH and temperature (see Chapter 3). A process facilitated by the

presence of colonic microflora, in the form of a faecal inoculum which provided the microorganisms for fermentation (15). This *ex-vivo* model allowed to us determine whether the processes of digestion and colonic fermentation enhanced the anti-cancer properties of berry phytochemicals as demonstrated in other studies (16). Unlike most other investigations, the anti-genotoxic potential of ileal fluid and ileal fluid fermentate (IFF) was assessed *in vitro* using models of both colon cancer (HT29) and normal colonocytes (CCD 841 CoN). The overall aim of this chapter was to assess whether the antigenotoxic properties previously assigned to berry (poly)phenolics is retained following digestion and colonic fermentation.

Methods:

Tissue Culture

Two colonic cell lines were used within this investigation to represent both normal and carcinogenic cells. CCD 841 CoN cells (normal colonic epithelial) were obtained from American Type Culture Collection (ATCC) and cultured in Minimum Essential Media (MEM) supplemented with 10% Foetal Bovine Serum (FBS), 100U/L penicillin/streptomycin, 1% sodium pyruvate, 1% non-essential amino acids. HT29 cells (colonic adenocarcinoma) obtained from European Collection of Cell Cultures (ECACC) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and 100U/L penicillin/streptomycin. Both cell lines were grown as monolayers in roux flasks incubated at 37°C and 5% CO_2 and sub-cultured every 3-4 days or when cells had reached 75% confluence. Cells were passaged by thrice washing with Dulbeco's phosphate-buffered saline (DPBS) and incubation with trypsin (0.25% trypsin-ethylenediaminetetraacetic acid) at 37°C for 5min. Trypsin was neutralised with equal volume of media and cells spun at 1200rpm for 3min, supernatant was removed and cells re-suspended in the fresh growth media. Both cells lines were used between passage 25-40.

Cell treatments

Three types of samples were used to treat the 2 cell lines in this investigation. 22 samples of ileal fluid; 11x 0hr baseline and 11x 8hr post-raspberry consumption were obtained from the raspberry feeding study described in Chapter 3. 44 samples of ileal fluid fermentate (IFF); 11x baseline 0hr fermentation, 11x post raspberry 0hr fermentation, 11x baseline 24hr fermentation and 11x post raspberry 24hr fermentation produced using *in vitro* batch culture (see Chapter 3). Four individual phenolics; 3-(3-Hydroxyphenyl) propionic acid (3HPPA), 3-

phenylpropionic acid (3PPA), 4-Hydroxbenzoic acid (4HBA) and Benzoic Acid (BA) with a concentration range of 10-100µM were chosen based upon compositional analysis of raspberry ileal fluid fermentate (see Chapter 3). Each powdered phenolic was dissolved in 0.5% DMSO v/v DPBS to produce a 5mM stock solution.

Cytotoxicity Assay

To establish a sub-cytotoxic dose for each treatment cell-viability was determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay (14). This assay is based upon the ability of mitochondrial dehydrogenase in living cells to metabolise the pale-yellow tetrazolium salt into dark blue formazan. The number of viable cells is directly proportional to formazan concentration (17). Cells were seeded into 96-well plates at a concentration of $1.5x10^4$ HT29 cells per well or $3.0x10^4$ CCD 841 CoN cells per well and incubated for 48h at 37℃. Media was then removed and replaced with treatment; 10, 25 or 40% v/v Ileal Fluid with media, 10, 25 or 40% v/v IFF with media or 10-100µM individual phenolic in media. After 24h treatment was removed, cells washed with DPBS and incubated for a further 4hr with 100µl fresh growth media and 15µl MTT dye (5mg/ml in DPBS). 100 µl of solubilising solution was added to each well, releasing the formazan product from cells. The concentration of the formazan was measured on a microtiter plate reader (Alpha, SLT Rainbow Thermo) at 570nm with a reference of 650nm. Each plate had an untreated control (growth media only) and a positive cytotoxic control (500 µM deoxycholic acid). Due to the limited volume of Ileal Fluid and IFF 1 subject was chosen to assess potential cytotoxic effects of each treatment. Only CCD 841 CoN cells were used to determine IFF cytotoxicity because of the finite amount of sample. Cytotoxicity of ileal fluid and individual phenolics was examined in both cell lines. Each treatment was replicated in

quadruple and each experiment repeated independently 3 times. Mean results were expressed as % cell survival normalised to the untreated control.

COMET Assay

The anti-genotoxic effects of each treatment was assessed using this well-established model for colonocyte DNA damage (18). Both HT29 and CCD 841 CoN cells were incubated for 24hr at 37℃ with either 20% Ileal Fluid v/v in media, 20% IFF v/v in media or 10-100µM individual phenolic in media. Cells were then harvested and placed under oxidative challenge by treatment with hydrogen peroxide (75 µM for HT29 and 25 µM for CCD 841 CoN) for 5min at 4℃. Each experiment included a negative control (DPBS) and positive known anti-genotoxic control (100nM CDDO). After centrifugation at 1500rpm, 4℃ for 10min, supernatant was discarded and cell pellet re-suspended in 0.85% low melting point agarose and added to pre-cast 1% normal-melting point agarose beads on fully frosted slides. Slides were placed in lysis buffer (2.5M NaCl, 100mM Na_2EDTA, 10mM Tris HCl) for 1hr at 4℃ to disrupt cellular and nuclear membranes. Immersion in an alkaline buffer (0.3M NaOH, 1mM EDTA) for 20min at 4℃ allowed DNA un-coiling prior to electrophoresis at 25V, 300mA for 20min. Gels were washed 3x 5min in a neutralisation buffer (0.4M Tris-HCl, pH 7.5) and stained with 20µl of ethidium bromide (2µg/ml in distilled water). Slides were analysed using Nikon eclipse 600 epi-fluorescent microscope at 400x total magnification. Images were scored using Komet 5.0 software (Kinetic Imaging Ltd, Liverpool, UK), a total of 50 cells per slide with a mean % tail DNA calculated from triplicate gels. Each experiment was repeated 3 independent times.

Statistical Analysis

Data was analysed as the mean of 3 independent experiments. D'Agostino & Pearson omnibus was used to test for normality. Analysis of variance was applied to test for significant differences between means and assessed with Dunnett's Multiple comparison post-hoc test. Significance was accepted at $p<0.05$. Analysis was performed using Prism 5 (version 5.01 for Windows).

Results:

Cytotoxicity

To establish a non-cytotoxic dose of each treatment a MTT cytotoxicity assay was performed, measuring cell viability after 24hr incubation with each treatment. Extracts which resulted in a loss of cell viability greater than 50% were considered highly cytotoxic.

No significant cell loss was observed for either baseline and post-berry ileal fluid at 10, 25 and 40% (v/v) when compared to media control (Abs= 1.45±0.05) (Figure 1A). However, when tested with CCD 841 CoN cells the Ileal Fluid demonstrated significant cell death at 40%. Baseline ileal fluid reducing cell viability to 54% and post-raspberry ileal fluid reducing cell viability to 62% when compared to media control (Abs= 1.39±0.03) (Figure 1B). Based on these results the sub-cytotoxic concentration of 20% ileal fluid (v/v) with media was selected for subsequent assays.

Raspberry IFF, both 0hr fermentation and 24hr fermentation showed no cytotoxicity upon CCD 841 CoN when incubated for 24hr at 10, 25 or 40% (v/v). Pre-berry 24hr ileal fluid fermentate had no significant effect on cell viability. In contrast pre-berry 0hr IFF at 40% (v/v) significantly decreased cell viability to 42% when compared to the media control (Abs= 0.87±0.08) (Figure 2). As a consequence, 20% was selected as the appropriated concentration to assess anti-genotoxicity.

The individual phenolics, 3HPPA, 3PPA, 4HBA and BA showed no evidence of cytotoxicity when pre-incubated for 24hr with HT29 (Abs=1.68±0.22) or CCD 841CoN (Abs=0.79±0.08) at any of the chosen concentrations of 10, 50 or 100μM (Figure 3). Further experiments could be performed using all the above concentrations.

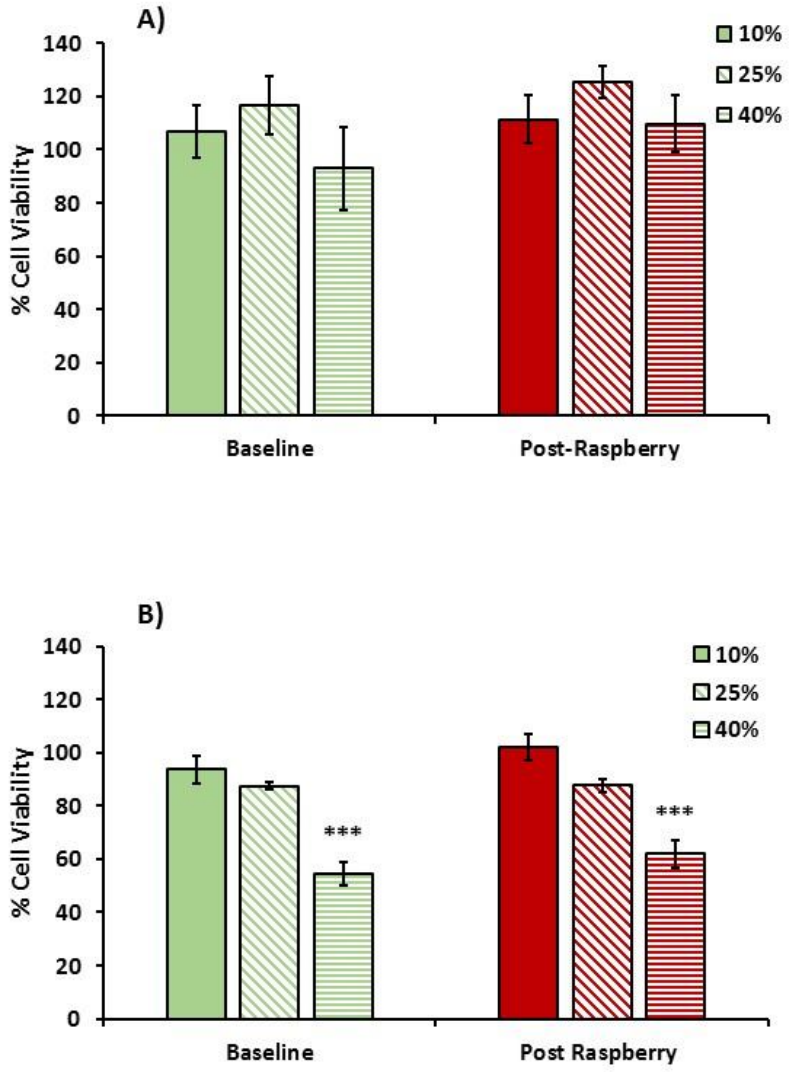

Figure 1: Cytotoxic effect of different concentrations of Raspberry Ileal Fluid (10, 25, 40%) on HT29 cells (A) and CCD841 cells (B). Data presented is mean of 3 independent experiments +SD. One-way ANOVA and Dunnett's Multiple Comparisons test, ***$p<0.001$, significance is compared to media control.

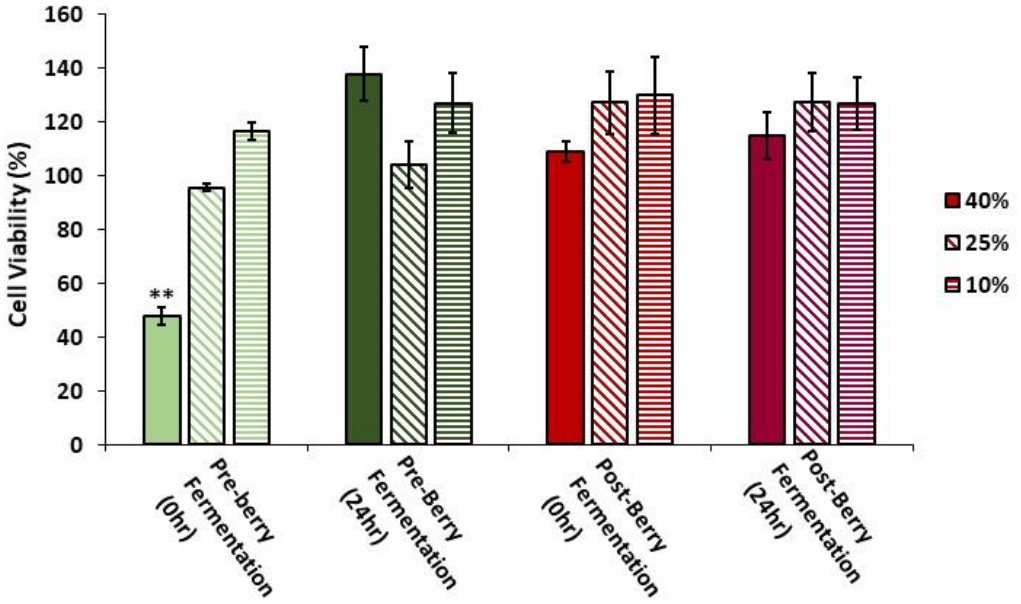

Figure 2: Cytotoxic effect of different concentrations of Raspberry Ileal Fluid Fermentate (10, 25, 40%) on CCD841 cells. Data presented is mean of 3 independent experiments +SD. One-way ANOVA and Dunnett's Multiple Comparison test, **$p<0.01$, significance is compared to media control.

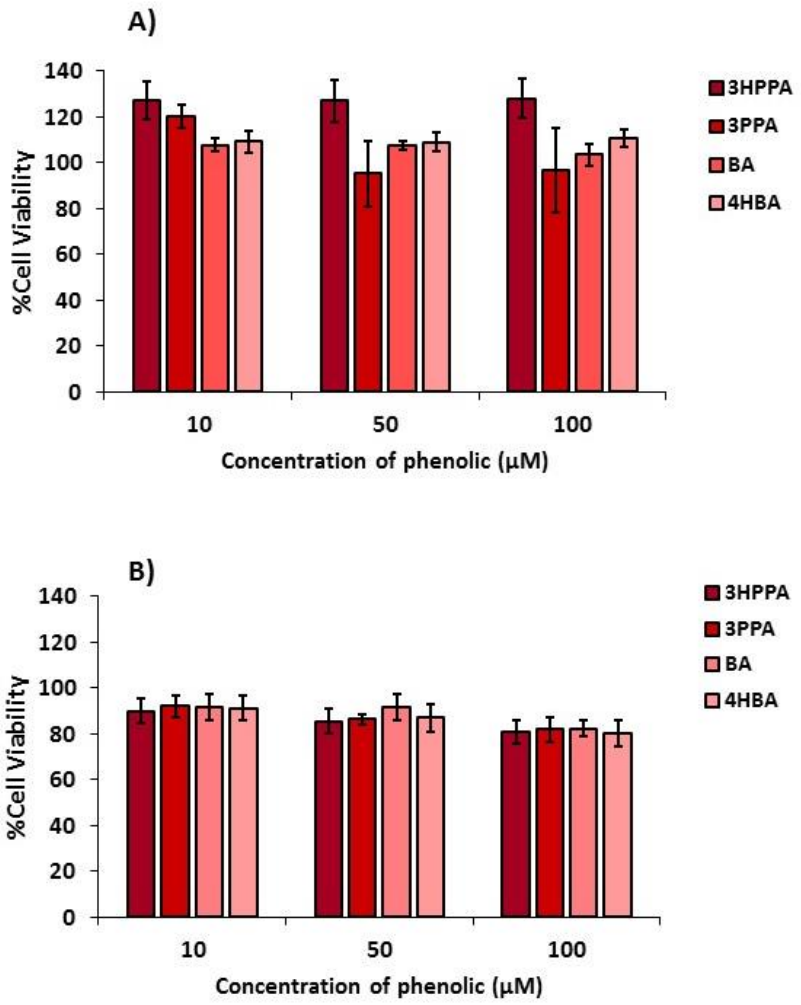

Figure 3: Cytotoxic effect of different concentrations of 4 individual phenolics on HT29 cells (A) and CCD841 cells (B). Data presented is mean of 3 independent experiments +SD. One-way ANOVA and Dunnett's Multiple Comparison test. No Cytotoxic effects found after 24hr pre-incubation when compared to media control.

Anti-genotoxic activity

All the treatments described above; post-berry ileal fluid, post-berry IFF and individual phenolics resulted in some degree of anti-genotoxic activity within both cell lines.

When compared to corresponding baseline ileal fluid five of the eleven post-raspberry ileal fluid samples demonstrated anti-genotoxic activity, in HT29 cells challengend with 75µM H_2O_2 (Figure 4A). Subjects 1, 3, 4, 8 and 10 all showed significant ($p<0.001$) decrease in % tail DNA. Treatment with subject 1 created the most dramatic reduction of damage ~50% compared to subjects 5 and 12 which demonstrated no anti-genotoxic activity. The average reduction of all 11 subjects ~23%. When used to pre-treat CCD 841 CoN cells for 24hr prior to challenge 25µM H_2O_2 , 8/11 post-berry ieal samples reduced DNA damage, compared to base line ileal fluid (Figure 4B). Subjects 1, 2, 4, 6, 8, 10 and 11 significantly decreased % tail DNA. As with HT29 cells the greatest reduction in damage was from subject 1 with a 40% decrease in tail DNA. The average reduction of tail DNA within this cell line was similar to that of HT29 at 25%. Four of the 11 subjects (S01, S04, S08 & S10) resulted decreased DNA damage in both cell lines.

Due to the limited volume of IFF the anti-genotoxic activity of these samples was only tested on CCD 841 CoN cells. A total of 7 out of the 11 subjects post- berry 0hr IFF samples reduced DNA damage when pre-incubated for 24hr prior to oxidative challenge (Figure 5A). Subjects (2, 3, 5, 8, 11) all significantly decreased % Tail DNA ($p<0.001$) with a further 2 subjects (S04 and S10) reducing damage ($p<0.01$) when compared to pre-berry 0hr IFF. (S08) post-berry showed the greatest reduction of DNA damage by 27, an average decrease of 20% across all 11 subjects. (S02, S04, S8, S10 and S11) retained their anti-genotoxic activity from post-raspberry ileal samples. Two samples proved to be cytotoxic, (S01) pre-berry 0hr IFF and (S12) post-berry IFF caused significant cell death, therefore no statistical analysis could be performed on these two.

After 24hr fermentation (Figure 5B) 9 out of the 11 post-raspberry IFF samples reduced DNA damage when compared to pre-berry IFF 24hr samples. (S01, S02, S03, S04, S05, S06, S08, S11 and S12) significantly decreased % tail DNA ($p<0.001$). (S12) produced the greatest reduction with a 47% decrease in % tail DNA and an overall average reduction of 30% for all 11 subjects. Four of the 11 (S02, S04, S08, 11) subjects maintained anti-genotoxic properties from 0-24hr fermentation and a further 2 maintained activity from ileal fluid (S01 and S08).

Antigenotoxic activity of the four individual phenolics; 3HPPA, 3PPA, 4HBA and BA, was assessed over a range of concentrations (10, 50 and 100µM) in both cell lines HT29 (Figure 6) and CCD 841 CoN (Figure 7). All four phenolics reduced DNA damage across both cell lines but to differing effects. When compared to medi control, pre-incubation of 3HPPA for 24hr at all 3 concentrations significantly reduced % Tail DNA in HT29 cells. The 50µM concentration was the most effective, reducing DNA damage by 31% ($p<0.001$). 3PPA treated HT29 cells only proved significantly antigenotoxic ($p<0.001$) at 50µM reducing DNA damage by 36%. 4HBA and BA reduced % Tail DNA across all 3 concentrations. 10µM 4HBA created 44% decrease in damage with 50 and 100µM 4HBA producing 40% reduction. BA at 100µM was the most effective of all the phenolic treatments for HT29 cells with 47% decrease in tail DNA, while 10µM BA reduced damage by 15% and 50µM by 32%. BA was the only individual phenolic that followed a dose dependent respond within HT29 cells.

CCD 841 CoN cells responded in a dose dependent manner when treated wih any of the individual phenolics, and as a consequence 100µM concentration produced the greatest antigenotoxic activity. Treatment with 3HPPA significantly decreased % Tail DNA at 10µM ($p<0.05$), 50µM ($p<0.05$) and 100µM ($p<0.001$), with 100µM producing a ~55% reduction in DNA damage. 50µM and 100µM concentrations of 3PPA resulted in signifcant

antigenotoxic activity ($p<0.001$) within CCD 841 CoN cells creating ~40% and ~50% decrease in tail DNA. 4HBA resulted in a significant reduction in DNA damage ($p<0.001$) at all 3 concentrations with a ~36% decrease of tail DNA at 10µM and 50µM, and ~50% decrease at 100µM. The pattern was repeated with BA 10µM ($p<0.01$), 50µM ($p<0.001$) and 100µM ($p<0.001$) all resulting in significant anti-genotoxic activity. 10µM produced a ~21% decrease, 50µM a ~32% decrease and 100µM a ~52% decrease of Tail DNA. All of the phenolics result in a greater decrease of % Tail DNA in CCD841 than HT29.

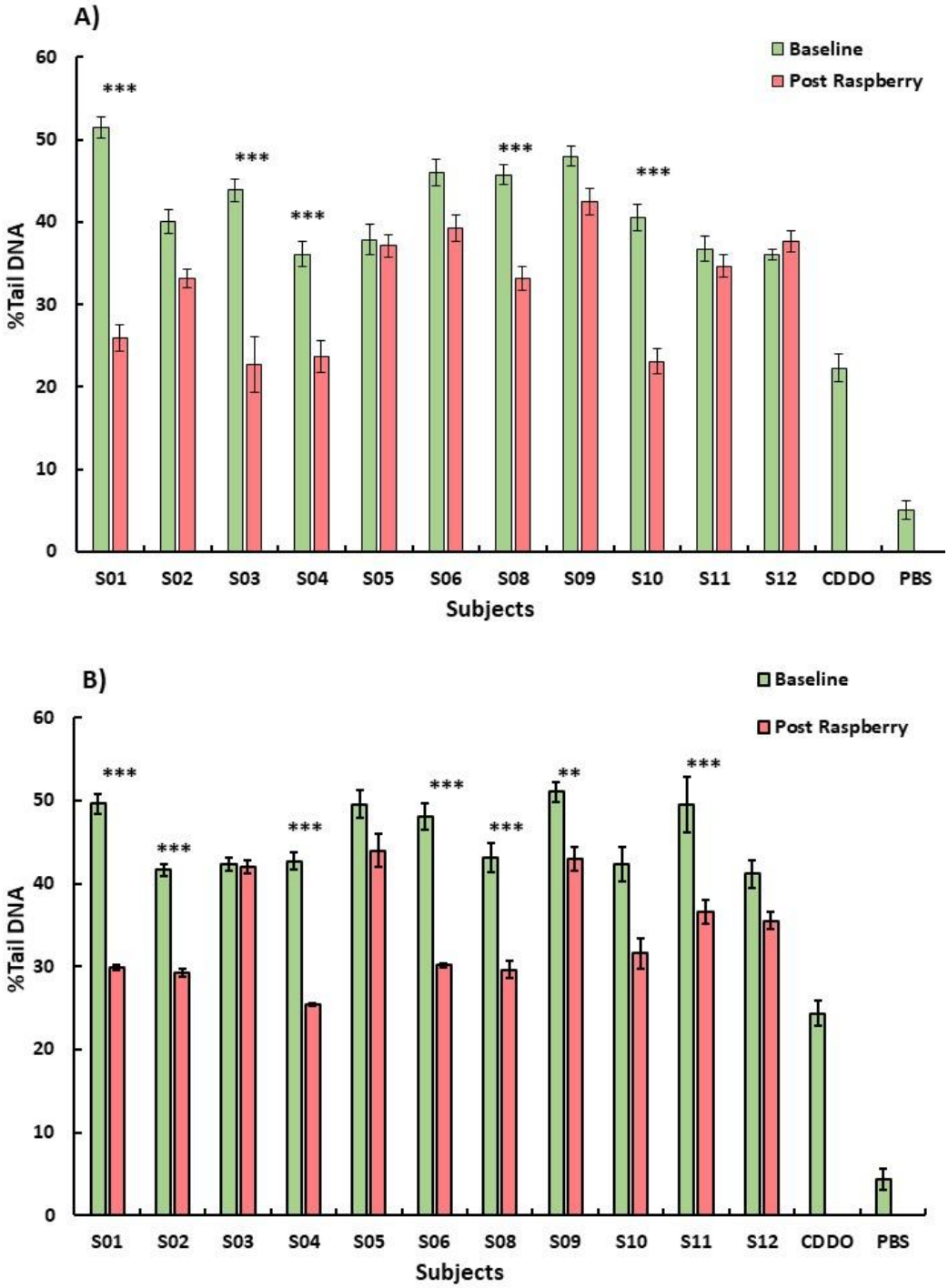

Figure 4: Anti-genotoxic effects of Ileal fluids both Baseline and Post-Raspberry feed at 20% ileal (v/v) in growth media, after 24hr pre-incubation at 37℃ on A) HT29 cells challenged with 75µM H_2O_2 and B) CCD 841 cells challenged with 25µM H_2O_2. Postive control CDDO, Negative control PBS. Data presented is as mean of 3 independent experiments +SD. One-way ANOVA and Bonferroni's multiple comparisons test, **p<0.01, ***p<0.001, significance is compared to baseline.

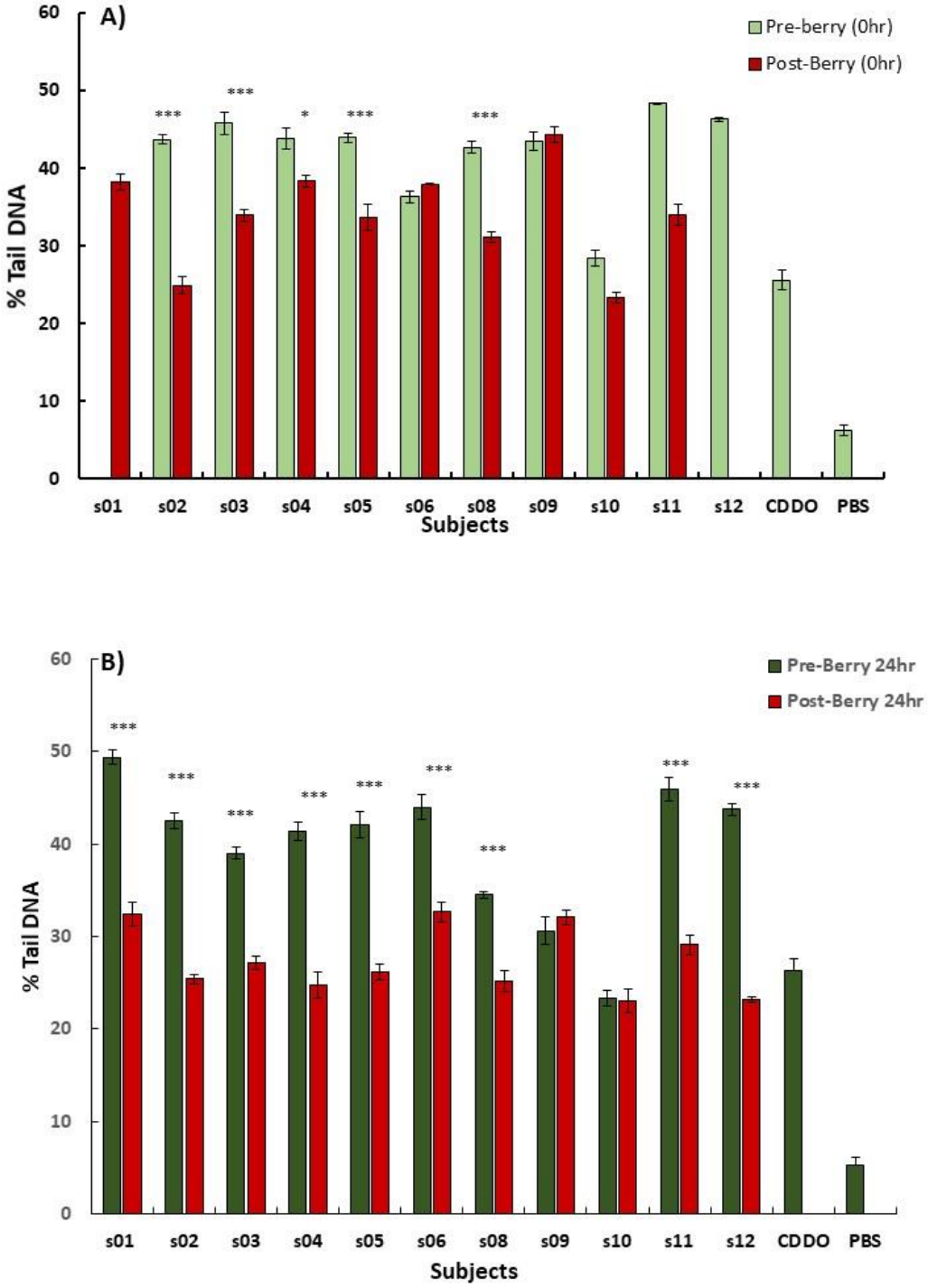

Figure 5: Anti-genotoxic effects of baseline and post-raspberry feed Ileal fluid fermentate A) 0h fermentation and B) 24h fermentation at 25% ileal (v/v) in growth media, after 24hr pre-incubation at 37℃ on CCD 841 cells challenged with 25µM H_2O_2. Postive control CDDO, Negative control PBS. Data presented is as mean of 3 independent experiments +SD. One-way ANOVA and Bonferroni post-tests, *<p0.05 **p<0.01, ***p<0.00, significance is compared to baseline.

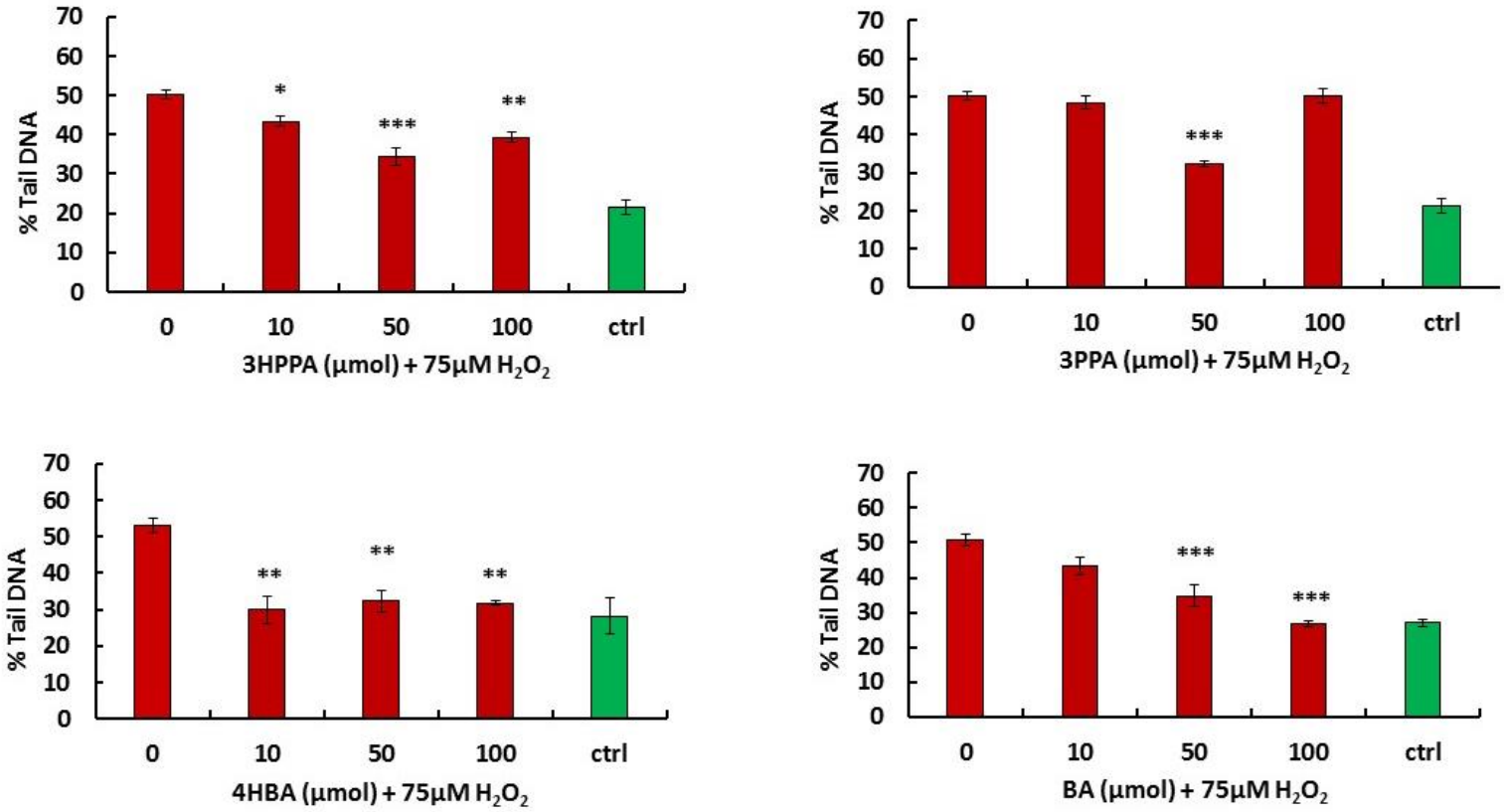

Figure 6: Anti-genotoxic effects of individual phenolics at a range of concentrations (0-100µM) after 24hr pre-incubation at 37℃ on HT29 cells challenged with $75H_2O_2$. Data presented is as mean of 3 independent experiments +SD. One-way ANOVA and Dunnett's Multiple Comparison test, *<p0.05 **p<0.01, ***p<0.001, significance is compared to media control.

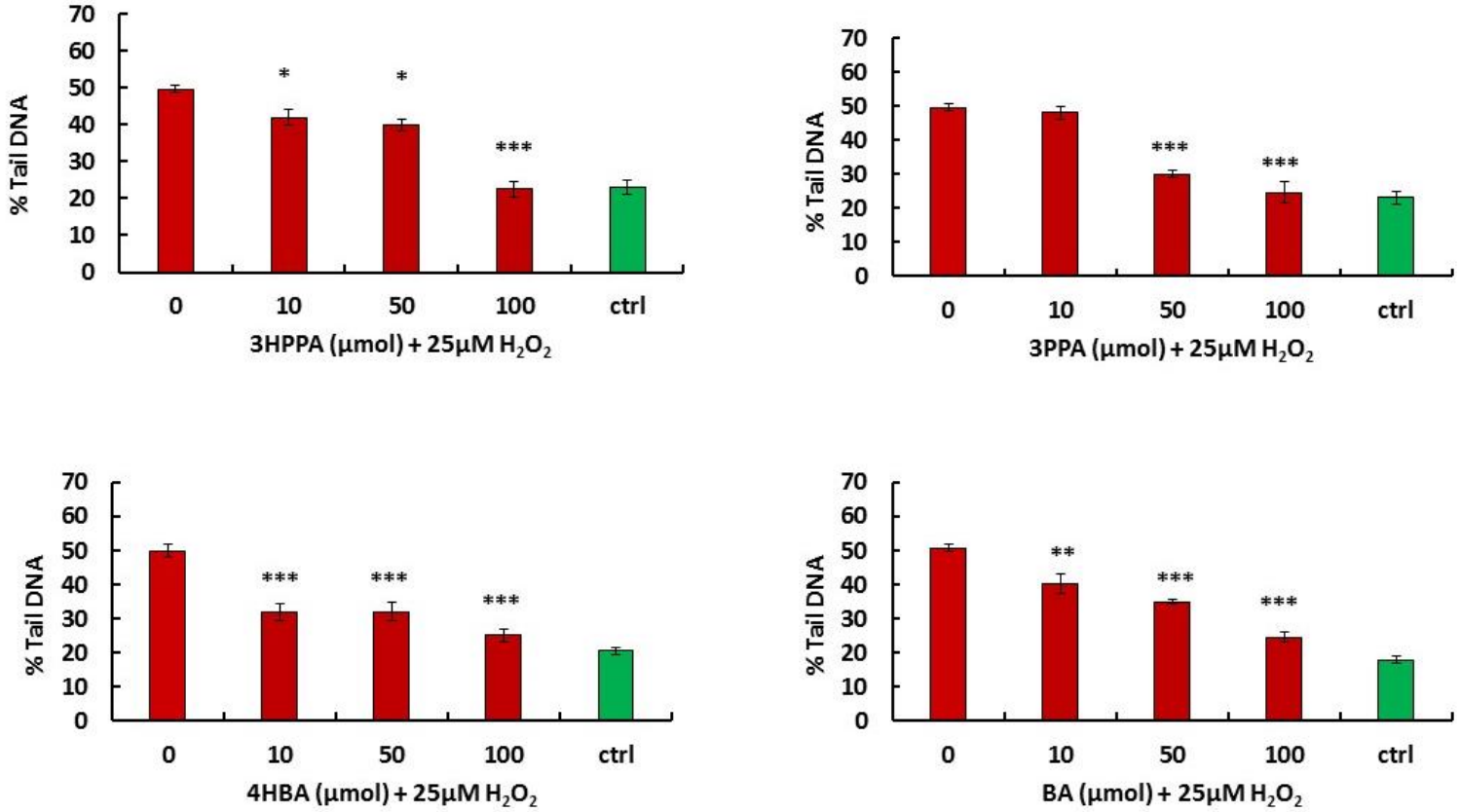

Figure 7: Anti-genotoxic effects of individual phenolics at a range of concentrations (0-100µM) after 24hr pre-incubation at 37℃ on CCD 841 cells challenged with 25H_2O_2. Data presented is as mean of 3 independent experiments +SD. One-way ANOVA and Dunnett's Multiple Comparison test, *<p0.05 **p<0.01, ***p<0.001, significance is compared to media control.

Discussion:

In this chapter we have demonstrated that the antigenotoxic potential of berry phytochemicals is retained following *in vivo* digestion and *in vitro* fermentation. These protective effects were observed in both models of colon cancer and normal colonic epithelium. Using the same model, we have illustrated the anticancer action of a range of berry phenolics at concentrations of 10, 50 and 100μM, comparable to those found after fermentation (19, 20). Previous studies have used a range of *in vitro* models to identify the chemopreventive and anti-genotoxic potential of purified phenolics and berry extracts (16). To the best of our knowledge this is the first study that utilised both *in vivo* digestion and an *ex vivo* fermentation model to assess the anti-genotoxic potential of these berries and their metabolites. Few investigations have utilised physiologically appropriate samples and therefore the data presented here can be considered more relevant for further investigation (21-23).

The human digestive tract can be sub-divided into 4 distinct processes which correspond to; the mouth, the stomach, the small intestine and the large intestine. As food passes through this system it is altered by a variety of mechanisms from physical cutting and grinding of the teeth, acid hydrolysis in the stomach, enzymatic reactions in the small intestines and anaerobic fermentation in the colon. This complex multistep procedure releases the nutrient from the food matrix making them more readily bioavailable (24). Many investigations have used *in vitro* models based on the work of Gil-Izquierdo *et al*. to simulate the process of berry digestion and the release of (poly)phenolic compounds (25). There have been discrepancies in the reported (poly)phenol bioavailability when utilising these *in vitro* models of digestion. McDougall *et al*. reported bioavailability of ~10% for (poly)phenols following *in vitro*

digestion of raspberries (26). In contrast the 2-step model utilised by Bermúdez-Soto *et al*. to digest chokeberries, recorded bioavailability of 15-30% for individual berry (poly)phenols (27). These approaches to *in vitro* digestion have provided a necessary starting point to gain an understanding of (poly)phenolic degradation, however such models can only go so far in simulating the dynamic nature of the human gastrointestinal tract. It was therefore decided a human feeding study would produce more biologically relevant samples and be of greater benefit to our investigation.

The human feeding studying (described in chapter 3) produced samples which more accurately represent the process of digestion. In the mouth, food is subjected to mechanical processing, cutting and grinding by the teeth, followed by interactions with salivary amylases. As our participants consumed a raspberry puree the meal would have been swallowed with little oral processing. The stomach is a highly acidic (pH 2) vessel into which a variety of gastric enzymes are secreted e.g. pepsin, lipases and amylases which act upon the food matrix (24). Most (poly)phenols are released from their plant structure within the gastric phase of digestion, digestive enzymes in conjunction with peristaltic movement and low pH break (poly)phenol crosslinks with carbohydrates, organic acids and other (poly)phenols (28). The digesta is subjected to further breakdown within the small intestine as pH is increased to 7 and a series of enzymes are secreted by the pancreas and bile (28). These changes in conditions convert anthocyanins into colourless chalcone pseudobases and phenolic compounds are deglycosylated and hydrolysed to produce aglycones (29). It is this stage of digestion which (poly)phenols may be absorbed across the brush border of the small intestine. Metabolites can pass through the portal vein to the liver, undergo further metabolism and may be recycled back into the small intestine (30). Although a variety of

(poly)phenols are absorbed in the small intestine analysis of ileal fluid (see chapter 3) indicates that a substantial portion passes into the large intestine.

This process of degradation continues within the large intestine, where conjugated moieties are cleaved by colonic microbiota and converted into simple and aromatic phenolics (12). This exposes the epithelial cells within the colon to a complex mixture of (poly)phenolic compounds. Unlike ileal fluid it is not possible to obtain a biological sample; in order to produce physiologically representative samples the collected ileal fluids where subjected to *in vitro* fermentation (see chapter 3). This batch culture model consisted of incubating the ileal fluids with basal media in the presence of 10% faecal slurry, at 37℃ and in anaerobic conditions. The faecal inoculum provided a source of colonic bacteria which acted upon the (poly)phenols present within each sample. This model produces samples with compositions comparabl to that found *in vivo.* Few studies have utilised batch culture models to produce biologically relevant samples, for example Mosele *et al.* used *in vitro* digestion and fermentation models to study the stability of pomegranate (poly)phenols, while Correa-Betanzo *et al.* assessed the biological activity of colon-available wild blueberries phenolics with a similar two-step *in vitro* process (31, 29). Other studies such as Pereira-Caro *et al.* simply assessed the colonic catabolism of individual (poly)phenols rather than the breakdown of the whole fruit (32). The compositional analysis described in chapter 3 demonstrated the difference between ileal fluid and ileal fluid fermentate while emphasising the significance of using both for further investigation and the need to assess antigenotoxic potential of both types of sample.

When compared to the original raspberry extract, compositional analysis of post-berry ileal fluids revealed an overall loss of total anthocyanin and total ellagitannin content after transit through the upper gastrointestinal (see chapter 3). Even with these changes in (poly)phenolic content antigenotoxic activity was observed in HT29 cells treated with 5/11 ileal samples and

in CCD841 CoN cells treated with 8/11 ileal samples. These results indicate that the antigenotoxic properties previously attributed to berries and berry extracts are retained following digestion (33).

The bioactivity of *in vitro* digested (poly)phenols has previously been examined by Chen *et al.* whom conducted 2 investigations, one with blackberry extracts and one with raspberry extracts (23, 34). They concluded that the berry digesta protected Caco-2 cells against Ethyl Carbamate induced damage, reduced nuclear fragmentation and scavenged ROS more effectively than their equivalent berry extract. In contrast to our results this study observed an increase in the total phenolic content from 118.43mg/GAE to 254.60mg/GAE following digestion and attributed the increased antigenotoxic activity to the additional bioactive components. Marhuenda *et al*. demonstrated a reduction in total (poly)phenolic and anthocyanin content following *in vitro* digestion of strawberries, blackberries, raspberries and blueberries (35). There was a correlation between the phenolic content of the samples and the antioxidant activity, the greater the concentration of phenolics the greater the antioxidant effects. Although the original extracts had greater antioxidant potential than the digesta, (poly)phenol concentrations remained high enough to exert a positive effect. As our study has no direct comparison to the antigenotoxic properties of the original raspberry extract it is not possible to make such a conclusion. However these results however do confirm that gastrointestinal digestion creates (poly)phenol breakdown products which have significant protective effects against oxidative challenge.

The antigenotoxic potential displayed by raspberry enriched ileal fluid samples was maintined following *in vitro* fermentation. Although the samples have been subjected to further degradation with the (poly)phenolics converted to simple and aromatic compounds, the fermentate samples produced following this process still possess anti-cancer activity. Post-berry 24hr IFF samples showed a similar reduction in DNA damage as post-raspberry

ileal fluid samples. As pre-treatment for CCD 841 CoN cells prior to H_2O_2 challenge, 9 out of 11 IFF samples significantly reduced DNA damage when compared to pre-berry IFF samples, with an average decrease of 29%. A total of 8/11 post-raspberry ileal fluid samples decreased single strand DNA breaks, with an average reduction of 25% when compared to corresponding baseline ileal fluid. On face value these results suggest that the fermentation process has had an insignificant effect upon the antigenotoxic potential of the ileal samples, however the pattern of samples which demonstrate protective benefits differed within the treatment groups. Only 6 of the 11 subjects produced both post-raspberry ileal samples and post-berry 24hr IFF samples which reduced DNA damage ((S01, 02, 04, 06, 08 and 11). A further 2 post-raspberry ileal samples (S09 and 10) significantly decreased %Tail DNA while, 3 other post-berry 24hr IFF samples proved to be cytoprotective (S03, S05 and S12). The variation found within the two sample groups can partly be explained on the differences in phenolic composition, as Chapter 3 demonstrated the post-berry ileal fluid is comprised primarily of anthocyanins, ellagic acid and ellagitannins while the post-berry 24hr IFF samples consisted of a range of simple phenolics including BA, 4HBA, 3PPA an 3HPPA.

Previous research by Brown *et al.* showed more dramatic antigenotoxic effects when assessing the bioactivity of lingonberry digest and fermentate (36). HT29 cells pre-treated with 13µg/ml GAE *in vitro* digest or 16µg/ml GAE *in vitro* fermentate reduced % tail DNA after H_2O_2 by ~50% when compared to untreated control samples, a much greater reduction than what the raspberry enriched samples from our study produced but when examining the antigenotoxic effects of lingonberry enriched ileal fluid they only produced ~14% reduction in DNA damage. It appears in this instance the *in vitro* digestion model may result in an over estimation of the antigenotoxic potential of berry metabolites. This is most likely due to the difference in (poly)phenol content, with ~3% total anthocyanin content in the ileal fluid but 25% in the *in vitro* digested sample.

The importance of phenolic content in relation to anti-carcinogenic activity was previously identified when comparing the bioactivity of *in vitro* digested and fermented blueberries to the original fruit extract (29). Following digestion, a decrease of ~50% in total (poly)phenols and ~80% anthocyanins corresponded with a decrease in DDPH and superoxide activity, while the fermentate sample lacked any significant scavenging activity. It is also important to note that the blueberry digest and fermentate inhibited cell proliferation in HT29 cells. A pattern that may reflect why the two 0hr IFF samples from our study demonstrated cytotoxicity. It is probable that the that additional components from the basal media, faecal inoculum and bile salts results in cellular inhibition and cell death.

The antigenotoxicity of 4 individual phenolics; BA, 4HBA, 3HPPA and 3PPA, which were increased in over half the raspberry enriched IFF samples was assessed in both the normal and carcinogenic cell lines. As the results above indicate all 4 phenolics had the potential to reduce H_2O_2 induced DNA damage. The concentration chosen for investigation were based on the values found after compositional analysis (see chapter 3) and those from previous raspberry studies (20, 21). The pattern above suggests that the individual phenolics are more effective at reducing DNA damage, with overall % reduction almost double that of the raspberry enriched ileal and IFF. The results from Taner *et a*l. who demonstrated the antigenotoxic potential of 2 individual phenolics, vanillic acid and cinnamic acid reflected our findings (37). After pre-incubation with either compound H_2O_2 induced damage in lymphocytes was reduced by 40-50%. These extracts, even when used at a physiologically relevant concentration, may produce greater anti-carcinogenic effects than the berry enriched ileal and IFF because they are pure samples and do not contain any biological contaminants. One possibility to consider for future study however is the synergistic effect of multiple pure phenolic extracts, which appears to play a key role in the action of ileal and IFF samples.

We have demonstrated that the berry-enriched ileal fluid, IFF samples and individual phenolics at biologically relevant concentrations, have the ability to protect against H_2O_2 induced damage in a model that reflects the initiation of tumour development. As cells where washed prior to Comet Assay, removing any remaining treatment, it is the phenolic compounds which have entered the cells are stimulating this protective effect. They are not acting as direct antioxidants but rather mediating a cellular response against the challenge. The potential mechanism by which this activity is not fully understood and is the subject of chapter 5.

In conclusion, this investigation has shown that biologically relevant dietary (poly)phenols and phenolic metabolites from berry enriched ileal fluid and IFF can modulate cellular processes in both colon cancer cells and normal colonocytes. The *ex-vivo* model which combined digestion and fermentation produced samples representative of those compounds found in the colon following raspberry consumption. We have demonstrated that although the berry phytochemicals undergo significant changes as digestion progresses they still retain their antigenotoxic capacity. Compositional analysis has also identified 4 colon-available individual phenolics which show greater anti-cancer properties than the IFF. Our investigation, supported by that of others, indicates that biologically relevant berry metabolites may be able to reduce the risk of CRC development.

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Chapter 5: The role of Nrf2-ARE pathway in antigenotoxicity

Introduction:

Dietary phytochemicals are compounds associated with many health benefits. This diverse group of bioactive compounds are found in a wide variety of fruit and vegetables and warrant considerable investigation as a strategy for reducing cancer incidence (1). (Poly)phenols are one category of phytochemical which have demonstrated anti-carcinogenic properties and the ability to promote antigenotoxic activity (2, 3). Chapter 4 demonstrated the ability of simple phenolics, berry enriched ileal and ileal fluid fermentate (IFF) to reduce H_2O_2 induced DNA damage. Normal colonic cells (CCD 841 CoN) and colonic adenocarcinoma cells (HT29) pre-treated with these samples showed significant decrease in single strand DNA breaks when compared to media control samples. This antigenotoxic activity is corroborated by several previous studies, including, Giampieri *et al*., Ceccarini *et al*., and Brown *et al*., who have shown the ability of a range of berry extracts (strawberry, goii berry and lingonberry) to protect cells in response to oxidative stress and prevent DNA damage (4, 5, 6).

Although many investigations have identified the antigenotoxic properties of berry phytochemicals, the mechanism of action behind these protective effects has yet to be fully elucidated. One pathway of particular interest is the activation of the Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) – Antioxidant Response Element (ARE) system. Nrf2 is a transcription factor that regulates the expression of a range of antioxidant and phase II detoxifying enzymes. ARE is the promoter region found within the DNA sequence coding for these protective proteins. This system is activated in response to oxidative or electrophilic stress and results in the up-regulation of a range of genes including; NAD(P) H:quinone oxidoreductase 1 (NQO1), heme oxygenase 1 (HO-1), glutathione S-transferase (GST) and glutamate-cysteine ligase (GCL), each of which help maintain cellular homeostasis (7). Due to the ability to regulate the expression of a variety of antioxidant and anti-genotoxic genes, Nrf2 is considered a promising target for the prevention of carcinogenesis (8).

NQO1 and HO-1 are two of the genes activated by the Nrf2-ARE pathway, which we have chosen to investigate. NQO1 is a cytosolic flavoenzyme with multiple biochemical roles, which can protect against cancer promotion and initiation (9, 10). The main role of this two-electron reductase is the detoxification of highly reactive quinone substrates that can form ROS and induce carcinogenesis and neurodegeneration. Quinones are reduced to more stable hydroquinones which undergo conjugation and are more easily excreted from the body (11). This reductase activity aids in maintaining endogenous antioxidants by reducing them to their active form, such as, α-tocopherolquinone to α-tocopherolhydroquinone which protects against lipid membrane peroxidation (10, 12). The tumour suppressor p53 is critical in the control of carcinogenesis, but under normal conditions it is rapidly degraded. NQO1 stabilises this protein, promoting the accumulation of p53 and maintaining anti-cancer action. A more direct role, is the ability of the NQO1 protein to scavenge superoxides in an NAPDH-dependent manner and therefore reduce the amount of cellular ROS (10, 13). HO-1 is one of two heme-oxygenase isoforms found in mammals which catalyse the degradation to heme to, biliverdin, carbon monoxide and iron. The combined effects of the removal of heme, a pro-oxidant iron chelate, and the generation of biologically active end products contribute to HO-1's cytoprotective activity (14, 15). The released carbon monoxide exerts potent anti-inflammatory properties, while bilverdin and bilirubin act as peroxy radical scavengers (16, 17). Under oxidative stress, HO-1 translocates to the nucleus, interacts with and stabilises Nrf2. This process results in further transcriptional activation of phase II antioxidant and detoxifying genes (18). There is considerable evidence that these two genes and their subsequent translated proteins are up-regulated in response to dietary phytochemicals (19).

Many (poly)phenols have been found to regulate the Nrf2/Keap1 pathway, either indirectly by activating other cellular modulators or by directly interacting with the protein complex. Phytochemicals can activate and up-regulate a range of different protein kinases and induce

post-translation regulation of Nrf2 (20). Quercetin (3,3',4',5,7-pentahydroflavone) is a flavonoid extract found in a range of fruit and vegetables, particularly in red wine and teas, which has previously demonstrated the ability to induce Nrf2 pathway. Treatment of L-02 (normal human liver) cells with 50μM quercetin induced the phosphorylation of JNK (c-Jun N-terminal kinase), ERK1/2 (extracellular regulated kinase) and p38 MAPK (mitogen-activated protein kinase) in a time dependent manner. Cells in which these kinases where inhibited did not respond in the same manner when treated with quercetin and showed a reduced expression of HO-1, GCLC (catalytic subunit of glutamate-cysteine ligase) and GCLM (modify subunit of glutamate-cysteine ligase), thus proving their importance in the Nrf2/ARE pathway (21). The true role of these kinases with regards to Nrf2 pathway is not fully understood, but appears to be dependent upon which specific protein kinase is activated. PKC (protein kinase C) activates Nrf2 by phosphorylating Ser40 located in the binding domain between Nrf2-ARE, disrupting this interaction and promoting translocation (22). Whereas, PI3K (phosphoinostide 3-kinase) which is activated in response to oxidative and electrophilic stimuli, causes the depolymerisation of actin microfilaments and facilitates the nuclear translocation of Nrf2 (23).

Alternatively, the (poly)phenolics can interact directly with the Nrf2-Keap1 binding complex. Both quercetin and Epigallocatechin-3-gallate (EGCG) can bind to and modify the cysteine residues within Keap1, resulting in dissociation of Nrf2, preventing proteasomal degradation and promoting nuclear translocation (7, 23, 24). Curcumin a diarylheptanoid found in the rhizomes of turmeric, is an extensively studied activator of Nrf2. This curcuminoid compound contains 2 unsaturated carbon groups that act as Michael acceptors and interact with the cysteine residues of Keap1, causing the dissociation of Nrf2 (25, 26).

As the evidence above illustrates, several studies have demonstrated the role of individual (poly)phenols or (poly)phenolic extracts upon the Nrf2-ARE system. Some investigations

have examined the effects of gut metabolites and berry breakdown products such as protocatechuic acid (3,4-dihydroxybenzoic acid) or phloroglucinol aldehyde (2,4,6-triddroxybenzaldhye) upon this pathway and established that these secondary metabolites retain the potential to activate Nrf2 and its down-stream genes. However, are these samples truly representative of the compounds which come in contact and interact with the colonic epithelium? Chapter 3 describes how we produced biologically relevant samples, comprised of compounds found after gastrointestinal digestion and colonic fermentation of raspberries. Compositional analysis revealed that they contained a wide range of berry metabolites including; benzoic acid (BA), 4-hydroxyphenyllatic acid (4HPL) and 3-(3-hydroxyphenyl)propionic acid (3HPPA) at various concentrations. Within this investigation we will determine whether the structural changes to the raspberry (poly)phenols that occurred during *in vivo* digestion and *in vitro* fermentation alters the ability to induce Nrf2 activity.

Real time PCR (qPCR) was used to examine the change in expression of Nrf2 and two of its downstream targets, NQO1 and HO-1. CCD 841 CoN cells were pre-treated with either raspberry enriched IFF or simple phenolics for 24hr. After this period of incubation RNA was collected, converted in cDNA and real-time PCR (qPCR) performed. The overall aim of this investigation was to establish if the Nrf2-ARE system was related to the antigenotoxic activity exhibited by our samples in Chapter 4.

Methods:

Tissue Culture

Tissue Culture was performed as described in Chapter 4.

Cell treatments

HT29 & CCD 841 CoN cells were seeded into 25cm^3 roux flasks at 1x10^6 or 8x10^5 cells respectively, incubated for 72hr prior to treatment with either IFF or simple phenolics. As a consequence of the limited volume of IFF, only CCD841 CoN cells were pre-treated with these samples prior to RNA extraction, while phenolics were used to treat both cell lines. Subsequently cells were treated for 24hr with either 20% ileal fluid fermentate (IFF) v/v in media or 10, 50 or 100µM individual phenolic in media.

Ileal Fluid Fermentate (IFF): Twenty-two samples of ileal fluid fermentate (IFF); n=11 pre-berry IFF 24hr and n=11 post raspberry IFF 24hr produced using *in vitro* batch culture (see Chapter 3) were only used to treat CCD 841 CoN cells due to limited volume.

Four individual phenolics; 4-Hydroxybenzoic acid (4HBA), Benzoic Acid (BA), 3-(3-Hydroxyphenyl)propionic acid (3HPPA) and 3-phenylpropionic acid (3PPA), with concentrations of 10, 50 or 100µM were chosen based upon compositional analysis of raspberry ileal fluid fermentate (see Chapter 3). Phenolic treatment was carried out on both cell lines.

In total, there were 3 independent experiments for each treatment, n=3 for each IFF sample and n=3 for each concentration of simple phenolic, carried out with a media control and known positive anti-genotoxic control (100nM CDDO).

RNA isolation and cDNA synthesis

After 24hr incubation with either IFF or individual phenolics, cells were collected and centrifuged at 1200rpm for 3min. Supernatant was removed and cell pellets placed on ice for 5min prior to homogenisation using QIAshredder (Qiagen, UK). RNA was extracted with the RNeasy Mini Plus Kit (Qiagen, UK) as per the manufacturer's instructions. The concentration and quality of RNA samples was determined via NanoDrop ND-100 UV/Vis spectrophometer and verified by gel electrophoresis. RNA samples with A_{260}/A_{280} and A_{260}/A_{230} between 1.8 and 2.0 were deemed acceptable for downstream use. Standard PCR of isolated RNA confirmed the absence of genomic contamination. Using 1µg RNA and anchored-oligo$(dT)_{18}$ in conjunction Transcriptor First Strand cDNA synthesis Kit (Roche) 20µl of cDNA was produced as per the manufacturer's instructions for reverse transcription. PCR using 1µl of cDNA as a template and the housekeeping gene, hypoxanthine phosphoribosyltransferase, HPRT (primer sequence shown below in Table 1) as a control, confirmed the absence of unspecific products. cDNA was stored a -20˚C until required.

Real-time PCR (qPCR)

Primers were designed for those genes of interest involved in the antioxidant response pathway (Nrf2, NQO1, HO-1) and 2 reference genes (HPRT, β-Actin) by utilising the online software OligoPerfect (http://tools.lifetechnologies.com/content.cfm?pageid=9716) in correlation NCBi Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table 1). Real-time qPCR was performed using the Lightcycler 480II (Roche) in accordance with the manufacturer's instructions. Each 10µl PCR reaction contained 0.5µM forward primer, 0.5µM reverse primer, 5µl LightCycler 480 SYBR Green I Master (Roche), 2µl nuclease-free

water, and 1µl cDNA template. Cycling conditions were as follows: 95˚C for 10min, 50 cycles of 95˚C for 10sec, 57˚C for 10sec, and 72˚C for 10sec. Product specificity for each gene was confirmed by melt curve analysis, only artefact free reactions were considered valid. Negative control reactions (no template control and negative reverse transcriptase) were run on the same plate for all experiments. cDNA standards (in triplicate) were produced by five-fold dilution of pooled cDNA, and used to create cDNA calibration curve slopes. LightCycler software (version 1.5) generated primer efficiencies for each gene (primers shown in Table 1), calculated by using the equation $E = 10[-1/\text{slope}]$. cDNA templates used for target runs were diluted to within the standard curve linear range, in this case 1:10. Only primers with an efficiency of 90–100% were used for target analysis (Table 1)

Expression was calculated using the following equation:

$$\frac{Conc.target\ gene}{Conc.reference\ genes}\ (control) : \frac{Conc.target\ gene}{Conc.reference\ genes}\ (sample)$$

Each cDNA target sample was normalized to two reference genes (HPRT and β-actin) and calculated as a ratio of the untreated control samples. All target cDNA samples were run as technical triplicates, n=3.

Statistical Analysis

Data was analysed as the mean of 3 independent experiments. D'Agostino & Pearson omnibus was used to test for normality. Analysis of variance was applied to test for significant differences between means and assessed with Dunnett's multiple comparison post-hoc test. Significance was accepted at $p<0.05$. Analysis was performed using Prism 5 (version 5.01 for Windows).

Table 1: Oligonucleotide primers used for PCR amplification and qPCR to assess the change in expression after treatment

Gene	Full Name	Forward Primer	Sequence (5'→3')	Reverse Primer	Sequence (3'→5')	Product Size (bp)
Nrf2	Nuclear factor (erythroid-derived 2)-like 2	Nrf2-F	AAACCAGTGGATCTGCCAAC	Nrf2-R	GCAATGAAGACTGGGCTCTC	190
HO-1	Heme oxygenase 1	HO-1-F	ATGACACCAAGGACCAGAGC	HO-1-R	GTGTAAGGACCCATCGGAGA	153
NQO1	NAD(P)H dehydrogenase, quinone 1	NQO1–F	AGGACCCTTCCGGAGTAAGA	NQO1-R	AGGCTGCTTGGAGCAAAATA	279
HPRT	hypoxanthine phosphoribosyltransferase	HPRT-F	AGCTTGCGACCTTGACCAT	HPRT-R	GACCAGTCAACAGGGGACAT	166
β-Actin	Actin, beta	β-Actin-F	GGACTTCGAGCAAGAGATGG	β-Actin-R	AGCACTGTGTTGGCGTACAG	234

Results:

Regulation of gene expression by raspberry enriched ileal fluid fermentate (IFF)

The investigation in Chapter 4 demonstrated the antigenotoxic potential of raspberry-enriched ileal fluid fermentate (IFF). When used to pre-treat CCD 841 CoN cells 9/11 post-berry IFF 24hr samples significantly decreased H_2O_2 induced DNA damage, when compared to their pre-berry IFF 24hr counterpart. To establish the mechanism by which this protective effect occurs qPCR was used to determine whether IFF samples activated the Nrf2/ARE system. We measured the change of expression in Nrf2 and two target genes, NQO1 and HO-1. As a consequence of the limited volume of IFF samples, only CCD841 CoN cells were pre-treated prior to RNA extraction. The majority of the post-berry ileal fluid fermentate (IFF) samples showed the ability to induce expression of one or more of the target genes within CCD 841 CoN cells (Figure 1).

Nrf2: (Figure 1A)

Nrf2 expression was significantly upregulated by 6 of the 11 post-berry IFF 24hr samples (S02, 3, 5, 6, 11 and 12), with (S03) post-berry IFF (24hr) producing the smallest change (1.42-fold, $p<0.05$) and (S06) post-berry creating the greatest increase (2.21-fold, $p<0.05$), when compared to media control. However 7 out the 11 pre-berry IFF 24hr samples (S01, 2, 3, 4, 5, 6 and 8) had the ability to significantly decrease Nrf2. Treatment with S01 pre-berry IFF resulted in the largest reduction in Nrf2 (1.60-fold, $p<0.05$) while S08 pre-berry caused a much smaller fold change (1.04-fold, $p<0.05$). In contrast to the other samples (S10) significantly down-regulated Nrf2 expression with both pre-berry (1.78-fold, $p<0.05$) and post-berry (1.34-fold, $p<0.05$).

NQO1: (Figure 1B)

In a similar pattern to the outcome of Nrf2 qPCR analysis post-berry IFF samples up-regulated NQO1 expression while pre-berry IFF samples resulted in down-regulation. A total of 8/11 post-berry IFF samples (S01, 2, 3, 4, 5, 6, 11, 12) and 10/11 pre-berry IFF samples (S01, 2, 3, 4, 5, 6, 8, 9 11 and 12) resulted in significant differential expression of NQO1. CCD 841 cells treated with 4/11 post-berry IFF 24hr samples demonstrated greater than 2-fold increase in NQO1, with S04 post-berry inducing the greatest induction (3.13-fold, $p<0.05$). While S05 pre-berry IFF created the largest reduction in NQO1 expression (2.27-fold, $p<0.05$) the remaining 9 samples resulted in at least 1-fold down-regulation. As seen with Nrf2, both (S10) post-berry IFF (1.27-fold, $p<0.05$) and (S10) pre-berry IFF (1.55-fold, $p<0.05$) significantly decrease the target gene.

HO-1: (Figure 1C)

Post-berry IFF 24hr treatment produced the most dramatic changes with HO-1 expression. Although only 7/11 (S02, 3, 4, 5, 6, 11 and 12) subjects significantly increased the target expression, greater fold changes where observed within these samples. (S05) post-berry resulted the largest up-regulation (6.87-fold, $p<0.05$) when compared to media control. This is more than double the expression change for NQO1 or Nrf2. Six out of eleven pre-berry samples (S01, 2, 8, 9, 11 and 12) induced a significant down-regulation of HO-1 after treatment, (S02) with the largest reduction (1.34-fold, $p<0.05$). Continuing the trend both (S10) samples reduced HO-1 expression, post-berry S10 (1.41-fold, $p<0.05$) and pre-berry (1.64-fold, $p<0.05$).

Six of the 11 post-berry ileal fermentate samples (S02, 3, 5, 6, 11 and 12) significantly up-regulated all 3 target genes after 24hr treatment, while two of the pre-berry samples (S8 and 9) consistently down-regulated all 3 target genes. The general pattern presented by these

results shows that berry-enriched ileal fluid fermentate samples increased target gene expression, while ileal fluid without the raspberry metabolites down-regulated the target genes.

Regulation of gene expression by simple phenolics -

The antigenotoxic activity of the individual phenolics, 4-hydroxbenzoic acid (4HBA), Benzoic Acid (BA),3-(3-hydroxyphenyl) propionic acid (3HPPA) and 3-phenylpropionic acid (3PPA) has been established in Chapter 4. All 4 simple phenolics demonstrated the ability to reduce DNA damage in both HT29 and CCD 841 CoN cells over a range of biologically relevant concentrations (10, 50 and 100µM). Real-time PCR (qPCR) was used to establish the role of Nrf2-ARE in this chemoprotection. Both cell lines were pre-treated with the individual phenolics for 24hr prior to collection of RNA and subsequent qPCR. Each of the tested phenolic acids induced the expression of target genes in both cell lines, however the patterns of up-regulation differed from phenolic compound to phenolic compound.

Nrf2: (Figure 2 and 5)

The individual phenolic compounds induced a differential pattern in the expression of Nrf2, a key gene in the ARE pathway. A significant dose response effect was evident for HT29 cells treated with Benzoic acid, as concentration increased from 10 - 100µM (Figure 2B), with the greatest change in expression (2.27-fold, $p<0.05$) was observed at 100µM. In contrast, 4-hydroxybenzoic acid displayed (figure 2A) an inverse dose response that mirrors the pattern of benzoic acid as maximum induction of gene expression (2.48-fold, $p<0.001$) observed at the lowest concentration (10µM) tested, while cells treated with 100 µM were not significantly different from the untreated control. 3HPPA induced significant changes in gene expression at 10 µM (1.73-fold, $p<0.05$) and 50 µM (2.15-fold, $p<0.01$) respectively (Figure

2C). For 3PPA only 50µM significantly increased Nrf2 expression in HT29 cells (1.71-fold, $p<0.001$), whereas 10µM (1.30-fold, $p<0.001$) and 100µM (1.46-fold, $p<0.01$) resulted in significant decrease in Nrf2 gene expression (Figure 2D).

CCD 841 CoN cells treated with the simple phenolics responded in dose dependent manner, with an increase in concentration directly correlating to the increase in Nrf2 expression. 4-hydroxybenzoic acid induced a significant up-regulation of Nrf2 from 10-100µM with the greatest increase shown at 100µM (2.57-fold, $p<0.01$) (Figure 5A). Treatment with BA significantly increased gene expression at 50µM (1.89-fold, $p<0.05$) and 100µM (2.29-fold, $p<0.01$) when compared to the untreated control (Figure 5D). 3PPA followed the same pattern up-regulating Nrf2 at 50µM (1.93-fold, $p<0.05$) and 100µM (2.05-fold, $p<0.001$) (Figure 5D). However, for 3HPPA only 100µM produced a significant increase in Nrf2 expression (2.26-fold, $p<0.01$) (Figure 5C).

NQO-1: (Figure 3 and 6)

The differential expression of NQO1, a downstream target of the Nrf2-ARE pathway, produced within HT29 cells after treatment with benzoic acid and 3PPPA mirrored the pattern seen with Nrf2 expression. Benzoic acid significantly induced NQO1 expression in HT29 cells at 50µM (1.64-fold, $p<0.05$) and 100µM (2.52-fold increase $p<0.001$) (Figure 3B). Treatment with 50µM 3PPA resulted in an increase of NQO1 expression (1.96-fold, $p<0.001$), while 10µM (1.35-fold, $p<0.001$) and 100µM (1.30-fold, $p<0001$) induced down-regulation (Figure 3D). In contrast, 4HBA and 3HPPA deviated from the results seen with Nrf2 expression. 4-hydroxybenzoic acid increased gene expression at 10µM (1.89-fold, $p<0.05$) and 50µM (1.91-fold, $p<0.05$), while 100µM (1.43-fold, $p<0.001$) reduced NQO1 expression (Figure 3A). Only 10µM 3HPPA (2.46-fold, $p<0.01$) treatment proved effective in significantly inducing gene expression (Figure 3C).

The dose dependent trend demonstrated by Nrf2 expression in CCD 841 CoN cells is repeated here with NQO1. Three of the individual phenolics significantly induced NQO1 expression at 50µM and 100µM with the higher concentration producing the greater up-regulation, 4HBA (1.51-fold, $p<0.01$) (Figure 6A), BA (2.44-fold, $p<0.001$) (Figure 6B), 3PPA (2.40-fold, $p<0.001$) (Figure 6D). As with HT29 cells, only 100µM concentration of 3HPPA significantly induced NQO1 expression (3-fold, $p<0.001$) (Figure 6C).

HO-1: (Figure 4 and 7)

Heme-oxygenase-1 the gene encoding for the cytoprotective enzyme which catalyses the degradation of heme, is another target of the Nrf2-ARE pathway which is regulated by (poly)phenols. All four of the individual phenolics significantly induced expression of this gene within HT29 cells when compared to untreated counterpart. BA and 4HBA proved effective at all three concentrations, with the greatest increases of HO-1 seen with 50µM 4HBA (3.17-fold, $p<0.001$) (Figure 4A) and 100µM BA (3.07-fold, $p<0.001$) (Figure 4B). 24hr pre-treatment of HT29 cells with 3HPPA significantly up-regulated HO-1 expression at 10µM (1.65-fold, $p<0.01$) and 50µM (1.39-fold, $p<0.05$) (Figure 4C). As demonstrated with the previous target genes, 50µM 3PPA significantly increased expression of HO-1 (2.04-fold, $p<0.001$) while, 10µM (1.31-fold, $p<0.001$) and 100µM (1.46-fold, $p<0.001$) resulted in a down-regulation of the gene (Figure 4D).

Benzoic acid, 4-hydroxybenzoic acid, 3-(3-hydroxyphenyl) propionic acid and 3-phenylpropionic acid significantly induced dose-dependent differential expression of HO-1 in CCD 841 CoN cells. 4HBA and BA produced significant up-regulation of HO-1 expression at 10, 50 and 100µM concentrations with the highest concentration creating the greatest up-regulation, 100µM 4HBA (2.07-fold, $p<0.001$) (Figure 7A) and 100µM BA (2.13-fold,

$p<0.001$) (Figure 7B). 3HPPA significantly increased gene expression at 50µM (1.62-fold, $p<0.05$) and 100µM (2.28-fold, $p<0.001$) (Figure 7C). CCD 841 CoN cells treated with 3PPA followed a similar trend with up-regulation of HO-1 after incubation with 50µM (1.87-fold, $p<0.001$) and 100µM (2.17-fold, $p<0.001$) (Figure 7D).

Treatment of CCD 841 CoN with individual phenolics was as effective as the positive control, 2-Cyano-3,12-dioxoolean-1,9-dien-28-oic acid or CDDO, a known activator of the Nrf2-ARE pathway. Treatment with 100nM of CDDO induced NQO1 expression by 1.71-fold, a change similar to most of the simple phenolics. However, 100µM of 3HPPA produced a greater 3.0-fold increase in NQO1. HO-1 induction after CDDO treatment induced a 2.08-fold increase which was equal to that shown by the individual phenolics. In contrast the positive control was much more effective than simple phenolic treatment in HT29 cells. 100nM CDDO induced NQO1 by 2.9-fold and HO-1 by 4.44-fold, while the greatest increase in NQO1 expression was produced by 10µM 3HPPA (2.46-fold) and 100µM BA created the largest HO-1 increase (3.07-fold).

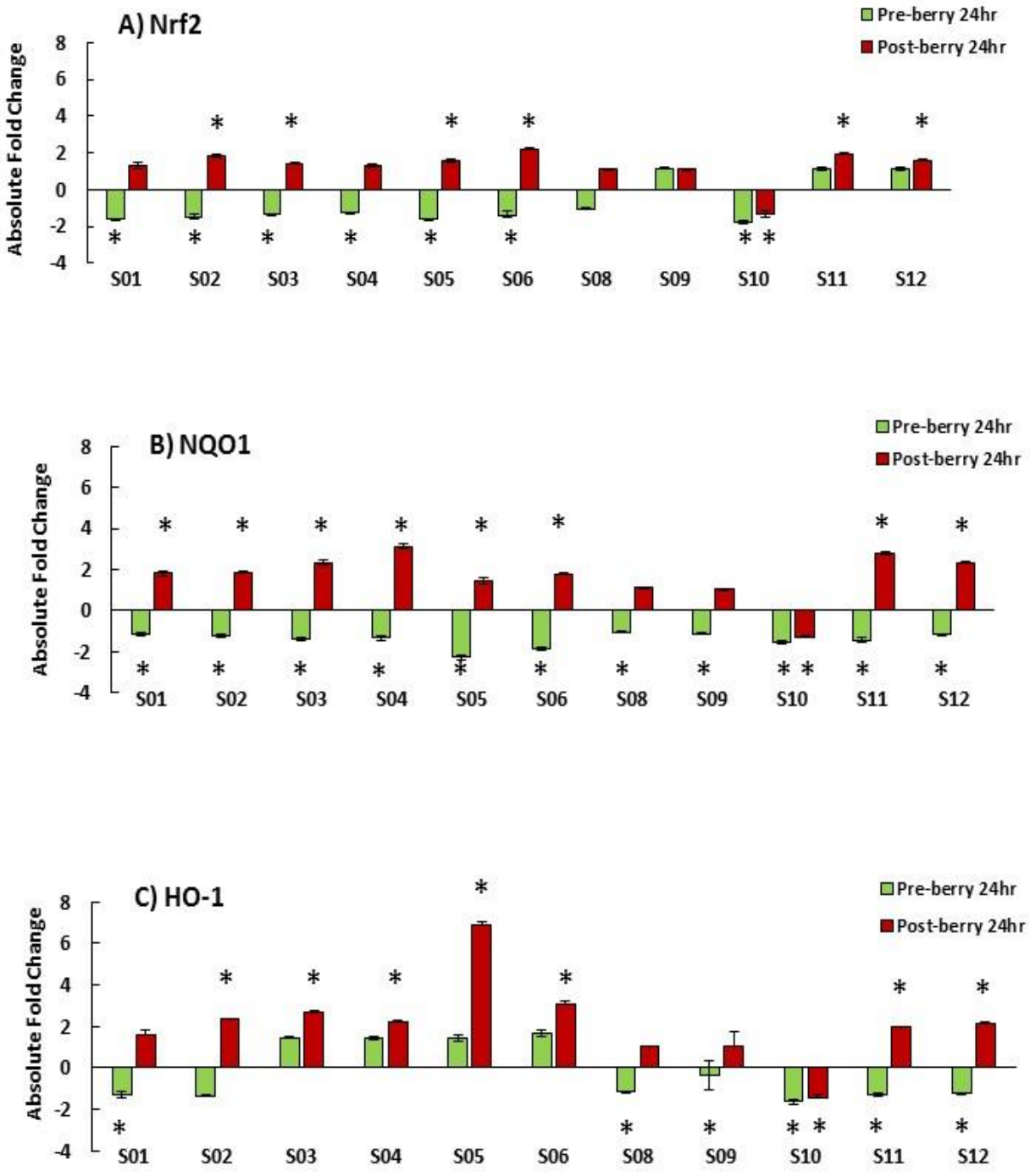

Figure 1: Effect of ileal fluid fermentates on ARE pathway in CCD841 cells after 24hr treatment. A) Nrf2, B) NQO1, C) HO-1. Data presented is mean of 3 independent experiments +SD. One-way ANOVA and Dunnett's Multiple Comparisons test. * $p<0.05$, significance is compared to media control.

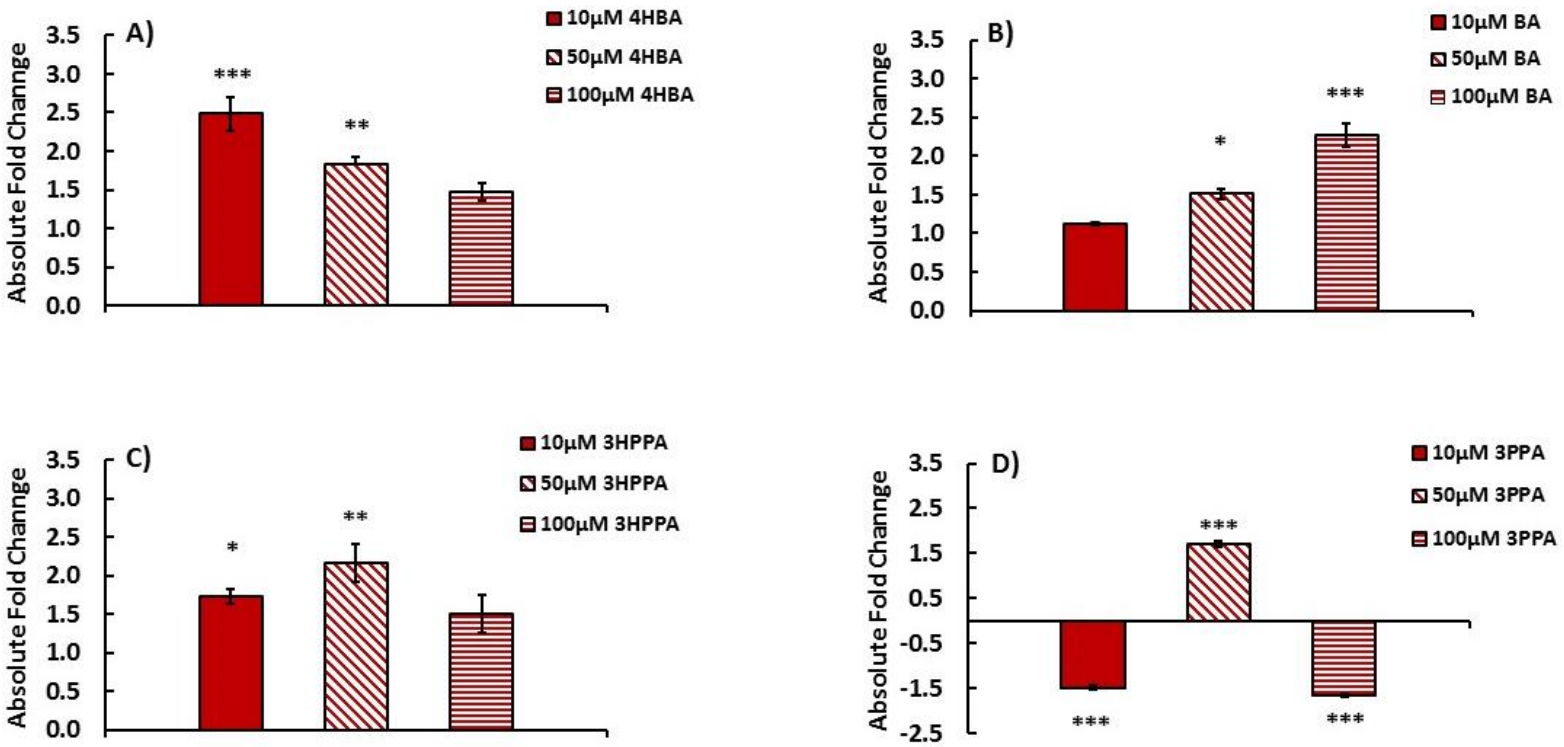

Figure 2: Change in Nrf2 expression in HT29 cells treated with either 10µM, 50µM or 100µM individual phenolic; A) 4HBA, B) BA, C), 3HPPA, D) 3PPA. Data presented is mean of 3 independent experiments +SD. One-way ANOVA and Dunnett's Multiple Comparisons test, *p<0.05, ** p<0.01, ***p<0.001, significance is compared to media control.

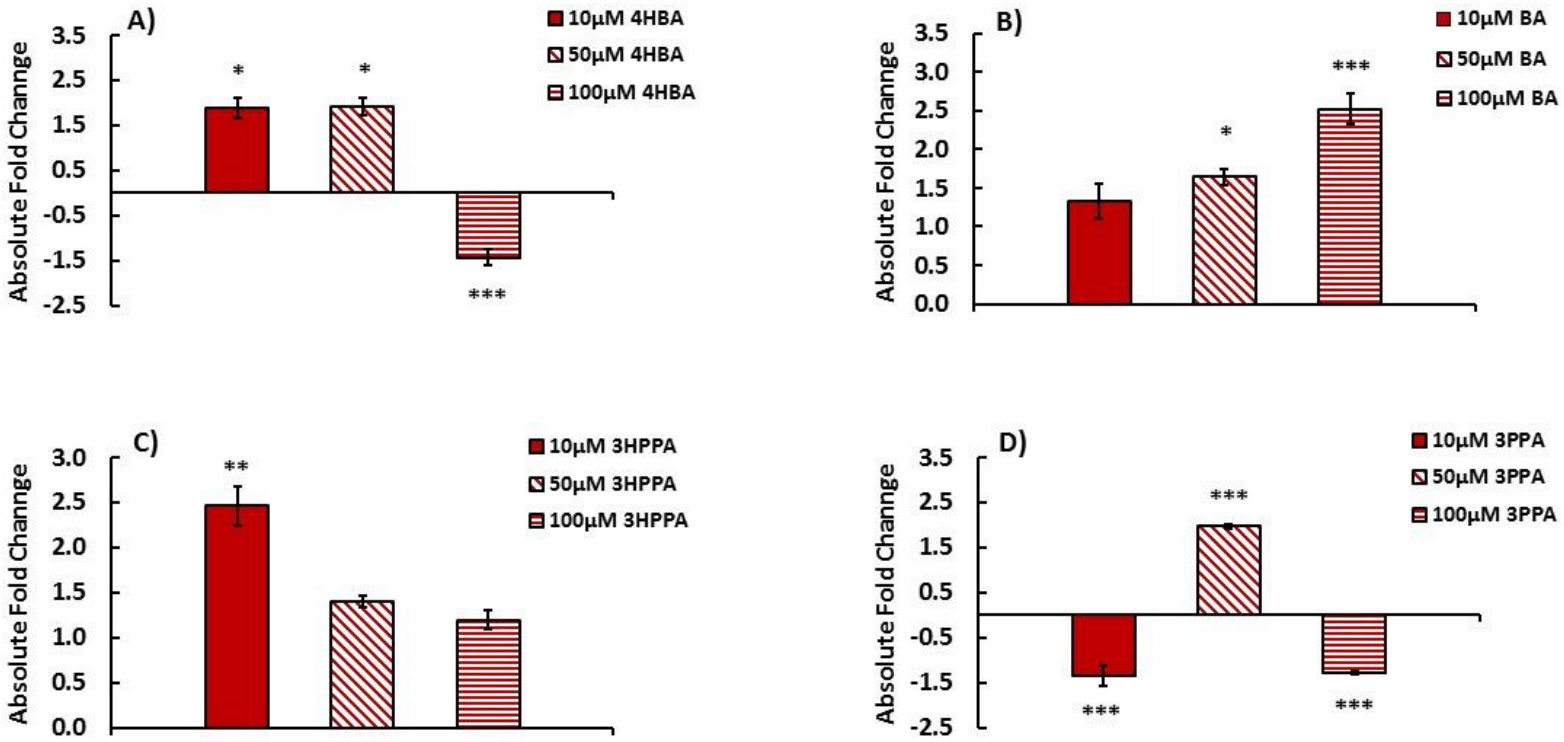

Figure 3: Change in NQO1 expression in HT29 cells treated with either 10µM, 50µM or 100µM individual phenolic; A) 4HBA, B) BA, C), 3HPPA, D) 3PPA. Data presented is mean of 3 independent experiments +SD. One-way ANOVA and Dunnett's Multiple Comparisons test, *$p<0.05$, ** $p<0.01$, ***$p<0.001$, significance is compared to media control.

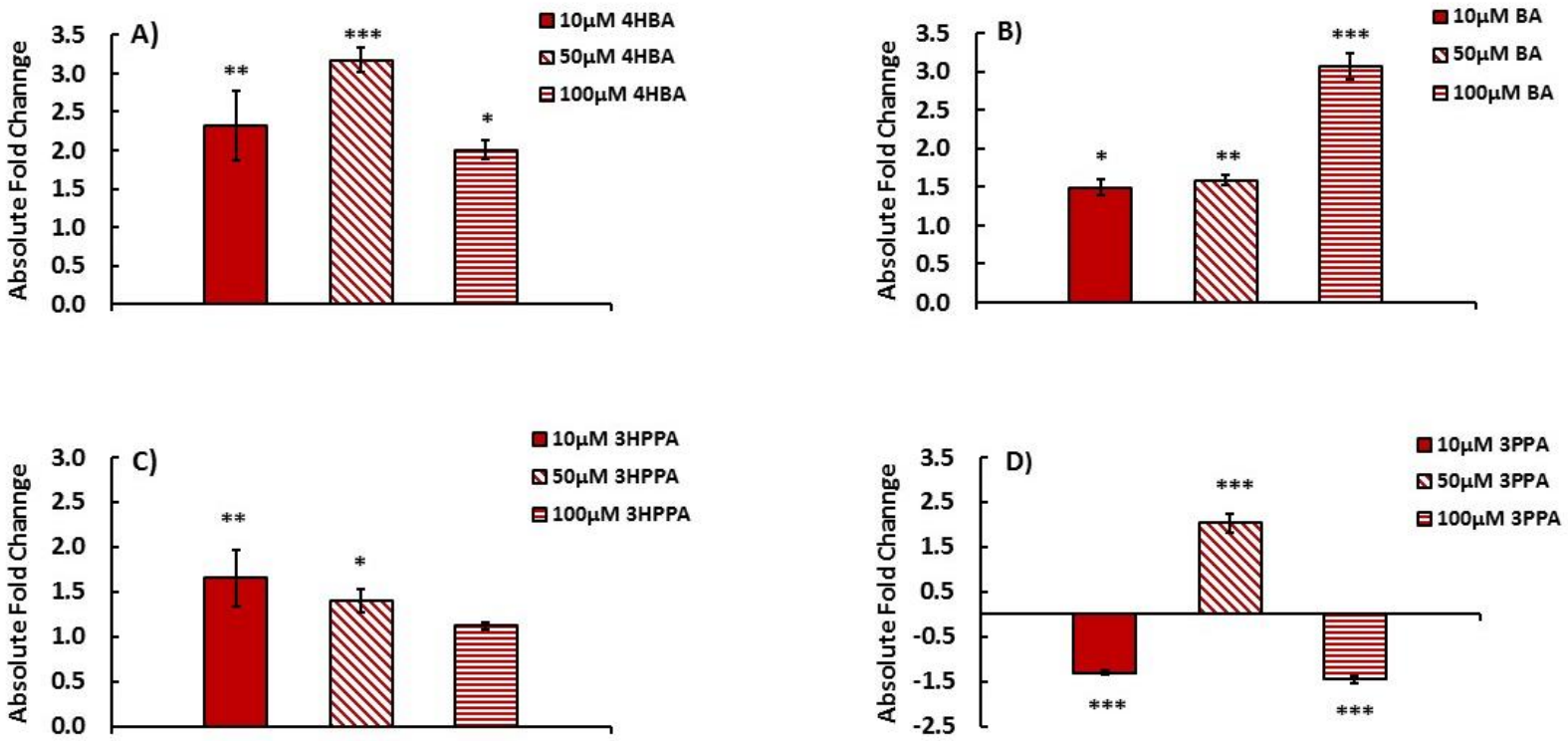

Figure 4: Change in HO-1 expression in HT29 cells treated with either 10µM, 50µM or 100µM individual phenolic; A) 4HBA, B) BA, C), 3HPPA, D) 3PPA. Data presented is mean of 3 independent experiments +SD. One-way ANOVA and Dunnett's Multiple Comparisons test, *$p<0.05$, ** $p<0.01$, ***$p<0.001$, significance is compared to media control.

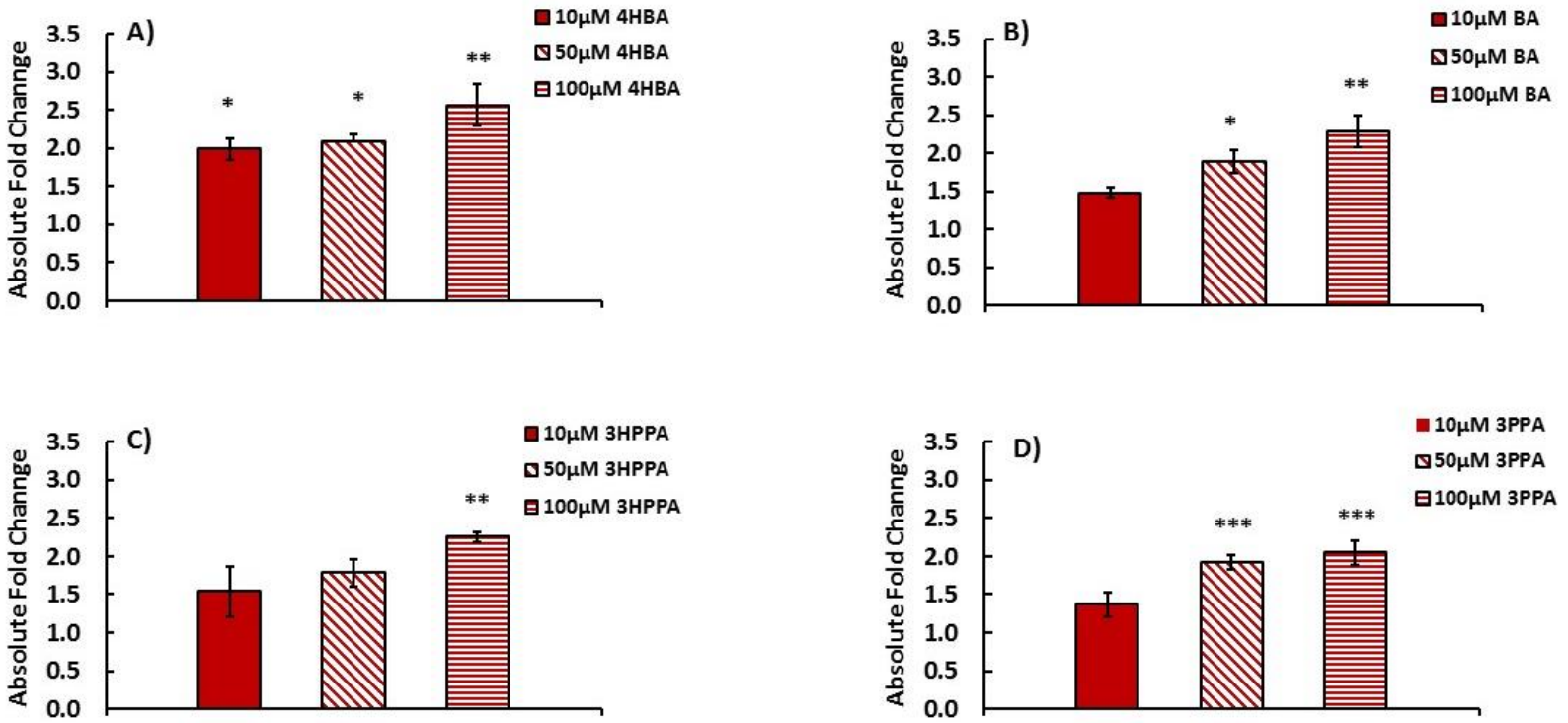

Figure 5: Change in Nrf2 expression in CCD 841 CON cells treated with either 10µM, 50µM or 100µM individual phenolic; A) 4HBA, B) BA, C), 3HPPA, D) 3PPA. Data presented is mean of 3 independent experiments +SD. One-way ANOVA and Dunnett's Multiple Comparisons test, *p<0.05, ** p<0.01, ***p<0.001, significance is compared to media control.

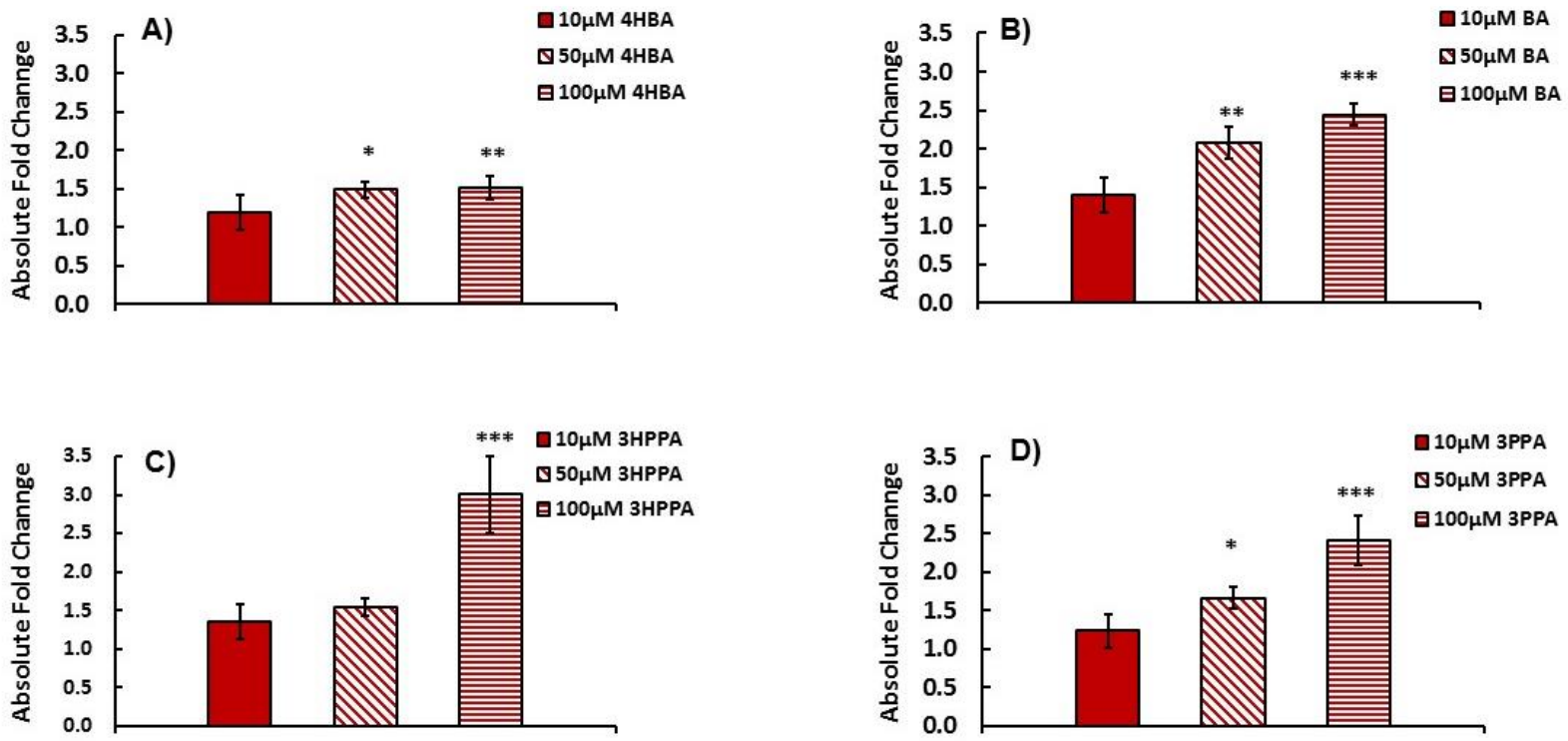

Figure 6: Change in NQO1 expression in CCD 841 cells treated with either 10µM, 50µM or 100µM individual phenolic; A) 4HBA, B) BA, C), 3HPPA, D) 3PPA. Data presented is mean of 3 independent experiments +SD. One-way ANOVA and Dunnett's Multiple Comparisons test, *p<0.05, ** p<0.01, ***p<0.001, significance is compared to media control.

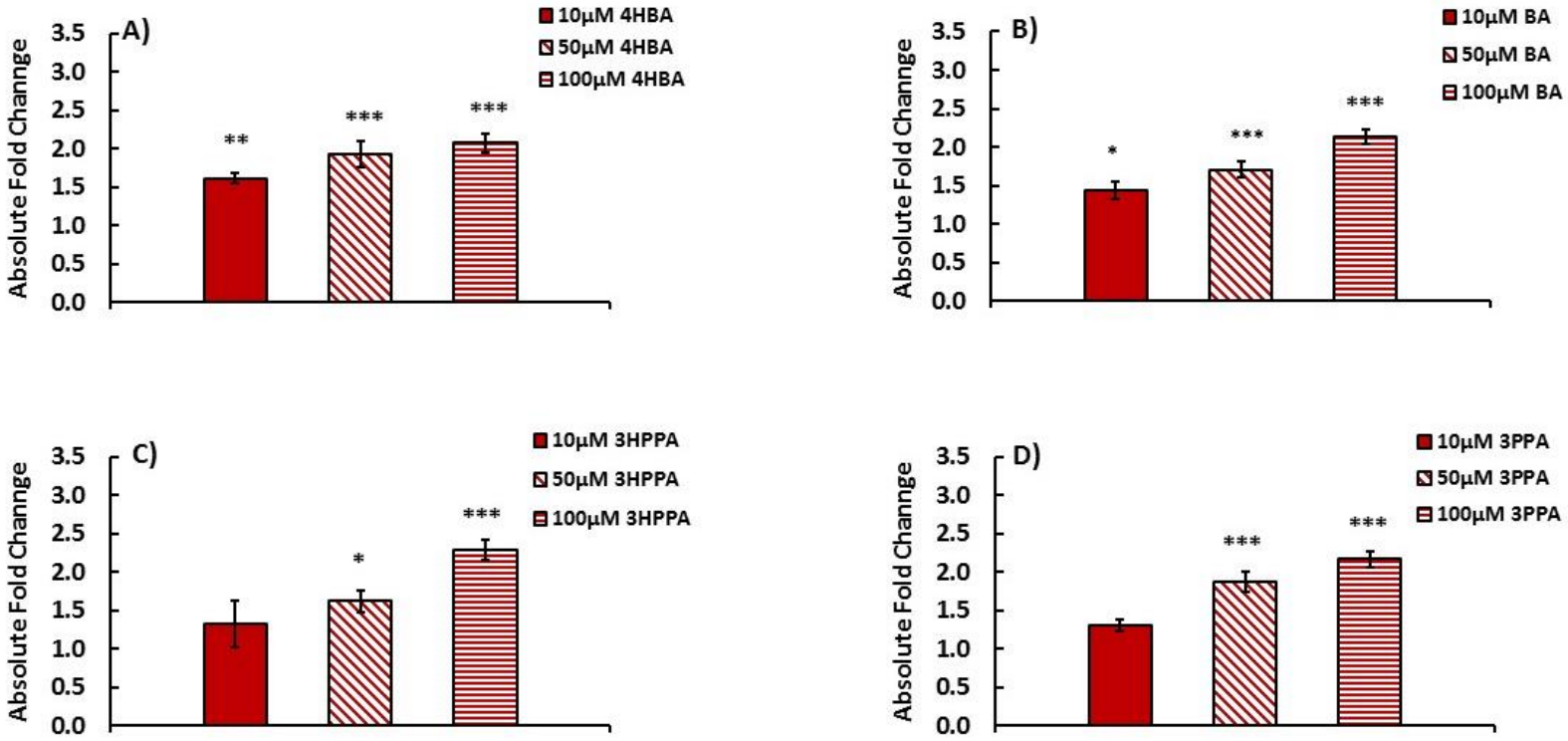

Figure 7: Change in HO-1 expression in CCD 841 CON cells treated with either 10µM, 50µM or 100µM individual phenolic; A) 4HBA, B) BA, C), 3HPPA, D) 3PPA. Data presented is mean of 3 independent experiments +SD. One-way ANOVA and Dunnett's Multiple Comparisons test, *p<0.05, ** p<0.01, ***p<0.001, significance is compared to media control.

Discussion:

Nuclear factor (erythroid-derived-2)-like 2 (Nrf2) is a ubiquitously expressed transcription factor which activates a range of antioxidant and detoxifying enzymes in response to cellular stress (27). As a consequence, Nrf2 is considered a key regulator of cytoprotection, cellular defence and cell survival. This transcriptional activator may be regarded as a tumour suppressor, as it has been shown to inhibit the progression of carcinogenesis and plays a role in cancer chemoprevention (28). Although the activation of the Nrf2-ARE system is a complex multistep pathway which is not yet fully understood, there has been much research based on identifying possible inducers. However, most of these studies have only investigated the potential of (poly)phenolic extracts or individual (poly)phenols to induce the Nrf2-ARE pathway, few have considered the implications of digestion upon these compounds. This chapter has examined the ability of raspberry enriched ileal fluid fermentates (IFF) to affect the Nrf2-ARE dynamic and activate the downstream target genes. These IFF samples represent the breakdown products of digestion and colonic fermentation each with a unique composition, comprised of a varied mixture of simple phenolics. The results from chapter 4 demonstrate the antigenotoxic ability of these IFF samples to reduce DNA damage, while the outcome from this study suggests that this may in part be due to the induction of Nrf2-ARE pathway.

A total of 6 out 11 (S02, 3, 5, 6, 11 and 12) post-berry IFF 24hr samples showed the ability to up-regulate all three target genes, increasing the expression of Nrf2, NQO1 and HO-1. The relationship between comet assay results, activation of Nrf2-ARE genes and the phenolic content of each sample is complex. When compared to the corresponding pre-berry IFF, (S06) post-berry IFF 24hr significantly induced all 3 target genes. This correlated with a significant decrease % tail DNA (25% reduction) (see Chapter 4), an increase in total

(poly)phenol content (1282 ± 306µM to 1893 ± 228µM) and an increase in the concentration of all 4 individual phenolics (BA, 4HBA, 3PPA and 3HPPA) (see Chapter 3). (S06) post-berry IFF 24hr was the only sample to activate Nrf2-ARE system, while reducing % tail DNA in-conjunction with an increase in total and individual phenolics. The other 5 samples induced all three target genes and reduced DNA damage but had various trends in phenolic concentration. Sample S12 post-berry IFF 24hr, when compared to (S12) pre-berry IFF 24hr, followed a similar pattern by significantly inducing all 3 target genes, in-conjunction with decreased % tail DNA (48% reduction) (Chapter 4) and increased total phenolic concentration (202 ± 19µM to 716 ± 42µM). In this instance only 1 of the individual phenolics (BA) was increased after berry enrichment (Chapter 3). CCD 841 CoN cells treated with (S02, 3, 5 and 11) post-berry IFF 24hr samples, demonstrated significantly increased expression of all 3 target genes and decrease in % tail DNA (Chapter 4) when compared to the corresponding pre-berry IFF. However, compositional analysis of these samples did not show an increase total phenolic concentration but did indicate increased amounts of individual phenolics. (S02 and S05) post-berry IFF 24hr showed a greater concentration of BA, 3PPA and 3HPPA; while (S03) exhibited an increase in the concentration of BA and S11 showed more 4HBA in the post-berry sample (Chapter 3). This illustrates the ability of post-berry IFF samples to induce expression of the target genes. These results combined with compositional analysis, imply there is a correlation between the total phenolic content, induction of Nrf2-ARE pathway and subsequent antigenotoxic activity.

In contrast, sample (S08) post- berry IFF 24hr significantly decreases DNA damage but does not significantly induce any of the target genes. This is potentially due to phenolic composition of the post-berry sample, as (S0)8 pre-berry IFF 24hr has greater total phenolic concentration (1032.9 ± 179.9µM) than its post-berry counterpart (161.1 ± 47.6µM) and has larger amounts of 2 out of the 4 individual phenolics (BA and 3PPA). Samples collected from

subject 10, both (S10) pre-berry and (S10) post-berry IFF 24hr, significantly reduced expression of Nrf2, NQO1 and HO-1 when compared to untreated control. These fermentates also failed to provide any antigenotoxic protection from H_2O_2 challenge. As with (S08),(S10) post-berry (532.4 ± 19.2µM) has a lower total phenolic concentration than its pre-berry counterpart (1502.3 ± 42.4µM), but does show a greater concentration in 3 out of the 4 phenolics (4HBA, BA and 3HPPA). These variations from the previous pattern shown with samples (S08 and S10) demonstrate that the relationship between phenolic content, Nrf2 activation and antigenotoxic activity requires further investigation. As the compositional analysis was concentrated upon phenolic compounds and only detected 18 simple phenolics of interest there is a chance we have not identified all the other components in the samples, some of which may also act upon the Nrf2-ARE pathway.

The synergistic actions of berry (poly)phenols potentially play a key role in the activation Nrf2. It is hypothesised that the complex mixture of phytochemicals found within the whole fruit is of greater benefit than an individual isolated compound (29). These purified compounds may lose their bioactivity or behave differentially than when they are present in a food complex or mixture. Saw *et al.* (2014) have attempted to demonstrate the significant of synergistic activity with their treatment of HepC2-C8 cells (hepatocellular carcinoma) with 3 berry constituents, quercetin, kaempferol and pterostilbene (30). Cells incubated with 25µM kaempferol for 24hr showed a significant induction of Nrf2 mRNA, while 25µM quercetin and 12.5µM kaempferol increased Nrf2 and HO-1 respectively but the induction was not significant. In contrast cells treated with multiple compounds increased mRNA expression of several genes at much lower concentrations; 3.12µM kaempferol/quercetin mixture significantly induced Nrf2 and GSTM2 (gluthione S-transferase Mu 2), while 6.26µM pterostilbene/kaempferol mixture increased HO-1 expression (30). These results suggest that the Nrf2/ARE system has some role in mediating the activity of berry constituents and their

synergism. The potential synergism of (poly)phenolics, combined with the structural changes which occur during digestion emphasise the importance of examining biologically relevant samples. As shown in Chapter 3 each of the IFF samples have a unique (poly)phenolic composition, containing a variety of simple phenolics at a range of concentrations. It is the diversity of phenolics which could explain the varying efficacies of the IFF samples

As the results from this study illustrate there is much potential for further investigation into the role of phytochemicals in the regulation of the Nrf2-ARE system. Several groups have demonstrated how this translates to *in vivo* models. The human intervention study performed by Kropat *et al*. (2013) used similar methodology to this chapter, to examine the effects of bilberry extracts upon the Nrf2-ARE pathway within peripheral blood mononuclear cells (PBMC) (31). The relative transcriptional levels of Nrf2, NQO1 and HO-1 of PBMC where compared in two cohorts, a control group and an ileostomy group, over 4 time points post-bilberry consumption. In the control group NQO1 was significantly increased 1hr after consumption. This induction continued to 142 ± 23% relative transcription level at 8hr post-consumption, while both Nrf2 and HO-1 expression were supressed at all 4 time points post-consumption. In contrast PBMC cells collected from the ileostomy group had significantly reduced Nrf2 expression at 2-8hr but produced no significant change in either NQO1 or HO-1 expression. Only the control group, the participants with intact colons, demonstrated the ability to activate the Nrf2-ARE pathway, a pattern seen again with the results from subsequent comet assay. Two-hour post-consumption the PBMC from the control group was able to reduce DNA damage, while PBMC samples from ileostomy group showed no significant change in DNA damage. This suggests that colonic fermentation and subsequent break-down of (poly)phenols within the lower intestine is necessary to produce metabolites capable of inducing antigenotoxic activity. On face-value when compared to the results of our study the bilberry extract in this investigation did not prove to be as effective at inducing

antigenotoxicity or activating the Nrf2-ARE pathway. However, Kropat *et al*. (2013) examined the *in vivo* consequences of bilberry extracts, concentrating on the effect of phytochemicals upon the PBMC, and as such have demonstrated the potential of bioavailable phenolics, i.e. those that have entered the bloodstream (31). The outcome of this investigation corroborates the low bioavailability of many (poly)phenols and when combined with the results from this chapter indicates that direct interaction between colonic cells and phenolic compounds is a more effective means of chemoprevention for CRC.

Few human feeding studies have been used to examine the relationship between dietary (poly)phenol the induction of Nrf2 and its target genes. Animal models are more a more common means of investigating this pathway *in vivo*. The anti-carcinogenic potential of cocoa (poly)phenols with regards CRC prevention was illustrated using a BALB/c mice model (32). After challenge with AOM/DSS (azoxymethane/ dextran sodium sulfate) mice which consumed a diet containing either 5% or 10% cocoa, showed reduced tumour size and tumour branching when compared to those fed a control diet. This correlated with a significant increase in Nrf2 protein levels within the colonic tissue and up-regulation of two Nrf2-ARE target genes, NQO1 and UDP-GT (UDP glucuronosyltransferase family 1 member). As this increase in Nrf2 target genes corresponds to the outcome shown in our *in vitro* investigation, this study emphasises the possible role of other dietary phytochemicals, e.g. raspberry (poly)phenols, have within *in vivo* models.

The impact of berry (poly)phenols upon the Nrf2-ARE system has been demonstrated in other models of chronic disease, including atheroscerlosis. Wild-type (WT) C57BL/6 mice fed on a high fat (21%) diet (HFD) supplemented with ellagic acid (0.5g/kg) have less atherosclerotic deposits than those fed on HFD alone. Western blot analysis illustrated that this protective effect was likely due to the up-regulation of Nrf2 and HO-1 protein levels within the aorta (33), which highlights the probable health benefits of berry extracts in

multiple disease states and the need to investigate this interaction on a protein level. While Sharma *et al.* (2016) demonstrated the potential benefits of apple (poly)phenols within liver disease. Mice were feed with apple pomace aqueous extract (APE) at 200mg/kg, 400mg/kg or a control diet prior to being treated with carbon tetra-chloride (CCl_4) to induce liver injury. Liver sections of APE treated mice were observed to have significant dose-dependent up-regulation of Nrf2 protein expression when compared to control mice (34).

Although the results from IFF samples have indicated that the complex mixture of simple phenolics plays a key role in the activation of the Nrf2-ARE pathway, it was important for us to attempt to identify any antigenotoxic potential within individual phenolics. Figures 2-7 illustrate that simple phenolics could significantly induce mRNA expression of Nrf2 and its target genes, NQO-1 and HO-1, in normal colonic epithelial cells (CCD 841 CoN) and carcinogenic colonic cells (HT29). The effect upon CCD 841 CoN cells was concentration dependent, with the 100µM concentration of each phenolic producing the greatest increase in mRNA expression for all three genes. This directly correlates with the reduction in DNA damage seen in Chapter 4. An increased phenolic concentration produced increased expression of protective genes and decreased % tail DNA after H_2O_2 challenge. These results are corroborated with the results of Li *et al*. (2016) and Ferrari *et al*. (2016) which demonstrated correlation between increased (poly)phenol concentration and increased mRNA expression (35, 36). The former study illustrated that incubating HAEC (human aortic endothelial cells) with quercetin for 18hr increased expression of HO-1 and NQO1 mRNA. As the concentration increased from 5-20µM the fold change doubled from 2 to 4-fold increase of HO-1 mRNA (35). While the later showed the effect of cyanidin-3-O-glucoside (C3G) upon TNF-α (tumour necrosis factor) within Caco-2 cells (colorectal adenocarcinoma). The increasing concentration of C3G from 20-40µM induced further

expression of HO-1 mRNA (from 1-3-fold increase) and NQO1 mRNA (from 3-7-fold increase) (36).

The pattern with HT29 cells is not so clear, with only Benzoic acid and 3-phenylpropionic acid demonstrating a direct correlation with the antigenotoxic activity observed during the Comet Assay (see Chapter 4). BA acted in the same concentration dependent manner as was seen with CCD 841 CoN cells. However, 3PPA resulted in an increase of mRNA expression for Nrf2, NQO1 and HO-1 at 50µM concentration, the only concentration which significantly reduced DNA damage after oxidative challenge (see Chapter 4). There was no definitive pattern with 4HBA and 3HPPA and no direct correlation with antigenotoxic activity.

Several studies have concentrated on the action of purified (poly)phenolics and (poly)phenolic extracts upon the Nrf2 pathway within colorectal cancer (CRC). Previous studies examined the effect of rosemary phenolic extracts upon colonic cells (37, 38). When used at 10µM concentration to treat HT29 and SW480 (colorectal adenocarcinoma) cells for 24hr, the (poly)phenol rich rosemary extract produced in the first investigation resulted in the upregulation of HO-1 and OSGIN1 (oxidative stress induced growth inhibitor), two downstream targets of Nrf2. Treatment proved more effective on SW480 cells with an increase in HO-1 (24.6-fold, $p<0.0001$) and OSGIN1 (3.6-fold, $p<0.0001$) (37). A more recent study investigated the effects of rosemary extract and carnosic acid (the predominant (poly)phenol in rosemary) upon HCT 116 (colorectal carcinoma) and SW480 cells connecting the up-regulation of anti-genotoxic genes with Nrf2-ARE pathway. The changes in protein expression were measured with the use of western blotting. Rosemary extract at 20µg/ml and carnosic acid at 30µM increased the expression of Nrf2 in both cell lines and promoted nuclear translocation of the protein. Rosemary extract also significantly up-regulated the expression Sestrin-2, a stress-inducible metabolic regulator regulated by Nrf2-ARE system, in HCT 116 and SW480 cells (37). The results from Valdés *et al*. mirrored the

findings of our investigation and identified the role of phase II detoxifying and antioxidant genes in the antigenotoxic effects of phytochemicals. This emphasises the chemopreventive potential of dietary (poly)phenols within a range of CRC cell lines. The outcome of the second study by Yan *et al*. suggested that the differential expression of the target genes seen within our results corresponds to an up-regulation of Nrf2 on a protein level and as such highlights the need for further investigation into the role of berry (poly)phenolics and their metabolites (38).

In summary, this chapter demonstrated the potential of raspberry (poly)phenols, both as individual phenolics and as berry metabolites within IFF to activate the Nrf2-ARE pathway. The induction of Nrf2 and its two target genes, NQO1 and HO-1 suggests that up-regulation of these antioxidant enzymes could be the underlying mechanism for the reduction in DNA damage, illustrated in Chapter 4. The Nrf2-ARE system and its downstream genes may contribute to the overall anticarcinogenic potential of raspberries. It would however be necessary to use proteomics to establish if this increase in mRNA expression translates to an induced protein expression and confirm the relationship between raspberry treatment and antigenotoxicity.

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Chapter 6: Antigenotoxic activity of colon-available triterpenoid

Introduction:

The health benefits associated with raspberries have primarily been attributed to the presence of anthocyanins and ellagitannins, within the fruit, and the bioactivity associated with these compounds (1-3). However, (poly)phenols are not the only components found within berries and other chemical constituents such as the many lipid groups (unsaturated fatty acids, sterols and terpenoids) may contribute to these protective effects (4, 5). One such group of interest are triterpenoids (TTPNs), a diverse group of isopentyl pyrophosphate oligomer metabolites which are present in a wide range of plant-based foods (6). TTPNs can be classified as either oleane (oleanolic acid, erythrodiol, β-amyrin), ursane (ursolic acid, uvaol) or lupane (lupeol, betulin, betulinic acid) triterpenes (7). Such compounds are primarily found in the skin of fruit and are prominent components of the waxy cutin-based tissue which forms a protective layer against the surrounding environment (8, 9). As with (poly)phenols the composition of TTPNs will differ from fruit to fruit but they are found in abundance within olives, tomatoes, grapes and apples (10). Olives contain a variety of TPPNs including; oleanolic acid, uvaol, erythrodiol and maslinic acid which are found in the fruit, leaves and within virgin olive oil (11, 12). The cutin of apple fruits is comprised of a range of TTPNs with over 30 constituent acids including ursolic acid ((3β)-3-hydroxyurs-12-en-28-oic acid) and oleanoic acid (3β-hydroxyolean-12-en-28-oic acid) derivatives (13-15). In contrast other fruits contain a predominant TPPN; for grapes this is oleanic acid derivatives, while lupeol (a lupane TTPN) is found within mangoes (16-18). Triterpenes have also been identified in a range of berries, specifically those from the *Vaccinium sp.*, which include blueberries, cranberries, bilberries and lingonberries (19-22). TPPNs are present in the fruit of several species from the *Rubus* family such as, *R. rosifolius* and *R. corneanus* and are also present in the as the non-edible leaves and roots from *R.imperialis* and *R. parvifolius* (23-26).

TPPNs have been linked with a range of anticancer activities, for example the tetracyclic and pentacyclic triterpenoids found within hawthorn berries demonstrate antiproliferative and antioxidant potential. A total of 15 TTPNs were isolated from the berries and included compounds such as ursolic acid, corosolic acid, uvaol, oleanolic acid and crataegolic acid, the majority of which produced potent antiproliferative effects when incubated with HepG2 (hepatocellular carcinoma), MCF-7 (breast adenocarcinoma) and MDA-MB-231(breast adenocarcinoma) cells. Three of these compounds, (3β,6β,18β-trihydroxy-olean-12-en-28-oic acid, 2α,3β,19α-trihydroxy-olean-12-en-28-oic acid and 2α,3β,19α,23-tetrahydroxy-olean-12-en-28-oic acid) showed further bioactivity and promoted potent peroxyl radical scavenging activity (PSC) (27). Similarly, the antioxidant potential associated with the "Stevens" and "Franklin" cultivars of cranberry (*Vaccinium Macrocarpon* L) correlated with high concentrations of TTPNS particulary oleanolic and urosolic acids (28).

These previous publications have demonstrated the potential health benefits of triterpenoids, however, for TTPNs to be of relevance within our model of investigation these compounds must be present within the ileal fluid that enters the large intestine. Targeted LC-MS analysis, described in Chapter 3 and previously published by McDougall *et al.,* identified a range of (poly)phenolic compounds consisting primarily of anthocyanins and ellagitannins which were present in post-raspberry ileal fluid samples (29). Non-targeted analysis of the same samples detected 2 previously unknown components (*m/z* 355 and *m/z* 679) which were up-regulated in all 11 post-berry samples. Further analysis was required to assess the chemical nature of these compounds, which concluded that both components are structurally related, with *m/z* 67900 most likely as dimer of *m/z* 355. Subsequent investigation putatively identified the unknown compound (*m/z* 355) as an ursolic acid-based triterpenoid most likely derived from the raspberry seed coat (30). Due to the ability to survive within the gastrointestinal tract it was important to ascertain whether this TPPN contributed to the

bioactivity and antigenotoxic activity ascribed to the raspberry enriched ileal fluid as previously discussed in Chapter 4 and 5.

Methods:

Bulk Raspberry Seed Extraction and Purification

Bulk raspberry seed extraction, performed at the James Hutton Institute, Glasgow, was used to produce sufficient quantities of the triterpenoid rich fraction. Batches (250g x 32) of raspberries where pureed and sieved to isolate the seeds (seed yield ~4%). 250g of seeds were extracted following incubation with 1L of 0.1% formic acid for 60min at 5°C, 90rpm (orbital rotation) and filtration through a glass sinter (porosity 3). This was followed by further purification; the entire methodology is described in full by McDougall *et al.* but for completeness it is briefly described here (30). Additional extractions were performed using a 70ml Sephadex LH20 column (GE Healthcare, Buckinghamshire, UK) and C18 solid phase extraction (Strata C18-E, GIGA units, 10 g capacity; Phenomenex, Ltd., Macclesfield, U.K), with the total phenolic content assessed using the Folin-Ciocalteu and the content in the final triterpenoid rich-fraction (TRF) was estimated as tenuifolin equivalents by peak areas.

Tissue Culture

Both HT29 (adenocarcinoma) and CCD 841 CoN (normal epithelial) were cultured and maintained as described in Chapter 4. The cell lines were treated with either a known inducer of the Nrf2/ARE pathway, 2-Cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO) or a triterpenoid-rich fraction (TRF) at 100nM.

Cytotoxicity Assay

The potential cytotoxic effects of CDDO and TRF upon both HT29 and CCD841 CoN cells were assessed using the MTT(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay, the protocol for which was previously described in Chapter 4. Concentrations of 100, 200 and 300nM of TRF and CDDO were determined, with each treatment performed in octuplet and 3 independent experiments carried out. Cell viability for media control samples was set a 100% with both treatments compared back to this value.

COMET assay

Single-cell gel electrophoresis or comet assay, the method for which was previously describes in Chapter 4, was used to determine the antigenotoxic potential of CDDO and TRF upon both HT29 and CCD841 CoN cells. The effect of 100, 200 and 300nM concentrations of each treatment were examined with each experiment repeated 3 independent times.

Real time PCR (qPCR)

Real-time qPCR was performed with the Lightcycler 480 in accordance with the manufactures instructions, the full methodology can be found in Chapter 5.

Statistical Analysis

Data was analysed as the mean of 3 independent experiments. D'Agostino & Pearson omnibus was used to test for normality. Analysis of variance was applied to test for significant differences between means and assessed with Dunnett's Multiple comparison post-hoc test. Significance was accepted at $p<0.05$. Analysis was performed using Prism 5 (version 5.01 for Windows).

Results:

Non-targeted LC-MS analysis revealed that the concentration of two unknown compounds was increased in all 11 post-raspberry ileal fluid samples (Figure 1), compound 1 (*m/z* 355) and compound 2 (*m/z* 679). Greater analysis determined that *m/z* 679 is a dimer of *m/z* 355, an ursolic acid-based triterpenoid derived from the seeds of the raspberry fruit. Bulk extraction of raspberry seeds isolated the triterpenoid rich fraction and produced an adequate amount to assess the bioactivity of this extract upon normal colonic epithelial cells (CCD 841 CoN) and colonic adenocarcinoma cells (HT29).

Prior to assessing the antigenotoxic potential of the purified TRF the cytotoxicity of the compound was determined using the MTT assay (Figure 1). Both cell lines were treated with three concentrations (100, 200 and 300nM) of the TRF for 24hr before cell viability was examined. The same pattern was seen with HT29 and CCD 841 CoN, an increased concentration of TRF increased correlated with a decreased cell viability. This dropped from 89% (100nM) to 72% (300nM) in HT29 and 90% (100nM) to 64% (300nM) in CCD 841 CoN cells, however this loss in cell viability was not significant. As a result, it was decided to proceed with 100nM concentration of the TRF for subsequent analysis.

The comet assay was used to examine any potential antigenotoxic properties, following 24hr incubation with HT29 or CCD 841 CoN cells, both the TRF and synthetic control (CDDO) displayed protective effects against H_2O_2 induced damage (Figure 2). In HT29 cells DNA damage measured by % tail DNA was significantly reduced from ~53% to 30% following treatment with TRF, which corresponded to 28% tail DNA after CDDO treatment. A similar pattern was seen with CCD 841 CoN cells pre-treated prior to challenge with 25µM H_2O_2, with a significant reduction of ~50-55% for both CDDO and TRF treatment.

To determine whether this reduction in DNA damage was due to activation of the Nrf2/ARE pathway, RNA was collected from CCD 841 CoN and HT29 cells after incubation with the TRF or synthetic CDDO. Following reverse transcription to produce cDNA, qPCR was performed to assess whether treatment altered the expression of Nrf2 (Nuclear factor (erythroid-derived 2)-like 2) and two of the downstream targets of the ARE pathway, NQO1(NAD(P)H:quinone oxidoreductase 1) and HO-1 (heme oxygenase 1) (31-33). A distinct pattern was evident after 24hr incubation with CDDO, with a significant increase in NQO1 (2.46-fold, $p>0.01$) and HO-1(5.73-fold, $p>0.01$) with HT29 cells (Figure 3A). With CCD841 CoN cells the changes in NQO1 (1.35-fold, $p>0.01$) and HO-1 (1.80-fold, $p<0.001$) expression were less dramatic but remained statistically significant. In contrast, Nrf2 expression within both cell lines was reduced significantly at 1.68-fold ($p<0.001$) for HT29 and 6.13-fold ($p<0.01$) in CCD 841 cells. These results are most likely a consequence of the time-frame of treatment selected for this study. The cells within this experiment were incubated with CDDO for 24hr to correspond with the exposure time used within the antigenotoxicity studies. As Figure 4 demonstrates cell lines treated over a shorter period demonstrate Nrf2 upregulation, with HT29 cells significantly up-regulated incubation of 2hr, 4hr, 6hr (3.23-fold, 8.24-fold and 3.29-fold, $p>0.001$) and as significant increase in target gene expression for CCD 841 CoN cells after 4hr and 6hr (7.07-fold and 3.02-fold, $p<0.001$). The changes in expression following treatment with TRF do not follow the same pattern as shown with the synthetic triterpenoid (Figure 3B). Incubation with 100nM of TRF induced an increase of Nrf2 expression in HT29 cells (1.84-fold, $p>0.001$) and a small but significant up-regulation of NQO1 activity in CCD 841 cells (1.19-fold, $p<0.0001$). However, NQO1 expression was significantly decreased in HT29 cells after TRF treatment (1.33-fold, $p>0.001$), while HO-1 was significantly decreased in CCD 841 cells (1.77-fold, $p>0.001$).

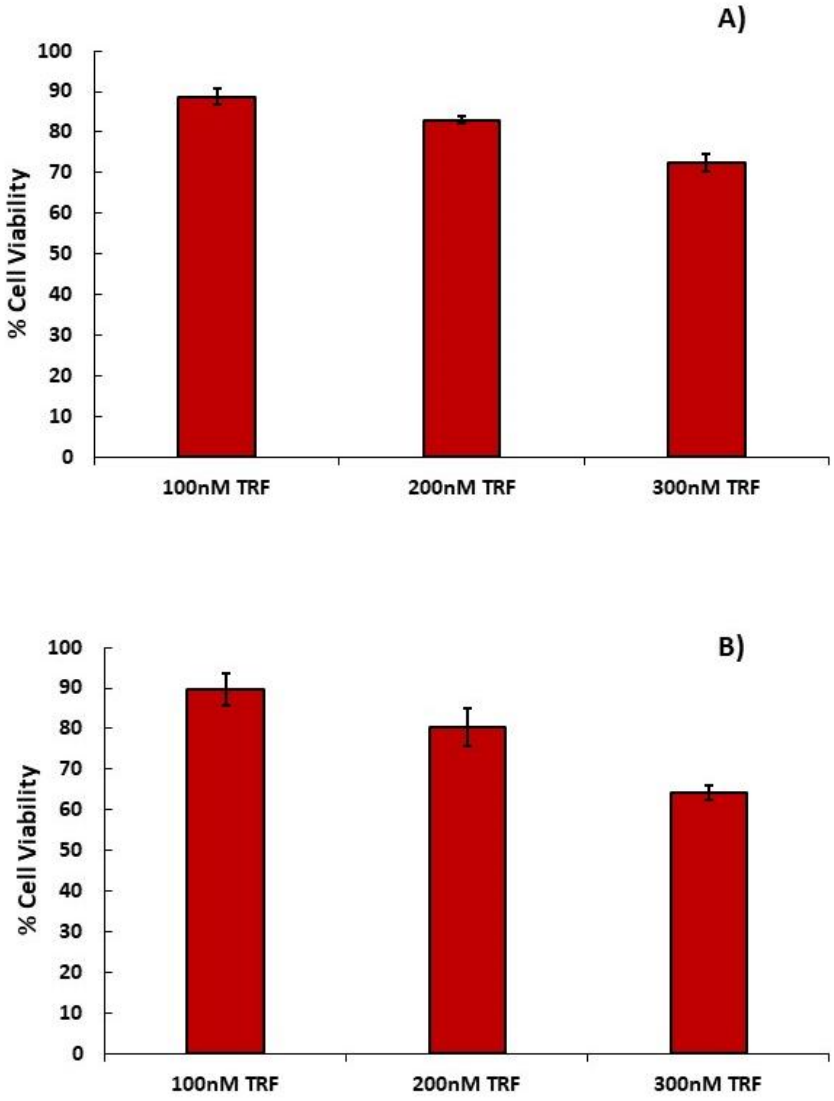

Figure 1: Cytotoxic effect of different concentrations of TRF on HT29 cells (A) and CCD841 cells (B). Data presented is mean of 3 independent experiments +SD. One-way ANOVA and Dunnett's Multiple Comparisons test, ***$p<0.001$, significance is compared to media control.

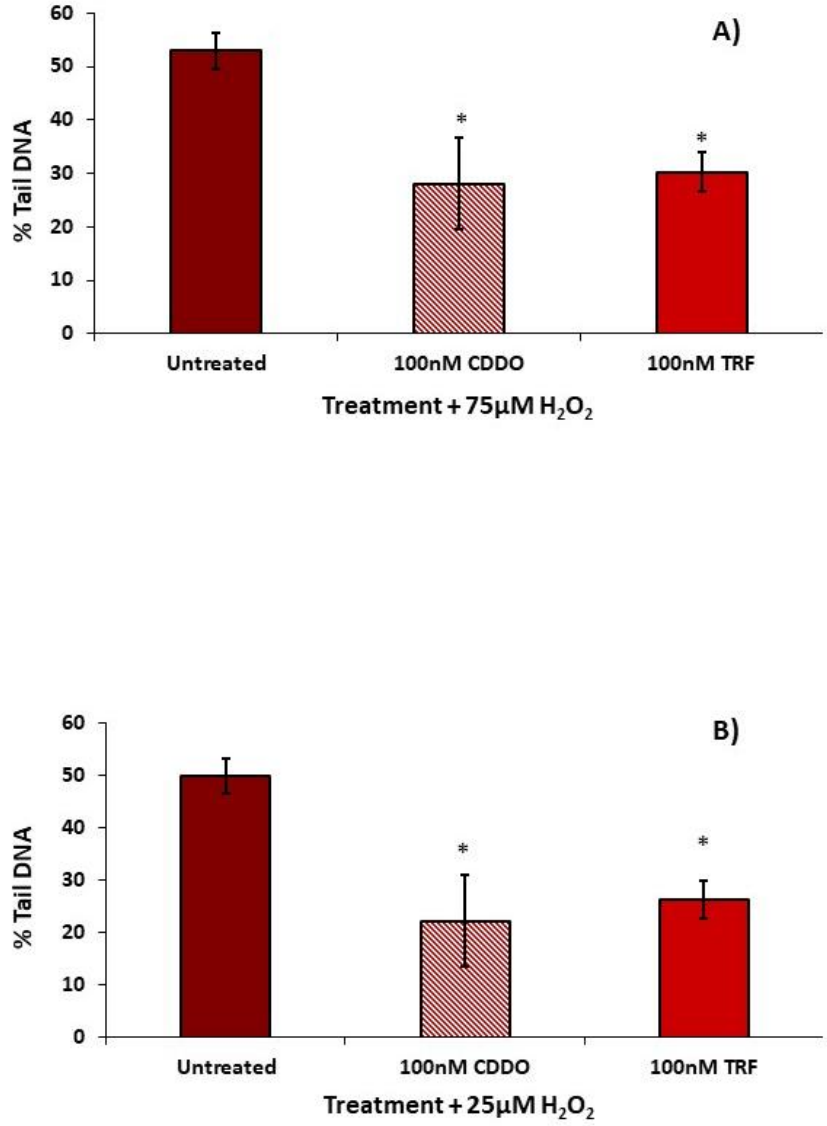

Figure 2: Anti-genotoxic effects of 100 nM CDDO and TRF after 24 h pre-incubation on DNA damage in (A) HT29 and (B) CCD 841 cells challenged with H_2O_2. Data is presented as mean of 3 independent experiments ± SD compared to the untreated cells as control. One-way ANOVA and Post Hoc test Dunnett's T * $p < 0.05$.

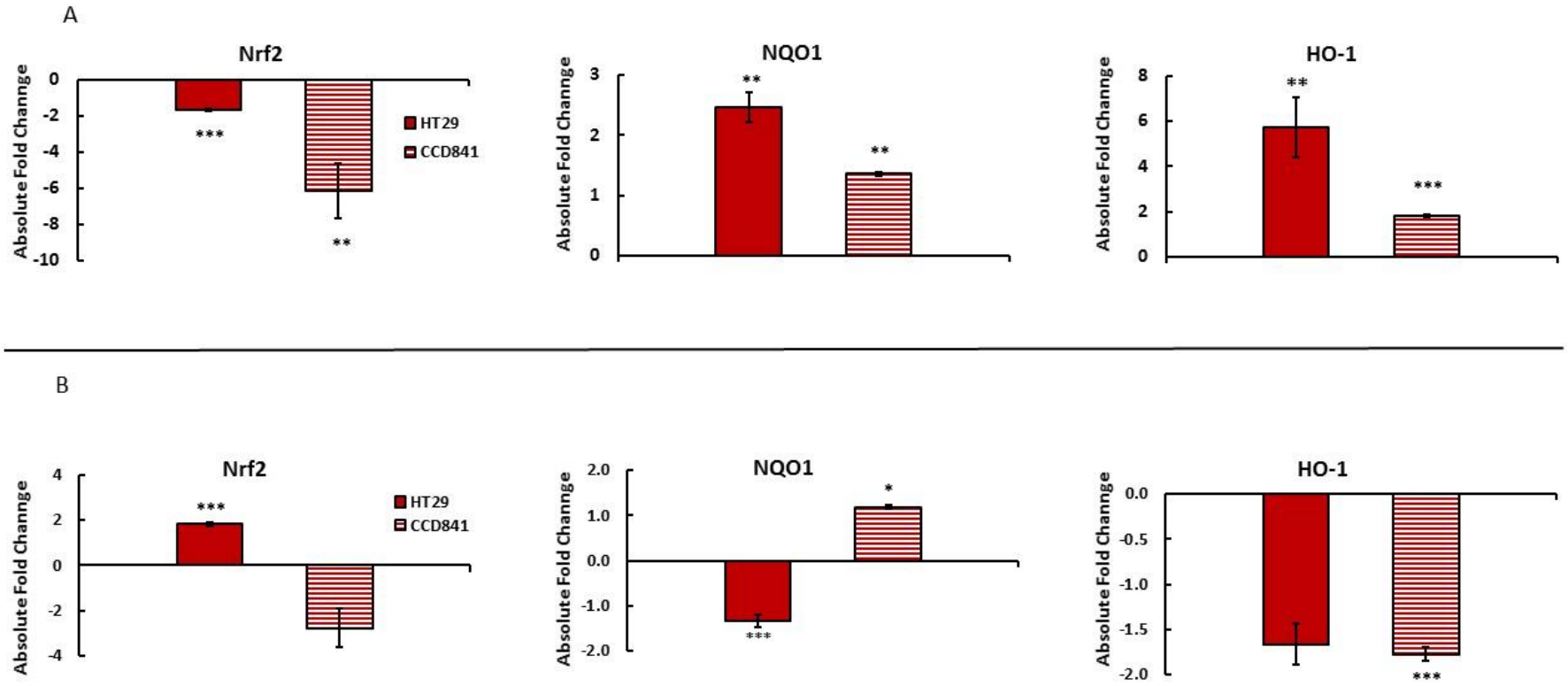

Figure 3: Effect of (A) CDDO and (B) TRF on ARE pathway in HT29 and CCD841 cells. Graphs show the change in gene expression when compared to the control normalised untreated cells. Data presented is mean of 3 independent experiments +SD. One-way ANOVA and Dunnett's Multiple Comparisons test. * $p<0.05$, ** $p<0.01$, ***$p<0.01$.

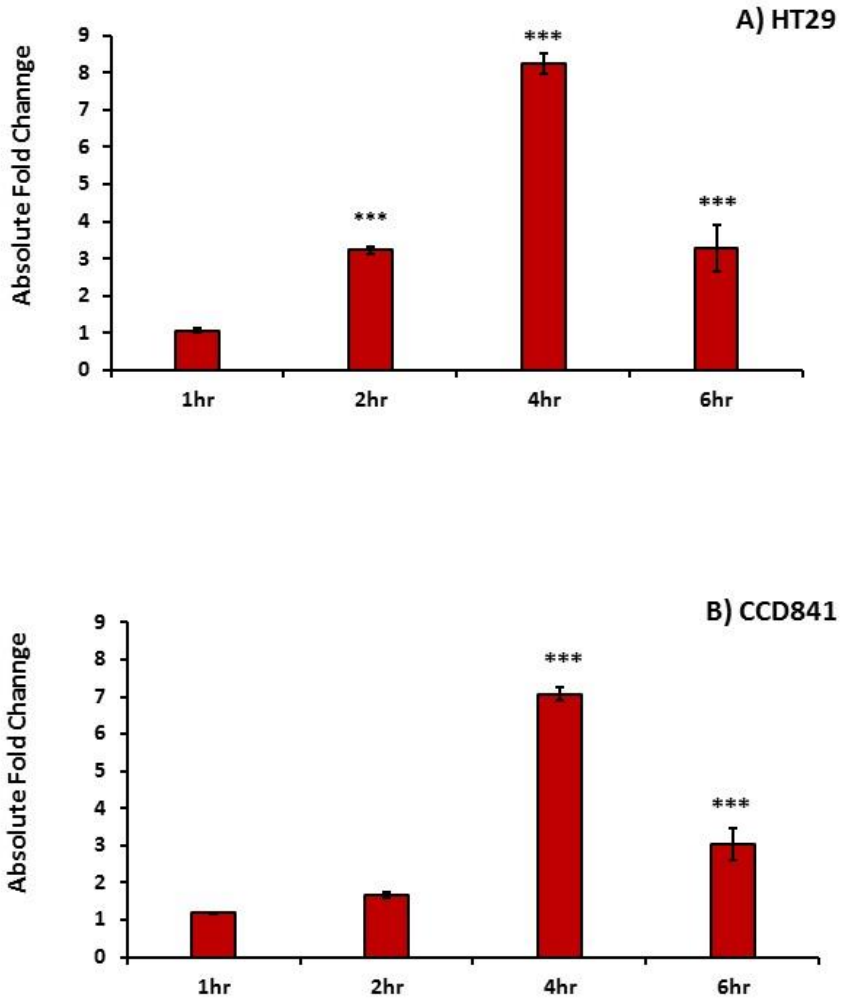

Figure 4: Time-course treatment of HT29 and CCD841 cells with 100nM CDDO. Graphs show absolute fold change values in Nrf2 gene expression, (i.e. change in gene expression when compared to normalised untreated cell as control). Data is presented as the mean of 3 individual experiments (n=3) ± SD. Student t-test. * $p<0.01$, ** $p<0.001$.

Discussion:

The initial targeted LC-MS^n analysis of ileal fluid samples collected post-raspberry consumption, as described in chapter 3 and by McDougall *et al.*, provided an insight into the metabolic pathway for anthocyanins and ellagitannins (29). We identified the phenolic compounds which, in an individual with an intact instestine, reach the colon and have the potential to interact with gut microbiota and colonic epithelial cells (34). To produce a more comprehensive overview of the chemical composition of the ileal fluid samples non-targeted LC-MS^n was performed. In this instance 2 unknown components where detected within all 11 of the post-raspberry ileal samples, *m/z* 355 and 679. Both compounds were present in whole raspberry puree and unseeded puree but where found at a greater concentration in within seed extracts. The combined results from analysis of the raspberry sample and ileal samples identifed *m/z* 679 as a dimer of *m/z* 355 and putatively identified this compound as an ursolic acid-based triterpenoid (30).

This novel TRF extracted from raspberries proved to be effective in reducing H_2O_2-induced DNA damage in both a normal colonic epithelial cell line (CCD 841 CoN) and a colonic adenocarcinoma cell line (HT29). We have attempted to elucidate the method by which this antigenotoxic occurs through use of qPCR analysis. Results from this investigation illustrated that, 24hr incubation with TRF resulted in a significant increase in Nrf2 expression within HT29 cells and significant up-regulation of NQO1 in CCD 841 cells. The synthetic triterpenoid control, CDDO, is a known inducer of the Nrf2/ARE pathway (35). This was confirmed by the results above as demonstrated by the statistically significant up-regulation in NQO-1 and HO-1 expression in both HT29 and CCD 841 cells. The discrepancy between the bioactivity of the two treatments could be attributed to the variation in concentrations of the pure CDDO and the fractionated mixture of the triterpenoid-rich extract. However, differences in the chemical structure of these two compounds could also contribute to the

divergence of results. The activation of the Nrf2-ARE pathway is time-dependent as shown above with CDDO time-course treatment (Figure 4) and therefore the actions of TRF upon this system have may not have been captured at the 24hr time-point. Few studies have established the role that triterpenoids play in the reduction of oxidative damage and up-regulation of the Nrf2/ARE pathway.

There is however considerable evidence to suggest that the naturally occurring triterpenoids and their synthetic counterparts can induce protective effects in a range of disease states (10, 36, 37). One compound of significance ursolic acid (UA), or 3B-hydroxy-urs-12-en-28-oic-acid, a pentacyclic triterpenoid which has demonstrated both anti-inflammatory and anti-cancer effects (38). This is of interest as the TRF investigated within this chapter has been putatively identified as UA-based. A range of herbs (peppermint, thyme, rosemary, lavender) and berries (blackberries, blueberries) contain UA, often found in conjunction with a second triterpenoid, oleanolic acid (OA, 3B-hydroxyolean-12-en-28oic acid) (39, 40). The chemopreventive effects of UA have been demonstrated across a range of cancer types, including colorectal carcinomas. The mechanism behind this action is not yet fully understood but the triterpenoid can induce apoptosis, inhibit proliferation and inhibit metastasis in both *in vitro* and *in vivo* models of CRC (41-43). One action of significance is the inhibitory effect of UA upon the activation NF-κB and therefore the has the potential to suppress carcinogenesis (44). It is therefore possible that the TRF in this chapter uses a similar mechanism of action, which we had not previously considered.

In contrast OA has been shown to increase the nuclear translocation of Nrf2 and subsequently induce the transcription of many antioxidant and phase II detoxifying genes (45). Activating both Nrf2-dependent and Nrf2-independent signalling cascades, OA inhibits proliferation of carcinogenic cells and induces apoptosis in a range of cancers including; hepatocellular, glioblastoma, breast, prostate and colorectal (46-50). Due to the anticancer

potential and pharmacological activity of OA, a variety of synthetic derivatives have been created, through modifications to the original compound a wide range of potent oleanane based triterpenoids have been produced (51). Two compounds of note are CDDO (2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid) and its methyl ester, CDDO-Me. These facts could also explain the discrepancies in antigenotoxic activity seen in the result above, as CDDO and the TRF are derived from different parent compounds therefore mechanisms of action may differ. It is possible that the TRF, as it is ursolic acid-based may also affect the NF-κB pathway, which is therefore a potential area for further research.

Many triterpenoid-based compounds have previously been linked with a range of chemopreventive properties which have proved effective upon models of colorectal cancer. Ursolic acid significantly reduced single strand DNA breaks in Caco-2 cells following 2hr incubation at concentrations of 5-10μM (52). Maslinic acid, the pentacyclic triterpene, isolated from the leaves and fruit of *Olea europaea* L., can inhibit HT29 cell proliferation and induce apoptosis through activation stress-related signalling as the p53 and JNK pathways (53). Fractions from *Rubus coreanus* Miquel (the Korean black raspberry) which contains 19α-hydroxyursane-type triterpenoids significantly reduced disease activity in the DSS-induced colitis mouse model (54). The triterpenoid rich fraction reduced macrophage infiltration and supressed pro-inflammatory cytokines within the murine model, while treatment of LPS-induced RAW macrophages downregulated activation of NF-κB and p38 MAPK signalling to reducing inflammation pathways. When combined with these previous publications the analysis from this investigation heavily suggests that the bioactivity of triterpenoids, both natural and synthetic, is an area of great potential with regards to the treatment and prevention of CRC.

In contrast to other these other studies the TRF we investigated is derived from the fruit and seeds of the raspberry, which are part of the normal diet and readily consumed. It is therefore

possible to conclude that the anticancer effects attributed to raspberry phytochemicals in previous investigations and described in Chapter 4 and 5 may in some part be due to the presence of triterpenoids (55, 56). Further investigation by McDougall *et al.* has resulted in the isolation of ursolic-based triterpenoid fractions within a range of raspberry genotypes, however the concentration and composition varied from berry to berry (57). The survival of the TRF within the gastrointestinal tract and their ability to induce anti-genotoxic activity at submicromolar concentration (100nM) further confirms the potential that these compounds have exert protective effects within the large intestine. However further investigation is required to provide a more comprehensive understanding of these novel raspberry components and their potential health benefits.

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Chapter 7: General Discussion

The evidence from previous studies has concluded that berries demonstrate a range of anticancer properties which included inducing the signalling pathways involved with; free radical scavenging, DNA repair, cell proliferation, apoptosis and invasion (1-4). This activity has been attributed to the abundant (poly)phenolic compounds found within berries. Chapter 2 reviewed the *in vitro* and *in vivo* studies which illustrated this anti-carcinogenic potential within models of colorectal cancer. The overall aim of this thesis was to build upon these findings, by producing a more physiologically relevant sample with which to identify the mechanism behind these anitgenotoxic effects.

Recently, a greater emphasis has been placed upon the significant effect digestion has upon the (poly)phenolic composition of the consumed berries (5, 6). The comparisons between the phenolic content of whole raspberries versus digested berries, as ileal fluid collected from 11 participants following a human feeding study demonstrated changes in (poly)phenol content (see Chapter 3). The crude berries contained a higher concentration and variety of anthocyanins than the post-berry ileal fluid, which in contrast had a greater amount of ellagic acids. Through use of these *in vivo* samples, the analysis of the post-raspberry ileal fluid more accurately represented the berry metabolites produced during digestion than the previous *in vitro* models. To further improve upon this system an *ex-vivo* model was developed as the ileal fluid samples were placed in 24hr batch culture and subjected to *in vitro* fermentation. In the presence of colonic microbiota, supplied in the form of a faecal inoculum the complex (poly)phenolic structures were converted to more simple metabolites. Anthocynanins were converted to simple and aromatic phenolics including catechol, 4- hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, tyrosol and 3-(3’-hydroxyphenyl)propionic acid, while ellagitannins were converted into ellagic acids. In contrast to previous studies the absence of ellagic acid metabolites, in the form of urolithins, indicates that the chosen faecal donor lacked the necessary species of *Gordonibacter* to further degrade these compounds (7, 8). The

compositional analysis from Chapter 2 confirmed the dramatic changes in phenolic content following digestion.

In order to assess if these changes in composition impact the anticancer activity of berry extracts it was necessary to assess the bioactivity of these samples. Other investigations which relied upon *in vitro* models of digestion have demonstrated that these samples maintain anticancer activity following (poly)phenolic metabolism. To ensure these findings translated to the more physiologically relevant samples used in this thesis a series of in *vitro* assays were utilised. The results from Chapter 4 showed that both the post-berry ileal fluid samples and ileal fluid fermentate samples have the ability to reduce H_2O_2 induced damage. When compared to baseline ileal fluid samples, post-berry ileal fluid significantly reduced DNA damage by ~25% in both colonic cell lines. This activity was also seen following *in vitro* fermentation with post-berry IFF 24h sample decreasing DNA damage in CCD 841 CoN cells by 30%. Using the findings from this chapter combined with the compositional analysis in Chapter 4 the bioactivity of the individual phenolics; 3-(3-Hydroxyphenyl)propionic acid (3HPPA), 3-phenylpropionic acid (3PPA), 4-Hydroxbenzoic acid (4HBA) and Benzoic Acid (BA) was also assessed. All 4 of these compounds showed the ability to reduce DNA damage in both cell lines. These results confirm that although berry phenolic composition changes dramatically following digestion and colonic fermentation these berry metabolites still possess the ability to induce anticancer activity and in turn reduce the risk of CRC.

The mechanism by which these chemoprotective actions occur was investigated in Chapter 5. Using the findings of previous studies which have indicated that the Nrf2/ARE pathway can be induced via (poly)phenolic compounds, the effect of these biologically relevant samples upon this system was assessed via real-time qPCR analysis. A total of 6/11 post-berry IFF 24hr samples demonstrated the ability to up-regulate Nrf2 and two of its downstream targets NQO-1 and HO-1. Such results suggest that the complex mixture

poly(phenols) found within the IFF samples plays a key role in the activation of Nrf2. It was also important to attempt to identify any antigentoxic potential within individual phenolics. All 4 individual phenolics (3HPPA, 3PPA, 4HBA, BA) showed the ability to increase mRNA expression across both cell lines. Further analysis of the results from Chapters 4 and 5 illustrate that there is a complex relationship between phenolic concentration, reduction in DNA damage and activation of the Nrf2 pathway. Only one IFF sample showed a significant decrease in % tail DNA and an increase all 3 genes of interest which also correlated with an increase in total (poly)phenol content. With the isolated phenolic compounds and CCD 841 CoN cells there was a direct correlation between concentration, reduction in DNA damage and increased expression of the target genes, with 100μM (the highest concentration) proving the post effective. This did not translate to HT29 cells and as such implies that further investigation is required to gain a greater understanding about this mechanism of action. Overall the findings from the Chapters 3-5 confirmed that the berry metabolites produced following digestion retain the bioactivity previously demonstrated by whole berry extracts. These samples can reduce DNA damage within colonic epithelial cells and colonic adenocarcinoma, with activation of the Nrf2/ARE pathway playing a key role in this antigenotoxic activity. Such results indicate berry (poly)phenols have the potential to act as dietary agents for anticarcinogenic activity.

Chapter 6 combined the techniques used within the previous chapters to assess the bioactivity of a novel triterpenoid which was detected following compositional analysis of post-berry ileal fluids. This triterpenoid-rich fraction (TRF) found within the raspberry seeds was putatively identified as a urosolic acid-based triterpenoid. TRF demonstrated the ability to reduce H_2O_2 induced DNA damage within both CCD 841 CoN and HT29 cells which correlated with up-regulation of NQO1 and HO-1 mRNA levels. To the best of our

knowledge this was the first study to identify a raspberry seed-derived triterpenoid which demonstrated antigenotoxic potential through activation of the Nrf2/ARE pathway.

The findings of this thesis have confirmed that berry (poly)phenols retain their chemoprotective activity following digestion and colonic fermentation. All the tested samples be that post-berry ieal fluid, post-berry IFF, individual phenolics or TRF demonstrated the ability to reduce DNA damage, an action which corresponded with the up-regulation of mRNA levels of Nrf2 or its target genes NQO1 and HO-1. These observations have highlighted the importance of understanding the role gastrointestinal digestion has upon (poly)phenolic composition and has ultised a unique *ex-vivo* model to produce physiologically relevant samples. As a consequence, we were able to identify a potential mechanism by which these compounds induce chemoprotective action.

Summary of findings:

- The phenolic content of raspberries changed significantly as a result of *in vivo* digestion and *in vitro* fermentation; complex (poly)phenols such as anthocyanins and ellagitannins were converted to simple phenolics and ellagic acids.

- Samples retained antigenotoxic potential following digestion with both post-berry ileal and post-berry IFF samples significantly inhibiting H_2O_2 induced DNA, in *in vitro* models of normal colon (CCD 841 CoN) and colonic adenocarcinoma (HT29).

- Post-berry IFF samples activated the Nrf2/ARE pathway; inducing increased expression of Nrf2, NQO1 and HO-1 mRNA.

- 4 individual phenolics (3HPPA, 3PPA, 4HBA, BA) increased in concentration following *in vitro* fermentation. These compounds decreased H_2O_2 induced DNA damage and up-regulated the target genes in both cell models.

- Raspberry seeds contain a ursolic-acid based triterpenoid, which reduced DNA damage and up-regulated NQO1 and HO-1 within CCD 841 CoN and HT29 cells.

Suggested Further Work

Due to the available volume of ileal fluid fermentate (IFF) the amount of experiments performed was limited. However, it would have been beneficial to assess antigenotoxic potential of these samples and the role Nrf2 plays upon a model of colon cancer (HT29 cells). If time had not played a limiting factor with this thesis qPCR analysis would have been used to assess the effect of ileal fluid samples upon the three target genes. This would allow for greater comparisons between the post-digestion and post-fermentation samples.

In addition to gene expression analysis, measurement of cellular protein changes would have provided a greater insight in to the cellular signalling pathways which played a role in the antigenotoxic activity. Western blot analysis of Nrf2, NQO1 and HO-1 would analyse if the up-regulation of mRNA translated to an increase in protein expression. In conjunction with this it would be valuable to quantify the induction of ARE via a luciferase reporter assay. Generation of Nrf2 knock-out cells through use of siRNA would confirm whether the Nrf2/ARE pathway was the only system activating induction of the phase II detoxify enzymes which reduced DNA damage within the cells.

Alternatively, it would be advantageous to repeat these experiments with another type of berry for example blueberries and assess how the different starting (poly)phenolic composition impacts the downstream results.

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List of Publications:

- Brown EM, **Latimer C**, Allsopp P, Ternan NG, McMullan G, McDougall GJ, Stewart D, Crozier A, Rowland I, Gill CI. In vitro and in vivo models of colorectal cancer: Antigenotoxic activity of berries. Journal of agricultural and food chemistry. 2014 Jan 21;62(18):3852-66.
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