INFLUENCE OF APPLE POMACE AND ITS FRACTIONS ON INTESTINAL TUMOR DEVELOPMENT IN APC$^{MIN/+}$ MICE

By

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ABSTRACT

INFLUENCE OF APPLE POMACE AND ITS FRACTIONS ON INTESTINAL TUMOR DEVELOPMENT IN APC$^{MIN/+}$ MICE

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Consumption of apples has been demonstrated to lower the risk of chronic diseases due to the presence of bioactive components such as fiber and polyphenols. The aims of this study were to determine the effects of apple pomace, a major waste product of apple juice processing, and its fractions on tumor development in APC$^{Min/+}$ mice. APC$^{Min/+}$ mice (21-28 days old) were separated into one of five dietary treatment groups consisting of powdered AIN-93G diet supplemented with either apple pomace, apple juice, apple ethanol extract or apple residue; cornstarch was used as the control diet. Mice consuming the apple juice-containing diet had the fewest adenomas in the small intestine (54.5 ± 5.6) and mice consuming apple residue the greatest number (77.6 ± 5.6), with the other dietary treatments resulting in intermediate tumor numbers. Additionally, consumption of diets containing apple juice led to the smallest ($P<0.05$) numbers of small intestinal adenomas for both female (57.3 ± 8.3) and male (51.7 ± 7.6) mice. In colon, no statistically significant differences in adenoma numbers were observed among mice consuming the different dietary treatments. However, male mice had higher incidence of colonic adenomas (79.7%) than female mice (59.3%). In cecum, mice consuming diets containing apple juice had the smallest average adenoma size and mice consuming apple residue the greatest average adenoma size. These results demonstrate that fractions derived from apple pomace differentially influence intestinal adenoma development in APC$^{Min/+}$ mice, but further research is required to determine the mechanisms for the observed effects.

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I. INTRODUCTION

Colorectal cancer is the second leading cause of deaths in the United States and the third most common cancer in both men and women. It is estimated that there will be 143,460 new cases from colorectal cancer diagnosed and 51,690 deaths due to this disease in the United States in 2012 (American Cancer Society, 2012). However, mortality rates for colorectal cancer have been dropping over the past two decades (American Cancer Society, 2012). This decline in mortality may be a consequence of improved cancer screening leading to earlier detection or to changes in diet and other life-style factors that impact cancer development.

Ecological studies suggest that consuming diets low in fat and high in fiber or calcium is associated with reduced mortality from colorectal cancer (Murtaugh et al., 2008), whereas high consumption of red meat (beef, pork, lamb, and processed meat) is associated with elevated cancer risk (Willett et al., 1990; Jemal et al., 2011). In addition, epidemiological studies have shown that diets rich in fruits and vegetables could reduce the incidence of colorectal cancers (Witte et al., 1996).

Fruits and vegetables contain a variety of polyphenolic compounds that have potential chemopreventative properties. Due to their widespread consumption, apples are one of the primary sources of flavonoids in diets consumed in the USA and in Europe (Boyer and Liu, 2004). It has been estimated that phenolics from apples account for twenty-two percent of the total phenolics consumed from fruits and vegetables in the USA, the largest dietary source of these compounds (Vinson et al., 2001; Boyer and Liu, 2004). Flavonoids likely prevent tumor growth by a variety of mechanisms including the alteration of signal transduction pathways associated with generating a stress response (Briviba et al., 2002; Lin 2002), and by inhibiting

proliferation of cancer cells and increasing apoptosis (Yanagihara et al., 1993; Kuntz et al., 1999).

There is ample experimental evidence that consumption of diets rich in polyphenolic compounds is associated with reduced cancer development in a variety of models. Apples are well-recognized as a rich source of these polyphenolic compounds in the diet. Despite this considerable evidence that apple phenolics could be associated with reduced cancer risk, relatively few studies investigating this hypothesis have been reported in the peer-reviewed scientific literature. Even fewer studies examining the potential impact of polyphenolic-rich apple processing waste products, such as apple pomace and fractions prepared from this by-product, have been reported. Therefore, this study was designed to determine the influence of apple pomace and fractions prepared from apple pomace on intestinal tumor development in $APC^{Min/+}$ mice, a model of human colon cancer.

II. REVIEW OF LITERATURE

A. Incidence of Colon Cancer

Colorectal cancer (CRC) is the third most common cancer diagnosed in men and women and is the second leading cause of cancer deaths in the United States (National Cancer Institute, 2012). There has been a decline in the past 20 years in mortality caused by colon cancer, but colorectal cancer is still a major health concern in the United States and globally. Incidence of CRC begins to rise after the age of 40 years and increases quickly at ages 50 to 55 years (National Cancer Institute, 2012). The prevalence of adenomatous polyps, the precursor lesions of most sporadic CRC, also increases with age. Incidence tends to be high in developed countries such as in North America, Australia/New Zealand, Western Europe and incidence is relatively low in Africa and Asia (Parkin et al., 2005).

B. Molecular Basis of Colon Cancer

B.1. Genetic alterations in colon cancer

Development of cancer or carcinogenesis is a microevolutionary process including three major stages – initiation, promotion, and progression. Initiation is the result of genetic change to a cell that provides it a growth advantage as compared to neighbor cells. Following initiation, cells go through a process referred to as promotion. Promotion is affected by factors that do not alter DNA sequences; it leads to increase a large number of daughter cells containing the mutation created in first stage. Progression requires additional mutations to transform a benign tumor to a neoplasm and malignancy (Pilot et al., 2004). Colon cancer typically begins as a benign tumor called a polyp. Polyps enlarge and require further mutations that turn them into

malignant carcinomas that are ultimately invasive and metastatic. The most frequent form of CRC is sporadic colorectal cancer, the second most common is hereditary non-polyposis colon cancer (HNPCC), and the least common is familial adenomatous polyposis (FAP) (Heyer et al., 1999). In general, colon carcinoma is the result of multiple sequential genetic alterations. These alterations can either happen in the sporadic forms or in genetic cancer predisposition syndromes such as HNPCC or FAP (Arnold et al., 2005). There are three general types of gene changes that can lead to colon cancer: 1) proto-oncogenes promote cell growth when mutated and become oncogenes; 2) tumor-suppressor genes are mutated and can no longer effectively regulate cell proliferation; and 3) mutations of DNA repair genes, which can result in irreparable DNA damage leading to mutations in proto-oncogenes and tumor-suppressor genes (Gryfe et al., 1997). Common genetic mutations in colon cancer include those in the adenomatous polyposis coli (APC), k-ras, deleted in colorectal cancer (DCC) and p53 genes, which are important events for development of colon cancer (Heyer et al., 1999; Arnold et al., 2005).

The APC gene is a tumor suppressor gene located on chromosome 5q21 in humans (Gryfe et al., 1997). Inactivation mutations of the APC gene are a common event leading to colon cancer development. The APC protein product can inhibit cell proliferation and stimulate apoptosis by inactivating β-catenin which, when activated, promotes cell proliferation. Thus, mutated APC enables elevated nuclear β-catenin concentrations, which can activate signaling pathways promoting formation of the adenoma (Arnold et al., 2005).

Mutations of k-ras, a proto-oncogene on chromosome 12, have been detected in 50% of large polyps and colorectal cancers (Vogelstein et al., 1988). This oncogene encodes for a protein which transmits extracellular growth signals to the nucleus. Mutations of k-ras are correlated with increased size of the lesions and progression to dysplasia. The mutation rate of

k-ras is 14% in small adenomas (< 2 cm) and is 33% in large adenomas (> 2 cm). In highly dysplastic adenomas, k-ras mutations are found up to 50% of the time as compared to 33% in moderately dysplastic lesions (Fearon et al., 1990).

DCC is a tumor suppressor gene located on chromosome 18q. DCC mutations mostly occur late in tumor progression wherein its frequent allelic deletion is important in the transition from an intermediate to a late adenoma (Vogelstein et al., 1988). Mutations in the p53 gene located on chromosome 17q also are common in colorectal cancer. The protein product of the p53 gene binds with specific DNA sequences and activates transcription of downstream genes, allowing repair of damaged DNA or leading to apoptosis (Gryfe et al., 1997). Thus, when p53 is mutated, it cannot inhibit the growth of rapidly proliferating cells.

B.2 Animal Model of Colon Cancer

The $APC^{Min/+}$ (multiple intestinal neoplasia) mouse, which has a mutation in the APC (adenomatous polyposis coli) gene (Su et al., 1992), has been used extensively as a model for human colorectal cancer in recent years. This mouse has a mutation at codon 850 of the APC gene resulting in production of a truncated protein of 850 amino acids (Heyer et al., 1999). The mutation in the APC gene of these mice is similar to that observed in human patients with familial adenomatous polyposis (FAP) and in many sporadic colon cancers (Nishisho et al., 1991; Powell, 1992). There are many similarities (86-90%) at the nucleotide and amino acid levels between the APC gene in $APC^{Min/+}$ mice and that in FAP patients (Su et al., 1992). Also, some studies have demonstrated that somatic APC mutations in FAP patients are present for about 75% of sporadic colorectal adenomas and carcinomas (Kinzler and Vogelstein, 1996; Arnold et al., 2005).

All cells in the $APC^{Min/+}$ mouse are mutated, and a subset of these cells accumulates additional mutations which ultimately lead to development of intestinal tumors. During tumor progression in this model, the loss of the wild-type APC allele is the earliest genetic change (Heyer, 1999). C57BL6/J mice carrying the $APC^{Min/+}$ mutation develop adenomas in the small intestine early in life and, if untreated, these adenomas lead to significant morbidity and mortality by 120-140 days of age (Hinoi et al., 2007). $APC^{Min/+}$ mice also develop polypoid adenomas in the colon and, to a lesser extent, in the cecum. Numerous studies have demonstrated that development of small intestinal and colonic adenomas in $APC^{Min/+}$ mice can be modulated by dietary factors and other compounds. Therefore, using the $APC^{Min/+}$ mouse model in intestinal tumorigenesis studies can provide insight into potential chemopreventative agents for intestinal carcinogenesis.

C. Dietary Polyphenols

Polyphenols are secondary metabolites produced by plants which are widely distributed in the plant kingdom. Polyphenols are classified into four main groups depending on the number of phenol rings and structure elements that bind these rings to one another. These four primary groups of polyphenols are phenolic acids, flavonoids, stilbenes and lignans (Pandey and Rizvi, 2009). Flavonoids and phenolic acids are the two main types of polyphenols which are common constituents of plant foods including cereals, fruits, and vegetables (Scalbert et al., 2005).

Polyphenols are well-known as strong antioxidants which can protect cells from oxidative damage due to their intrinsic hydrogen atom donating capacity (Rice-Evans et al., 1996). Interest in these compounds is increasing because polyphenols possess potent biological

properties including anti-carcinogenic, anti-inflammatory, neuroprotection, anti-allergic, antidiabetic, and cardioprotection activities (Scalbert et al., 2005; Han et al., 2007; João et al., 2011).

The anti-carcinogenic effect of polyphenols is one of the most documented properties. Several mechanisms have been suggested to explain the effect of polyphenols on tumor growth. Polyphenols can potentially influence the three major stages of carcinogenesis: initiation, promotion and progression. First, polyphenols may reduce initiation by modulating the expression of cytochrome P450 enzymes and thereby influencing the activation of procarcinogens to carcinogens. Polyphenols may increase the expression of phase II conjugating enzymes for detoxification of xenobiotics, inducing a general boosting of body defense against toxic compounds (Baez et al., 1997; Scalbert et al., 2005; Pandey and Rizvi, 2009). Polyphenols may also inhibit cancer initiation by stimulating DNA repair. Secondly, polyphenols may act as suppressing agents and limit tumor development from initiated cells via mechanisms such as inhibition of cell proliferation (Kuntz et al., 1999). It has been observed that some polyphenols express anti-tumor effects by inhibiting activator protein 1 (AP1), a transcription factor commonly up-regulated in tumor promoter-induced neoplastic transformation (Dong et al., 1997; Barthelman et al., 1998). Certain polyphenols also can suppress protein kinase C activity and oncogene expression (Lin et al., 1997) and decrease the activity of ornithine decarboxylase (Schneider et al., 2000), a key enzyme in the synthesis of polyamines associated with cancer growth. Collectively, these effects of polyphenols on activity of cell signaling enzymes can inhibit tumor promotion. Lastly, polyphenols can reduce the growth of tumors by inhibiting angiogenesis or inducing apoptosis of tumor cells (Scalbert et al., 2005, Pandey and Rizvi, 2009).

However, the potential beneficial effects of polyophenols may not be fully realized *in vivo* because of their limited absorption and metabolism. Polyphenols can be present in bound and free forms in plants. Generally, the aglycone forms of polyphenols can be absorbed from the small intestine, but conjugated polyphenols in the form of esters, glyscosides, or polymers cannot be absorbed in the native form (Pandey and Rizvi, 2009). Before absorption, these compounds must be hydrolyzed by intestinal enzymes, such as β-glucosidases and lactase-phlorizin hydrolase, or by the colonic microflora (Nemeth et al., 2003; Aura, 2008). As a result, the forms of polyphenols which reach the blood and tissues *in vivo* may differ from native forms in the plant. Such complexity is a challenge to elucidating the potential biological effects of polyphenols and their metabolites *in vivo*.

D. Dietary Fiber

Epidemiological studies have shown that frequent consumption of fruits and vegetables is associated with reducing the risk of chronic diseases such as cardiovascular disease and cancers (American Dietetic Association, 2008). Dietary fiber and antioxidants (vitamin E and C, carotenoids, polyphenols) are two nutritional components having a significant role in the prevention of these diseases (Saura-Calisto, 2011). Dietary fiber is defined as "plant polysaccharides and lignin which are resistant to hydrolysis by the digestive enzymes of man" (Trowell et al., 1976).

Fiber can be categorized into soluble and insoluble fiber. Soluble fiber includes some hemicelluloses, pectins, gums and mucilages which can delay gastric emptying, decrease glucose absorption, and slow down the process of digestion. In contrast, insoluble fiber components such as lignin, cellulose, and some hemicelluloses can decrease internal transit time and increase fecal

bulk (Groff and Gropper, 1999). Because of these properties, it has been suggested that fiber may protect against cardiovascular disease and colon cancer. Fatty acids, cholesterol and bile acids bound with fiber cannot be absorbed and these will be passed into the large intestine for degradation (by bacteria) or excretion in the feces (Story et al., 1976; Kritchevsky, 1978). Therefore, fiber can diminish absorption of lipid and lower serum cholesterol. The action of fiber on bile acid metabolism may also be associated with reduced colon cancer risk. Bile acids can be converted in the intestine to harmful secondary bile acids which can promote colon carcinogenesis. These secondary bile acids can bind to fiber which promotes their excretion (Story et al., 1976). In addition, insoluble fiber can increase fecal bulk and reduce intestinal transit time, decreasing potential contact time of carcinogens with the colonic mucosa (Rose et al., 2007).

The other potential mechanism whereby fiber may protect against colon cancer is via its fermentation by intestinal bacteria, producing short chain fatty acids. For example, butyric acid, a product of insoluble fiber fermentation, has been demonstrated to slow the proliferation of tumor cells (Hijova and Chmelarova, 2007). Proliferation of bacteria in the colon can also play an important role in detoxification, resulting in the increased microbial scavenging and sequestering of toxic substances and promoting their excretion. Furthermore, some bacteria (e.g. *Lactobacillus acidophilus*) can decrease the activities of enzymes that catalyze the conversion of procarcinogens to carcinogens (Groff and Gropper, 1999).

However, the potential effect of fiber intake on colon cancer is still controversial as the results of epidemiological studies have not consistently supported a protective role of fiber against colon cancer. A prospective study of 88,757 women in the USA followed during a 16 year period found no significant correlation between fiber intake and the risk of colorectal

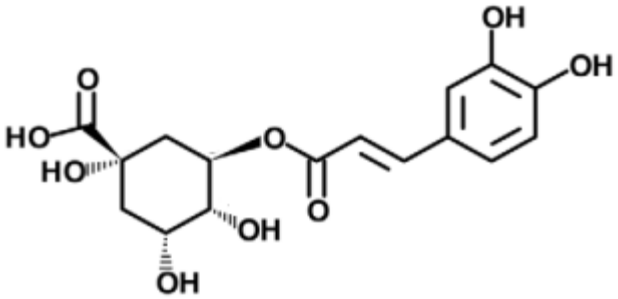
adenoma incidence (Fuchs et al., 1999). More recent studies have found that a high fiber intake was inversely correlated with the risk of colon cancer. In one study, Bingham and colleagues found that persons with low average intakes of dietary fiber could decrease their risk of colorectal cancer by 40% if they increased their total fiber intake from foods by a factor of two (Bingham et al., 2003). These beneficial effects of fiber were also confirmed by another study in which the group that consumed the highest quantities of dietary fiber had a 27% lower risk of developing colorectal adenomas as compared to the group with the lowest dietary fiber intake (Peters et al., 2003). Similarly, a case-control study conducted in men and women found that groups with high intakes of fruits and vegetables had reduced risk of colorectal adenomas (Millen et al., 2007).

E. Apple Polyphenols and Their Bioactivities

Polyphenols are important components of vegetables and fruits, and apples are a particularly rich source of these compounds. Apples (*Malus* sp., *Rosaceae*) have been studied around the world because of their high levels of polyphenols and other phytochemicals (Gerhauser, 2008 and Hyson, 2011). Five types of polyphenols are commonly present in apples – hydroxycinnamic acids, flavan-3-ols/procyanidins, anthocyanins, flavonols, and dihydrochalcones (Oleszek et al., 1988; Mazza et al., 1992; Schieber et al., 2001). Monomers such as (+)-catechin and (-)-epicatechin, chlorogenic acid, quercetin 3-glycosides (galactoside, glucoside, xyloside, arabinoside, and rhamnoside) and their dimers, phloridzin, and cyanidin 3-glycosides (mainly galactoside) are the significant individual polyphenols in apple (McRae et al., 1990; Mazza et al., 1992; Guyot et al., 2002). The chemical structures of several polyphenols in apple and apple products are shown in Figure 1.

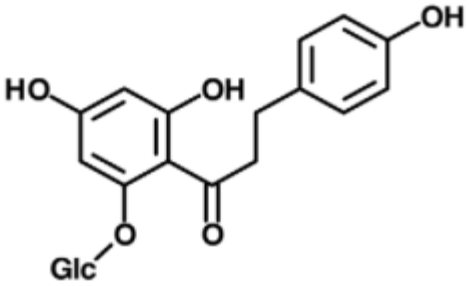
Hydroxycinnamic acids

5- Caffeoylquinic acid (Chlorogenic acid



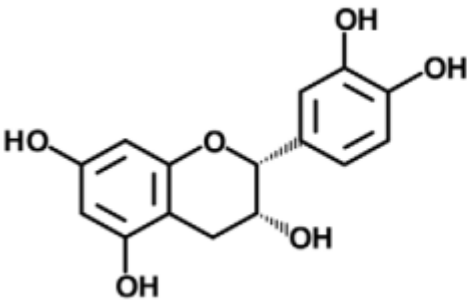
Dihydrochalcones

Phloretine - 2'- glucoside (Ploridzin)



Flavan-3-ols

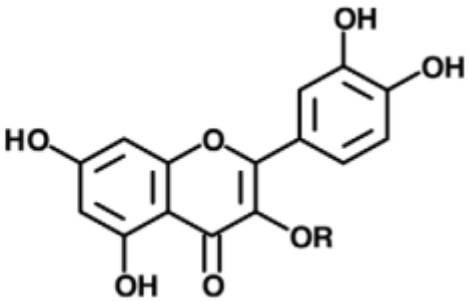
(-)- Epicatechin



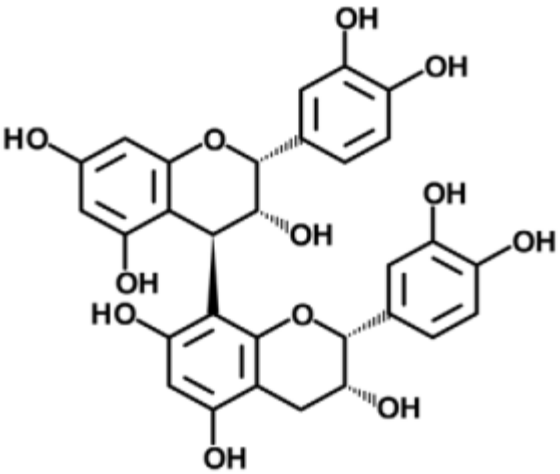
Flavonols

Quercetin (R = H) and

Quercetin glycosides (R = sugar moiety)



Procyanidin B_2



Triterpenoids (from apple peel)

Ursolic acid

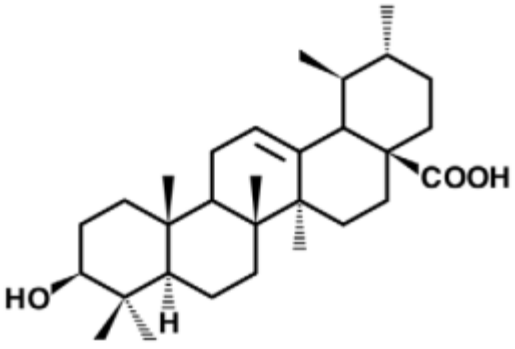


Figure 1. The chemical structures of several polyphenols in apple and apple products. Adapted from Gerhauser (2007).

Concentrations of the phenolic compounds in the flesh and in the skin are different, and polyphenol composition also strongly depends on the apple variety. Total polyphenolic content of apples may range from 0.1 to 10 g/kg fresh weight (Guyot, 1998; Manach et al., 2004). Out of ten varieties commonly consumed in the US, Fuji apples had the highest total phenolic and total flavonoid contents (Boyer and Liu, 2004). One report studying four varieties of apple – Ida Red, Rome Beauty, Cortland and Golden Delicious – also showed that Ida Red and Rome Beauty apples had the highest concentrations of total phenolic compounds and flavonoids (Wolfe et al., 2003). A recent report found that Granny Smith (a green apple variety) contained more total polyphenol and flavonoid compounds than Royal Gala apples – a red variety (Alberto et al., 2006). Depending on the variety of apple, procyanidin concentration accounts for about 69-87% of total polyphenols, which makes procyanidins the predominant polyphenols in apples. Procyanidins are typically concentrated in the skin but are also present in the flesh. The procyanidin concentration in the skin has been reported to be 3-7 times higher than in the flesh (Guyot et al., 2002).

Because they are a good source of antioxidants and polyphenolic compounds, apples are a standout for containing health-supportive nutrients. Apple consumption is also associated with reduced risk of cancer. There are many studies which have demonstrated the potential anticancer activity of apple phytochemical compounds *in vitro* and *in vivo*. These will be reviewed in the subsequent sections.

E1. *In Vitro* Studies Using Cancer Cell Lines

Wolfe et al. (2003) found that apple extract greatly inhibited HepG2 human liver cancer cell growth. He and Liu (2008) likewise reported that triterpenoids such as ursolic acid and the

flavonoid glycoside quercetin-3-O-β-D-glucopyranoside from polyphenol extracts of Red Delicious apples had antiproliferative effects against HepG2 cells and MCF-7 human breast cancer cells. Apple polyphenolic compounds also have demonstrated inhibitory effects on colon cancer cells. Schaefer and colleagues (2006) reported that apple juice extracts including rutin, epicatechin and caffeic acid can reduce oxidative DNA damage in human colon cancer cell lines (HT-29, Caco-2). Reduction of DNA damage potentially would be indicative of protective effects against cancer development since DNA single strand breaks are commonly the first step in chemical carcinogenesis (Barth et al., 2005). These authors also found that chlorogenic acid in juice extracts significantly decreased cellular concentrations of reactive oxygen species (ROS). Similarly, one study in which the anti-cancer properties of apple phenolics were tested on different human colon cancer cell lines showed that apple extract prevented hydrogen peroxide-induced damage in HT-29 cells, improved the barrier function in CaCo-2 cells, and inhibited the invasion of HT115 cells in an *in vitro* Matrigel invasion assay (McCann et al., 2007).

Collectively, these studies have demonstrated that polyphenols extracted from apples can protect against colon cancer from initiation through later stages of cancer promotion and progression. Decreased DNA damage by apple polyphenols can be considered to be indicative of potential to reduce the initiation stage of tumor development. Increased barrier function can be related to potential to reduce tumor promotion by preventing abnormal cellular interactions with the surrounding extracellular environment. Reducing the invasion of cancer cells can be indicative of potential inhibition of the process of tumor metastasis. Therefore, *in vitro* studies demonstrate considerable potential for apple polyphenols to inhibit colon cancer development.

E2. Animal and Human Studies Related to Cancer

In a study using rats which had been injected with 1, 2-dimethylhydrazine (DMH) to induce colonic adenoma development, Barth et al. (2005) investigated the potential anticancer effect of cloudy apple juice. Rats that were watered with cloudy apple juice and clear apple juice had reduced colonic crypt epithelial cell proliferation, but only cloudy apple juice significantly decreased the number of large aberrant crypt foci (ACF), which are premalignant lesions that can progress to adenomas and carcinomas in this model. Even though the cloudy and clear juices had similar concentrations of polyphenolic monomers, the authors speculated that cloudy apple juice had a greater effect because it contained higher concentrations of procyanidin B2/B1 and pectin. A different study, using rats administered whole apple extracts two weeks before being injected with 7, 12-dimethylbenz[a]anthracene (DMBA), found inhibition of cumulative mammary tumors after continuously administering apple extracts for 24 weeks. The mammary tumor onset was delayed with increasing dose of apple extracts. Also, the tumor numbers and tumor burden were reduced significantly (Liu et al., 2005).

The addition of apple phenolic compounds (mainly polymeric molecules) to the drinking water can inhibit the growth of human metastatic colon cancer cells (Gosse et al., 2005). In addition, the extract inactivated protein kinase C and also down-regulated polyamine biosynthesis which may induce apoptosis in tumor cells. Furthermore, the fraction with high polymeric polyphenol content demonstrated a protective effect when it was administered through drinking water to rats that had been treated with azoxymethane to induce colon cancer. It was found that the numbers of ACF were reduced significantly in the colons of mice administered the apple extracts.

Recently, the administration of an apple polyphenol extract (APE) rich in phytochemical compounds has been tested for its effects in on colorectal tumors in the $APC^{Min/+}$ model of human colon cancer (Fini et al., 2011). $APC^{Min/+}$ mice administered APE had reductions in polyp load of 52% and 60% for Western Diet and Balanced Diet, respectively. This finding supports the concept that apple polyphenolics represent promising chemopreventive agents against colon cancer development. However, polyphenols and flavonoids are not stable during storage. Depending on their structures, pH, temperature and presence of complexing agents, polyphenols can degradation over time in storage (van der Sluis et al., 2005; Bakowska et al., 2003). Therefore, it is difficult to correlate apple polyphenols administered by APE in the drinking water with colon cancer risk. In addition, although several animal studies have demonstrated an association between administration of APE and reduced colon cancer risk (Barth et al., 2005; Schaefer et al., 2006; McCann et al., 2007), there is little published research on the comparative effects of apple and its component fractions on colon cancer risk. Because each fraction contains different polyphenolic compounds with varying physiochemical properties, it is anticipated the fractions will have different effects on colorectal cancer development.

There is considerable evidence from epidemiological studies suggesting that consumption of apples and apple products is associated with reduced colorectal cancer risk. In a case control study in Italy with 1,953 patients having colorectal cancer and 6,629 matched cases without cancer, persons who consumed more than one apple per day had significantly reduced colorectal cancer in comparison with those who consumed less than one apple per day (95% confidence interval = 0.71-0.90) (Gallus et al., 2005). In another larger study with more than 34,000 women in the Nurses' Health Study, it was reported that the odds ratio (OR) for colorectal adenomas was

0.83 (95% CI= 0.70-0.98) when comparing the women in the highest and lowest quintiles of apple consumption (Michels et al., 2006). Collectively, these *in vivo* studies in animal models of colon cancer and human epidemiological studies support the hypothesis that consumption of apples and apple polyphenols can protect against colon cancer development.

III. RATIONALE AND AIMS

Colon cancer is one of the leading causes of cancer deaths in Western countries. Certain dietary components are recognized to increase or reduce colon cancer risk. The potential of polyphenols in apples and apple products to decrease colon cancer risk has been demonstrated in cell and animal models of human colon cancer. However, despite considerable evidence supporting the potential protective effects of apples and apple polyphenols on colon cancer risk, no systematic studies have been reported which examined the potential impact of different apple fractions on cancer risk. For this research we were particularly interested in the potential anticancer effects of apple waste streams such as apple pomace from juice manufacturing, as this material is naturally rich in polyphenolics and other compounds such as dietary fiber that may reduce colon cancer development. In addition to testing the effects of intact apple pomace on colon cancer risk, we were interested in fractionating this material to further elucidate the fractions that are most responsible for the putative anticancer effects of apples.

The overall goal of this research was to determine the potential of apple processing waste products to reduce colon cancer risk using a mouse model of human colon cancer development ($APC^{Min/+}$ mice). The specific aims of this research were to:

1) fractionate apple pomace into components that differ in concentration of polyphenolic compounds and other bioactive constituents such as dietary fiber, and characterize the polyphenolic composition of these fractions, and
2) determine the effect of these fractions (apple pomace, juice pressed from apple pomace, ethanol extract of apple pomace, and pomace residue after ethanol extraction) and a corn starch-based control diet on intestinal adenoma development in $APC^{Min/+}$ mice.

The first specific aim was addressed by obtaining waste apple pomace from a commercial food processor, subjecting this material to further processing to obtain the indicated fractions, and characterizing the polyphenolic composition of the resulting fractions by high-performance liquid chromatography. The second specific aim was addressed by incorporating the apple pomace and its fractions in experimental diets administered to $APC^{Min/+}$ mice for nine weeks and assessing the impact of these diets (as well as a corn starch control diet) on body weight and adenoma development in the small intestine, cecum and colon of these mice.

IV. MATERIALS AND METHODS

A. Apple Pomace Fractionation

Apple pomace produced in the 2011 season from apple juice manufacturing at Gerber Products Company (Fremont, MI). The pomace was a by-product resulting from the commercial processing of equal parts of Red Rome and Golden Delicious apple varieties. Briefly, the apples were culled to remove damaged or decayed fruit, washed in a flume system, mechanically scrubbed, hammer-milled, and pressed in a rotary filter press to extract apple juice. The apple pomace was the residue retained by the filters in the press.

The apple pomace was stored in refrigerator until being subjected to a fractionation procedure (Figure 2). One portion of the original apple pomace (45 kg) was dried in a forced-air oven (Proctor & Schwartz Inc., Philadelphia, Pennsylvania) at 60^{o}C (140^{o}F) and then ground in a FitzMill (The W.J. Fitzpatrick Co., Chicago, Illinois) using a 0.5 mm screen. The rest of the apple pomace (113 kg) was placed in woven polypropylene plastic bags and pressed using a hydraulic press (The W.J. Fitzpatrick Co., Chicago, Illinois) to collect residual apple juice that remained in the pomace. This apple juice was stored at -20^{o}C until used to prepare mouse diets. Next, the apple pomace remaining after pressing was transferred to a stainless steel vat and subjected to ethanol extraction first using 70% ethanol (EtOH) for 24 hours followed by 100% EtOH for 24 hours (60 liters of EtOH were used in each extraction). Combined EtOH extracts were stored at 4C until being concentrated by rotary evaporation. The residue remaining after EtOH extraction was dried in a forced-air oven for 48 hours, ground in a FitzMill using a 0.5 mm screen, and stored at room temperature. Following concentration by rotary evaporation, the EtOH extracts were stored at 4^{o}C until being used to prepare diets. Throughout this

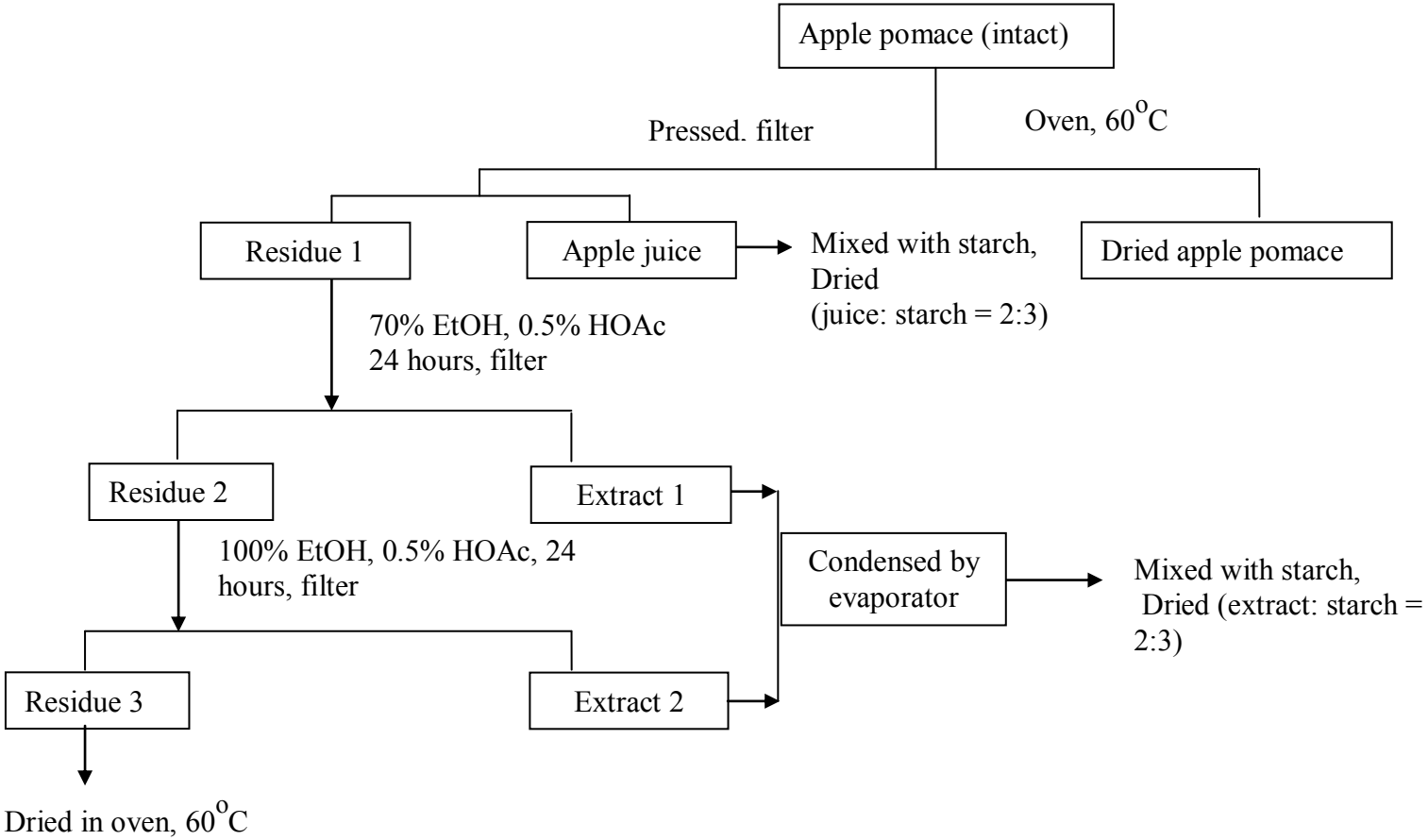


Figure 2. Fractionation procedure for apple pomace.

fractionation procedure, fraction weights and/or volumes were measured and sub-samples were obtained to determine dry matter yields.

The recoveries of dry matter from the original apple pomace in the resulting fractions was quantified and used to formulate the experimental diets in the manner such that the fractions incorporated into the diets were proportional to their contributions to the original apple pomace. The dry matter recoveries for these fractions were 13.9% (wt/wt) in the apple juice fraction, 19.7% (wt/wt) in the EtOH extract fraction, and 66.4% (wt/wt) in the apple residue.

To remove excess water or EtOH and facilitate incorporation into diets, known quantities of the apple juice and EtOH extract fractions were combined with corn starch (ratio of two parts juice or EtOH extract to three parts corn starch on a wt/wt basis) and mixed thoroughly in a Hobart mill (The Hobart MFG. Co., Troy, Ohio). Before incorporation into the diet together with other ingredients, these mixtures were dried in a forced-air oven at 60^{o}C until dry and then powdered using a FitzMill. These dried fractions were then added into diets used for the mouse feeding study.

B. Mouse Breeding Colony and Animal Care

Due to their high cost and limited commercial availability, the mice used in the feeding study (C57BL/6J APC$^{Min/+}$ mice) were produced in a breeding colony maintained at Michigan State University (MSU). All animals were housed in research facilities overseen by MSU University Laboratory Animal Resources located in the G. M. Trout Building and Biochemistry Building at MSU.

The breeding colony was maintained as follows. Normal C57BL/6J female mice were mated to males who carried one mutated allele of the APC gene (C57BL/6J APC$^{Min/+}$). Both

the normal females and carrier males were purchased from The Jackson Laboratory (Bar Harbor, ME). Male $APC^{Min/+}$ breeders were given sulindac (200 mg/L) in their drinking water to prevent morbidity due to intestinal adenomas.

All pups generated by the colony were genotyped to determine if they were heterozygous for the APC mutation, and only C57BL/6J Min/+ mice were used for the experiment. These mice were weaned at three to four weeks of age and then randomly assigned to dietary treatments (n=24-25 mice per treatment). The mice were assigned to five different treatment diets: control (also designated as the corn starch diet), dried apple pomace, apple juice, ethanol extract from apple pomace, and the dried residue from apple pomace following ethanol extraction.

All mice were given *ad libitum* access to food and water and were weighed weekly in order to track growth and morbidity. Mice were housed in plastic cages (1-3 mice/cage) in temperature ($23^{\circ}C \pm 2^{\circ}$) and humidity (40-60%) controlled rooms with a 12 hour light-dark cycle. This study was approved by the Michigan State University All-University Committee on Animal Use and Care.

C. Diets

The composition of diets is presented in Table 1. All diets were based on AIN-93G diets with slight modification. The fat content of each diet was increased to 15% fat (wt/wt) to imitate a more typical human diet. The concentrations of essential nutrients were also increased compared to standard AIN-93G diets to compensate for reduced intakes consequent to the increased energy density of these diets.

The five treatment diets differed in the composition of apple fractions as follow: corn starch (control), dry apple pomace, apple juice, apple extracted with ethanol, and residue of apple

Table 1. Composition of the modified AIN-93G diets. The compositions are presented as g/100 g diet.

Ingredient	Corn Starch (Control)	Apple Pomace	Apple Juice	Apple EtOH Extract	Apple Pomace Residue
Casein (85% protein)	22.12	22.12	22.12	22.12	22.12
Soybean Oil (TBHQ Stabilized)	15.00	15.00	15.00	15.00	15.00
AIN-93G-MX (Mineral Mix)	3.87	3.87	3.87	3.87	3.87
AIN-93G-VX (Vitamin Mix)	1.11	1.11	1.11	1.11	1.11
L-Cystine	0.33	0.33	0.33	0.33	0.33
Choline Bitartrate (41.1% Choline)	0.28	0.28	0.28	0.28	0.28
Tert-Butylhydroquinone (TBHQ)	0.003	0.003	0.003	0.003	0.003
Cellulose	5.00	5.00	5.00	5.00	5.00
Corn Starch	52.29	31.44	5.08	10.16	38.44
Dried Apple Pomace		20.00			
Apple Juice / Starch			45.27		
Apple EtOH Extract / Starch				40.07	
Dried Apple Pomace Residue					13.05

pomace after ethanol extraction. The apple fractions were substituted for corn starch on an equivalent dry matter basis. Apple pomace was included at a level of 20% (wt/wt) of the diet. The other treatments (apple juice, apple EtOH extract, and apple pomace residue) were included at concentrations equivalent to their yield from the original apple pomace. As stated previously, apple juice and apple EtOH extract were mixed with corn starch and dried to facilitate incorporation into the diets. All additions of apple fractions were at the expense of an equal quantity of dry matter from corn starch. All other dietary ingredients were added at levels identical to that in the control diet

D. Experimental Design

Mice at 21-28 days old were assigned to treatment groups after weaning. Due to the relatively short life span of these mice (120 ± 31 days), they were fed experimental diets for a total of nine weeks. At nine weeks after the beginning of dietary treatments, the mice were sacrificed by CO_2 asphyxiation followed by exsanguination from the heart via cardiac puncture. Due to diurnal variation in intestinal epithelial cell cytokinetics and other parameters, all mice were sacrificed between 6:00 and 10:00 in the morning. Samples of liver and abdominal fat and plasma collected from blood were frozen (-20^{O}C) and saved for future analyses if necessary. The small intestine, colon and cecum were removed and rinsed with tepid tap water. The small intestines were then cut into three sections that were approximately equal in length (proximal, medial, and distal), opened longitudinally, and rinsed with tap water to remove digesta. All tissues were placed in a phosphate-buffered saline solution (PBS, pH 7.4) immediately following cutting open until they were pinned flat on cardboard. Tissues were then fixed in a 10% neutral-buffered formalin solution (pH 7.4) for 24 hours. After fixation, all tissues were stored in 1%

neutral- buffered formalin, and then stained with 0.3% methylene blue in PBS for 2-3 minutes to facilitate tumor counting. Before staining with methylene blue, a one-centimeter section of the medial part of the colon was removed and saved for later histological analysis if necessary.

E. Quantification of Intestinal Tumors

All tumor quantification was performed by one person who was blinded to treatments. Tissues were viewed with a Nikon SMZ stereo microscope to determine the numbers and sizes of tumors. All adenomas in the small intestine were flat, two-dimensional tumors and their sizes were quantified using the formula: a (area) = (π * w * l) / 4 (where w = the width and l = the length of each tumor). Colonic and cecal tumors were polypoid so three dimensional measurements – the width (w), length (l), and height (h) – of each tumor were determined. Tumor size for colonic and cecal tumors was calculated using the formula: a (area) = (π * w * l * h) / 6. For the small intestinal sections, tumor number, average tumor size and the total tumor area (burden) were reported. For colon and cecum tumors, the same parameters are reported as well as the number of tumors per tumor-bearing mouse and tumor incidence (% of mice with solid tumors).

F. Quantification of Chemical Composition

F.1. Quantification of Polyphenols

Each apple fraction – dried apple pomace, apple juice, EtOH extract, dried apple residue – was analyzed for polyphenol profile by high-performance liquid chromatography (HPLC) using a Waters HPLC system consisting of dual model 510 pumps, an autosampler (model 717),

and a photodiode array detector. The column used was a C18 (25 cm x 0.46 cm ID) with 5-µm packing. Absorption spectra were monitored between 210 and 400 nm and polyphenol quantification was performed using absorbance at 280 nm. The elution solvents used were A (95% phosphoric acid 0.01 M and 5% acetonitrile) and B (30% phosphoric acid 0.01 M and 70% acetonitrile). The samples were eluted using the following gradient: 0% B as initial condition; 15% B for 35 min; 92.7% B for 30 min. The flow rate was 1 ml/min. The sample injection volume was 20 µl. Polyphenolic compounds were identified by comparing the retention time and UV spectra with known standards. The standards (+)-catechin, (-)-epicatechin, procyanidin, chlorogenic acid, rutin and quecertin were acquired from Sigma Chemical Company (St. Louis, MO).

Samples were prepared for HPLC analysis by solid phase extraction (SPE). Samples (0.5 g) of dried apple pomace and dried apple residue were extracted with 4 ml of 70% methanol in an ultrasonic bath for 30 min at room temperature (Kosmala et al., 2011). After extraction, samples were centrifuged at 10,000 x g, and the supernatant was collected. The residue from centrifugation was extracted two additional times with 70% methanol (3ml). All supernatants were collected and made up to 10 ml. These sample solutions were used for SPE extraction. The apple juice and EtOH extract fractions were subjected to SPE directly.

Samples of each of the four fractions were subjected to SPE to enrich polyphenols prior to HPLC analysis. The SPE columns were conditioned by washing with 2 ml of 100% methanol followed by 6 ml of deionized water. Samples (1 ml) were then loaded on SPE columns which were then washed with 15 ml of phosphoric acid (0.01 M). Polyphenols were then eluted from the SPE columns using 5 ml of 80% methanol. The solution was collected and evaporated to dryness under nitrogen gas. Samples were then dissolved in 1 ml of 80% methanol and filtered

through a membrane filter (0.5 μm) prior to injection on the HPLC. Each sample extracted and quantified in triplicate.

F.2. Carbohydrate Assay

For solid fractions (apple pomace and residue), samples (100 mg) were extracted three times with 10 ml of 80% ethanol by boiling the sample in a 95^{o}C water bath for 10 min for each extraction. After each extraction, samples were centrifuged at 2,000 x g for 5 min and the supernatants of three extractions combined and made up to 30 ml for sugar analysis. Ethanol-soluble carbohydrate was determined by the phenol-sulfuric acid method (Fournier, 2001). Samples (0.75 ml) of each solution were mixed with 0.5 ml of 4% phenol, followed by addition of 2.5 ml of concentrated sulfuric acid. After 10 min of color development in the dark, absorbance was measured at 490 nm. Ethanol-soluble carbohydrate was quantified by comparison to absorbance by a standard curve generated using a mixture of glucose, fructose, and galactose (1:1:1). Each sample was tested in triplicate.

F.3. Protein Determination

Crude protein of fractions was determined by nitrogen analysis (Yasuhara and Nokihara, 2001). Samples were weighed and put into a 100 ml Hach digestion flask, and then sulfuric acid (3 ml) was added. After swirling the flask to disperse sample in the acid, the flasks were placed on the heating block with a stainless steel weight on the neck and the fractionating column and capillary funnel were placed on the top of the flasks. Samples were heated until fumes filled the flask and rose into the fractionating column, at which time 10 ml of hydrogen peroxide was added to the capillary funnel. After decomposition was complete (all the black organic material

gone and volume was about 2 ml), the mixture was cooled to room temperature and neutralized with 1 M sodium carbohydrate (pH 7.5). Nitrogen content of each sample was then determined. Two reagents: reagent 1 (10 g phenol, 50 mg sodium pentacyanonitrosyl ferrate dihyrate in 1 L) and reagent 2 (15 g sodium hydroxide, 10 ml sodium hypochlorite in 1 L) were added in the sample tubes, which were vortexed and placed in a heating block for 40 min at 50^{o}C. Absorbance was read at 640 nm and compared to an ammonium chloride standard to measure nitrogen content in samples. Nitrogen contents were multiplied by 6.25 to estimate crude protein contents.

F.4. Ash Determination

Ash content was determined by sample ignition using a muffle furnace (Fisher Scientific) at 600^{o}C. Triplicate samples (2 g) of apple pomace and apple residue were weighed into pre-weighed porcelain crucibles. Samples of apple juice and EtOH extract solution were dried in an oven (100^{o}C) and the remaining dry matter (approximately 2 g) was used to determine ash content. After ignition for four hours in the muffle furnace (600^{o}C for 4 hours), crucibles were cooled in a desiccator and weighed. Each sample was subjected to triplicate analysis. The ash content was calculated as follows:

Ash (dry basis) = (weight after ashing / dry matter weight) x 100

G. Statistical Analyses

Statistical analyses were performed using SAS statistical software (SAS Institute, Inc. Cary, NC, Version 9.2). Body weight, tumor number, tumor average size and tumor burden of

each tissue were analyzed using analysis of variance with a 2 x 5 factorial arrangement of treatments (2 sexes and 5 dietary treatments). When significant effects were detected ($P<0.05$), appropriate means were compared using the Least Significant Difference procedure. All statistical differences were detected using a critical value of $P < 0.05$ with the F statistic. Results in tables are presented as least-square means ± SEM (standard error of the mean). The cecal and colon adenoma incidence for mice consuming different dietary treatments were analyzed using the GENMOD procedure of SAS (version 9.2).

V. RESULTS

A. Chemical Composition of Apple Fraction Preparations

Chemical content and the polyphenolic compound content of apple fraction preparations are presented in Table 2. The highest content of ethanol-soluble carbohydrate was found in ethanol extract (1,207 mg/g), whereas apple residue exhibited the lowest level of carbohydrate (29 mg/g). Among four apple fraction preparations, apple juice had the highest ash content (115.2 mg/g), the preparation apple pomace and apple residue had the lowest ash contents (~20 mg/g), and the ethanol extract was intermediate in ash content (37.3 mg/g). Crude protein contents were 55.9, 20.9, 14.5 and 85.3 mg/g for apple pomace, apple juice, EtOH extract and pomace residue, respectively. The residue fraction after ethanol extraction had increased crude protein content as compared to apple pomace, but the crude protein content of juice and ethanol extract was low compared to the initial material.

All preparations were analyzed for polyphenolic compounds. Apple pomace contained the following polyphenolic compounds: chlorogenic acid (0.14 mg/g), rutin (0.47 mg/g), quercetin (0.21 mg/g), and flavonol glycosides (0.46 mg/g). Apple juice had lower concentrations of chlorogenic acid and quecertin, but higher concentrations of rutin and flavonol glycosides, when compared to apple pomace. The greatest concentrations of flavonol glycosides were found in the ethanol extract, which contained increased concentrations of rutin (3.32 mg/g), quecertin (0.52 mg/g), and other flavonoid glycosides compared to apple pomace. No chlorogenic acid was detected in this fraction although it was present in the apple pomace. The apple residue contained only 0.31 mg/g of rutin, 0.03 mg/g of quecertin and 0.32 mg/g of other flavonol glycosides. No chlorogenic acid was detected in the residue.

The contribution of chemical constituents of apple pomace and its fractions to the overall diets is presented in Table 3. These values were calculated based on the dry matter percentage of each fraction and the dry matter yield of each fraction as a proportion of the original apple pomace (13.9, 19.7 and 66.4% for apple juice, EtOH extract, and apple residue, respectively). The apple pomace, apple juice and EtOH extract diets contained considerable quantities of additional ethanol-soluble carbohydrates that were contributed by the fractions. A modest quantity of additional ash and protein was contributed by these fractions, but the quantities were likely insufficient to significantly influence mouse growth. As anticipated, the EtOH extract fraction and original apple pomace contributed the greatest quantities of polyphenolics to the final diets.

B. Mouse Body Weights

Weekly body weights of female and male mice are presented in Figures 3 and 4, respectively. There were no statistically significant differences ($P>0.10$) in mouse weights due to treatment or sex at the beginning of the experiment (week 0). After the first week of dietary treatment, significant differences in average body weight were found for male and female mice, Averaged across all dietary treatments, male mice weighed significantly more than female mice, ($P<0.05$). This significant difference was consistent throughout the feeding period.

Averaged across both sexes, dietary treatment significantly influenced mouse body weights beginning in week 3. Apple residue-fed mice weighed significantly less than apple juice-fed mice, with the other three treatments being intermediate. This difference was maintained through the conclusion of the feeding study. Male and female mouse body weights were affected differently by dietary treatments (diet X sex interaction; $P<0.05$). After one week of treatment, diet started having significant effects on the body weights of male mice. At day 7, the apple

residue-fed male were smaller than apple juice-fed male mice, with the other treatments being intermediate ($P<0.05$). This trend continued similarly through the end of feeding experiment. In female mice, dietary treatments started having significant effects on body weight after four weeks of treatment. The mice consuming diets containing apple residue weighed significantly less than mice consuming diets containing the ethanol extract. These differences persisted until weeks 8 and 9 of treatment, when there were no significant differences in body weight among female mice consuming the different dietary treatments.

C. Small Intestinal Tumors

Tumor numbers, average sizes and total burdens were assessed in the proximal, medial, and distal thirds of the small intestine (SI). These values were summed to obtain aggregate values for the overall small intestine. Small intestinal adenomas were not evenly distributed throughout the small intestine, but were relatively more abundant in the medial and distal thirds of the SI. Averaged across all mice, the proximal, medial and distal thirds accounted for 16.8, 38.6 and 44.6 percent of all SI tumors, respectively.

Overall SI adenoma numbers, average sizes and burdens are presented in Table 4. Female mice had significantly higher total SI tumor numbers (73.2 ± 3.6) than male mice (61.7 ± 3.4). Total SI adenoma number was also significantly influenced by diet. Mice consuming apple juice had the lowest number of adenomas in the SI (54.5 ± 5.6) and mice consuming apple residue the greatest number (77.6 ± 5.6), with the other dietary treatments resulting in intermediate tumor numbers. The average adenoma size in the SI was significantly greater for males (1.07 ± 0.03) than females (0.98 ± 0.03). Mice consuming apple pomace and apple residue had the smallest average SI adenoma size, whereas mice consuming apple juice had the greatest average size

($P<0.05$). Mice consuming the corn starch and EtOH extract treatments had intermediate adenoma average sizes. Total adenoma burden in the SI was not influenced by sex or dietary treatments and averaged 70.3 mm^2 across all mice. There was a sex X diet interaction ($P<0.05$) detected for adenoma number but this interaction was not apparent for adenoma total burden and average size. This interaction was due to slightly different effects of diets on total SI adenoma numbers. Female mice consuming apple residue diets (98.4 ± 8.3) and male mice consuming apple pomace diets (77.8 ± 7.6) had the greatest ($P<0.05$) tumor numbers within females and males, respectively (data not shown). Consumption of diets containing apple juice led to the smallest ($P<0.05$) numbers of SI adenomas for both female (57.3 ± 8.3) and male (51.7 ± 7.6) mice.

Proximal SI adenoma number (Table 5) was significantly influenced by sex, being greater ($P<0.05$) in females (12.6 ± 0.6) compared to males (10.1 ± 0.6). Diet also significantly influenced the number of tumors in the proximal SI, with starch-fed mice having the highest number of adenomas in this section (15.4 ± 0.9) when compared to adenoma numbers in mice consuming the other dietary treatments. The average size of adenomas in the proximal SI was not influenced by sex or diet and averaged 1.78 mm^2 across all mice. Total adenoma burden in proximal SI was not influenced by sex, but was significantly impacted by diet. Mice consuming apple pomace and EtOH extract had the lowest ($P<0.05$) adenoma burden in the proximal SI and mice consuming starch the greatest burden, with the other treatments being intermediate. No sex X diet interactions were detected for any parameters in the proximal SI.

Medial SI adenoma number (Table 6) was not influenced by sex, but was significantly influenced by diet. Mice consuming diets containing apple juice had significantly fewer adenomas in the medial SI (19.1 ± 2.5) than mice consuming apple pomace (30.1 ± 2.5), starch

(27.3 ± 2.4) or apple residue (28.8 ± 2.5), with mice consuming diets containing the EtOH extract having intermediate numbers (24.4 ± 2.4). Sex did not significantly influence the average size of adenomas in this SI section, but this parameter was significantly influenced by diet. The average size of adenomas in the medial SI was significantly smaller in the mice consuming apple pomace and apple residue than mice consuming any of the other dietary treatments. Total adenoma burden in the medial SI was not influenced by sex, but was significantly influenced by diet. Mice consuming apple pomace and apple residue had the smallest medial SI burdens and mice consuming starch had the greatest burden, with mice consuming apple juice and EtOH extract being intermediate. No sex X diet interactions were detected for these parameters in the medial SI.

Distal SI adenoma number (Table 7) was greater ($P<0.05$) in females (33.1 ± 1.9) versus males (27.2 ± 1.8). Distal SI adenoma number was also significantly influenced by dietary treatment. Mice consuming apple juice had the lowest number of distal SI adenomas (24.2 ± 2.9) and mice consuming apple residue the greatest number (37.3 ± 2.9), with the other treatments being intermediate. Sex and diet also significantly influenced average adenoma size in the distal SI. The average adenoma size in this section was significantly greater for male (0.96 ± 0.04) versus female mice (0.74 ± 0.04). Mice consuming apple pomace and apple residue had the smallest distal SI average adenoma size, whereas mice consuming EtOH extract the greatest average size, with the other treatments having intermediate adenoma sizes. Total adenoma burden in the distal SI was not influenced by sex or by dietary treatments and averaged 26.4 mm^3 across all mice. There was a sex X diet interaction detected for distal SI adenoma number but not for burden and average size. This interaction was due to different effects of diets on distal SI adenoma numbers. Female mice consuming apple residue diets (49.4 ± 4.3) had significantly

greater numbers of adenomas in the distal SI compared to female mice consuming any other diet, which all had statistically similar distal SI adenoma numbers (data not shown). Male mice consuming apple pomace diets (36.3 ± 4.0) had the greatest ($P<0.05$) distal SI tumor numbers compared to male mice consuming the other treatments (data not shown).

D. Colonic Tumors

Colonic tumor incidence, numbers, average sizes and total burdens are reported in Table 8 and Table 9. Colonic adenoma number per mouse was significantly influenced by sex, with males (1.65 ± 0.17) having greater colonic adenoma numbers than females (1.12 ± 0.18). However, when comparing adenoma numbers only among mice that had colon tumors, there was no difference in tumor numbers between females and males. There were no statistically significant differences in colonic adenoma numbers per mouse or number per tumor bearing mouse for mice consuming the different dietary treatments. The average size of adenomas in the colon was not influenced by sex or dietary treatment. Total tumor burden in the colon was not influenced by sex, but was significantly influenced by diet. Mice consuming starch had the smallest total adenoma burden, whereas mice consuming apple pomace the greatest. Colonic adenoma incidence was statistically significantly different among the sexes, with an incidence of 79.7% in male mice and 59.3% in female mice. Dietary treatment did not significantly influence the incidence of colonic adenomas.

E. Cecal Tumors

Cecal tumor incidence, numbers, average sizes and total burdens are reported in Table 10 and Table 11. Cecal adenoma number per mouse and adenoma number per tumor-bearing mouse

were not influenced by dietary treatment, but tended ($P<0.10$) to be influenced by sex. Male mice (0.81 ± 0.10) tended to have more cecal tumors per mouse than female mice (0.52 ± 0.10). Similarly, tumors per tumor-bearing mouse tended to be greater for males (1.54 ± 0.10) compared to females (1.23 ± 0.13). The average size of adenomas in the colon was not influenced by sex, but was significantly influenced by diet. Mice consuming diets containing apple juice had the smallest average adenoma size and mice consuming apple residue the greatest average adenoma size. Total tumor burden was significantly influenced by sex, with male mice (4.19 ± 0.74) having greater burden than females (2.17 ± 0.97). Diet also significantly influenced total burden of tumors in the cecum, with apple residue-fed mice having the greatest total adenoma burden and apple juice-fed mice the smallest. The incidence of cecal adenomas was not significantly influenced by sex or by diet.

Table 2. Chemical composition of apple fraction preparations.*

	Apple fraction preparations			
Ingredients (mg/g)	**Apple pomace**	**Apple juice**	**EtOH extract**	**Apple residue**
Ethanol-soluble Carbohydrate	360.85	1161.22	1206.61	28.94
Ash	23.7	115.2	37.3	21.0
Protein	55.9	20.9	14.5	85.3
Polyphenol				
Chlorogenic acid	0.14	0.11	N.D	N.D
Rutin	0.47	0.63	3.32	0.31
Quecertin	0.21	0.07	0.52	0.03
Flavonol glycosides [b]	0.46	0.75	4.05	0.32

* Values are expressed on a dry matter basis. Each sample was analyzed in triplicate.
N.D = not detected.
[b] Quantified as rutin

Table 3. Estimated chemical constituents contributed by apple pomace and its fractions to the final diets.

	Apple fraction preparations			
Item	**Apple pomace**	**Apple juice**	**EtOH extract**	**Apple residue**
Ethanol-soluble Carbohydrate (mg/g)	67.42	30.16	44.41	3.59
Ash (mg/g)	4.43	2.99	1.37	2.60
Protein (mg/g)	10.4	0.6	0.5	10.6
Polyphenols (mg/100g)				
Chlorogenic acid	2.54	0.28	N.D	N.D
Rutin	8.71	1.62	12.23	3.88
Quecertin	3.98	0.18	1.92	0.36
Flavonol glycosides	8.50	1.94	14.89	3.97

Values are expressed on a dry matter basis.

N.D = not detected

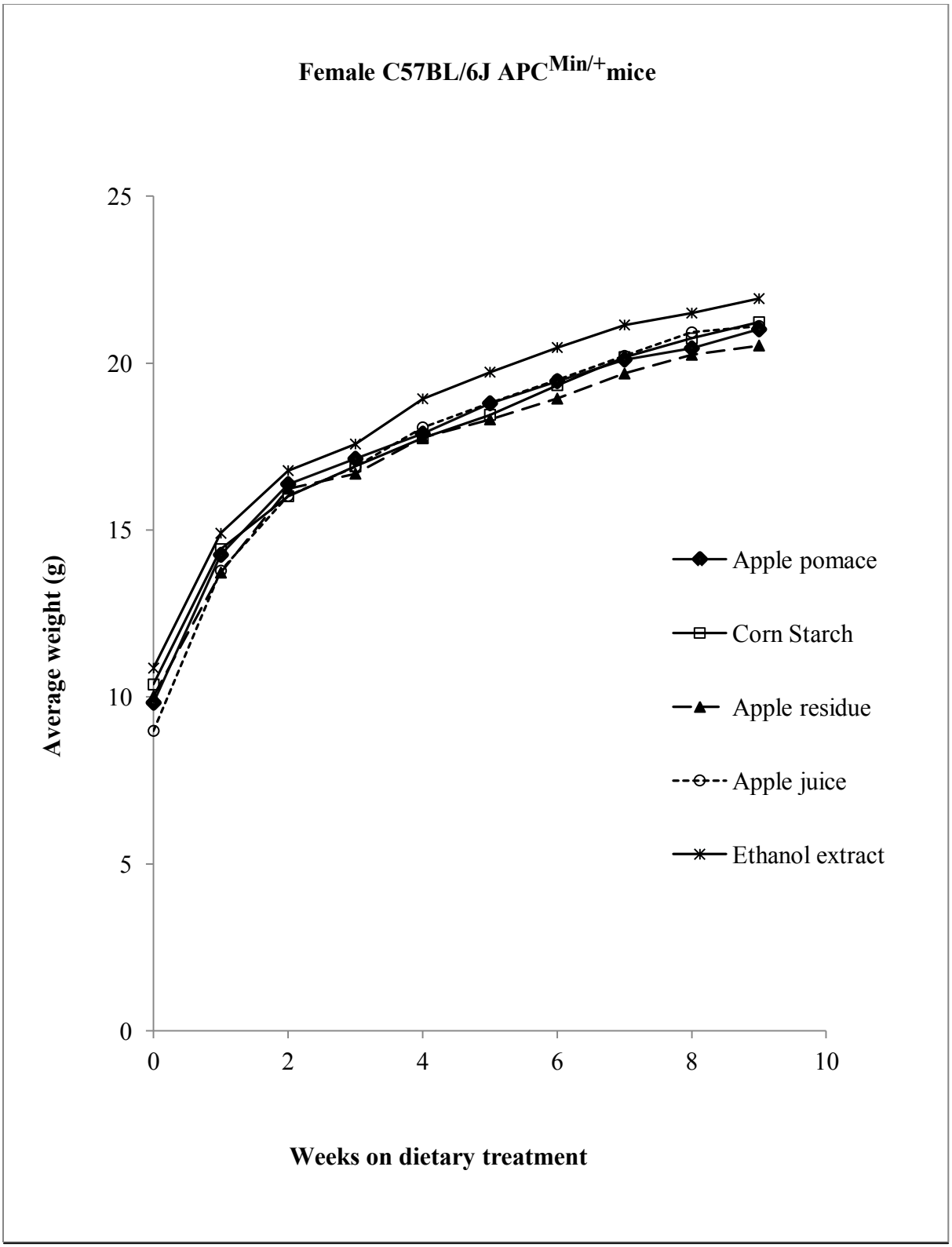


Figure 3. Weekly body weights of female C57BL/6J APC$^{Min/+}$ mice when fed diets containing apple pomace, corn starch, apple residue, apple juice, or ethanol extract.

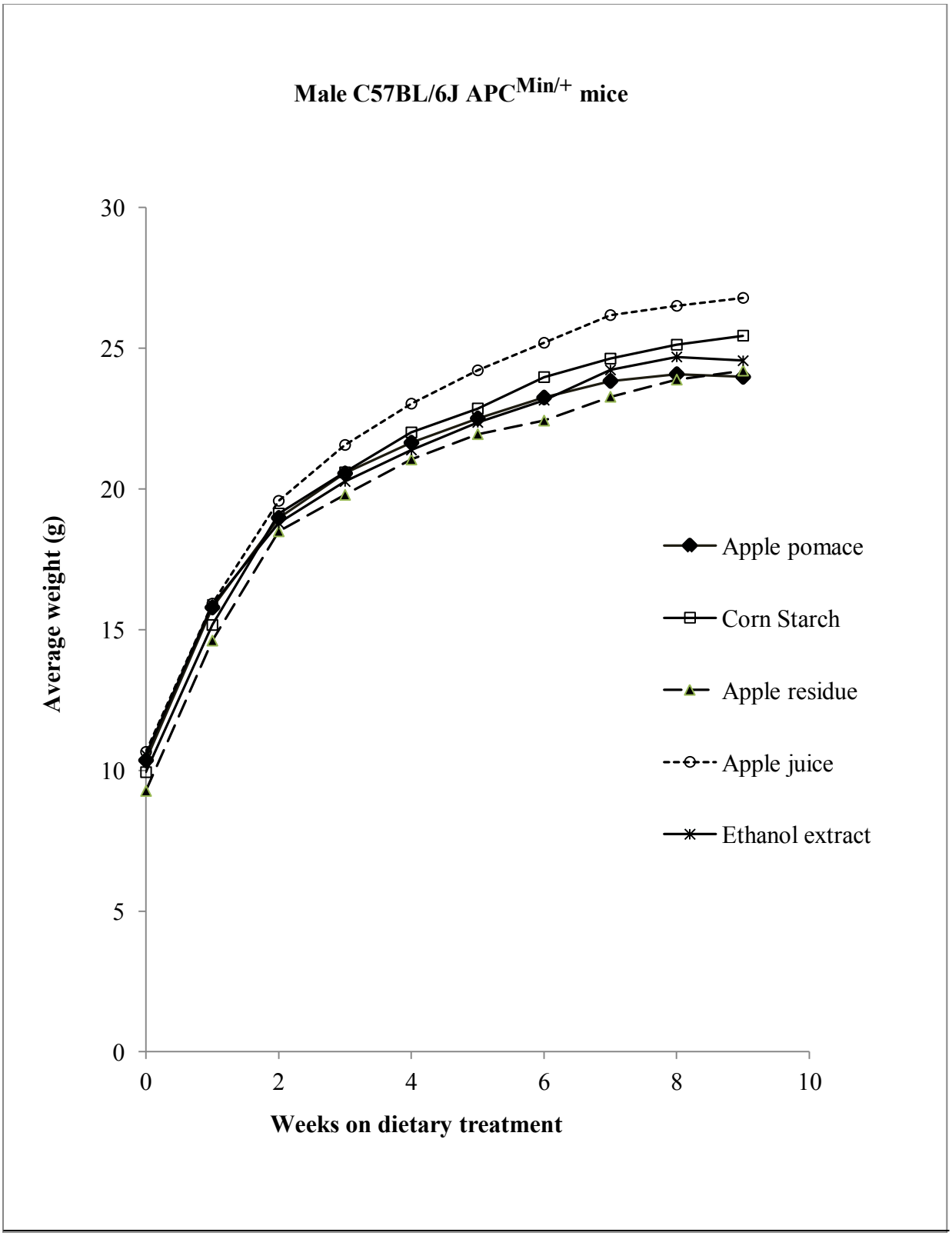


Figure 4. Weekly body weights of male C57BL/6J APC$^{Min/+}$ mice when fed diets containing apple pomace, corn starch, apple residue, apple juice, or ethanol extract.

Table 4. Total small intestine tumor number, average size, and burden in C57BL/6J APC$^{Min/+}$ mice fed diets containing apple pomace, corn starch, apple residue, apple juice, or EtOH extract.[+]

TOTAL SMALL INTESTINE	**Tumor Number**	**Average Size (mm^2)**	**Total Burden* (mm^2)**
Sex			
Female	73.2 ± 3.6 [b]	0.98 ± 0.03 [a]	71.9 ± 5.2
Male	61.7 ± 3.4 [a]	1.07 ± 0.03 [b]	67.9 ± 5.0
Treatment			
Apple pomace (intact)	72.2 ± 5.6 [bc]	0.85 ± 0.05 [a]	61.7 ± 8.2
Corn starch	72.5 ± 5.4 [bc]	1.06 ± 0.05 [b]	79.6 ± 7.8
Residue	77.6 ± 5.6 [c]	0.86 ± 0.05 [a]	66.8 ± 8.2
Apple juice	54.5 ± 5.6 [a]	1.22 ± 0.05 [c]	68.8 ± 8.2
EtOH extract	60.5 ± 5.5 [ab]	1.15 ± 0.05 [bc]	72.7 ± 8.0

[+] Data presented as Least Square Mean ± SEM.

*Total burden was calculated by summing the total tumor area within the proximal, medial, distal SI.

[abc] Means not sharing a common superscript within a column and category are different ($P<0.05$).

Table 5. Proximal small intestine tumor number, average size, and burden in C57BL/6J $APC^{Min/+}$ mice fed diets containing apple pomace, corn starch, apple residue, apple juice, or EtOH extract.[+]

PROXIMAL SMALL INTESTINE	**Tumor Number**	**Average Size (mm^2)**	**Total Burden* (mm^2)**
Sex			
Female	12.6 ± 0.6 [b]	1.72 ± 0.11	20.8 ± 1.5
Male	10.1 ± 0.6 [a]	1.84 ± 0.11	18.3 ± 1.4
Treatment			
Apple pomace (intact)	9.0 ± 0.9 [a]	1.76 ± 0.17	15.7 ± 2.3 [a]
Corn starch	15.4 ± 0.9 [b]	1.50 ± 0.17	23.5 ± 2.3 [b]
Residue	11.5 ± 1.0 [a]	1.92 ± 0.17	19.9 ± 2.4 [ab]
Apple juice	11.1 ± 1.0 [a]	1.94 ± 0.17	21.9 ± 2.4 [ab]
EtOH extract	9.8 ± 0.9 [a]	1.77 ± 0.17	16.6 ± 2.3 [a]

[+] Data presented as Least Square Mean ± SEM

*Total burden was calculated by summing the total tumor area within the proximal of the SI.

[ab] Means not sharing a common superscript within a column and category are different ($P<0.05$).

Table 6. Medial small intestine tumor number, average size, and burden in C57BL/6J APC$^{Min/+}$ mice fed diets containing apple pomace, corn starch, apple residue, apple juice, or EtOH extract.[+]

MEDIAL SMALL INTESTINE	**Tumor Number**	**Average Size (mm^2)**	**Total Burden* (mm^2)**
Sex			
Female	27.6 ± 1.6	0.96 ± 0.04	25.8 ± 1.9
Male	24.3 ± 1.5	0.94 ± 0.04	22.7 ± 1.8
Treatment			
Apple pomace (intact)	30.1 ± 2.5 ^{b}	0.73 ± 0.07 ^{a}	21.9 ± 3.0 ^{a}
Corn starch	27.3 ± 2.4 ^{b}	1.07 ± 0.06 ^{b}	29.5 ± 2.8 ^{b}
Residue	28.8 ± 2.5 ^{b}	0.69 ± 0.07 ^{a}	19.9 ± 3.0 ^{a}
Apple juice	19.1 ± 2.5 ^{a}	1.19 ± 0.07 ^{b}	23.4 ± 3.0 ^{ab}
EtOH extract	24.4 ± 2.4 ^{ab}	1.08 ± 0.06 ^{b}	26.5 ± 2.9 ^{ab}

[+] Data presented as Least Square Mean ± SEM.

*Total burden was calculated by summing the total tumor area within the medial of the SI.

^{ab} Means not sharing a common superscript within a column and category are different ($P<0.05$).

Table 7. Distal small intestine tumor number, average size, and burden in C57BL/6J APC$^{Min/+}$ mice fed diets containing apple pomace, corn starch, apple residue, apple juice, or EtOH extract.[+]

DISTAL SMALL INTESTINE	**Tumor Number**	**Average Size (mm^2)**	**Total Burden* (mm^2)**
Sex			
Female	33.1 ± 1.9 [b]	0.74 ± 0.04 [a]	25.4 ± 2.5
Male	27.2 ± 1.8 [a]	0.96 ± 0.04 [b]	27.0 ± 2.4
Treatment			
Apple pomace (intact)	33.1 ±2.9 [bc]	0.72 ± 0.06 [a]	23.8 ± 4.0
Corn starch	29.9 ± 2.8 [abc]	0.85± 0.06 [ab]	27.1 ± 3.8
Residue	37.3 ± 2.9 [c]	0.71 ±0.06 [a]	27.0 ± 4.0
Apple juice	24.2 ±2.9 [a]	0.93 ±0.06 [bc]	23.6 ± 4.0
EtOH extract	26.3 ± 2.9 [ab]	1.03 ±0.06 [c]	29.6 ± 3.9

[+] Data presented as Least Square Mean ± SEM.

*Total burden was calculated by summing the total tumor area within the distal of the SI.

[abc] Means not sharing a common superscript within a column and category are different ($P<0.05$).

Table 8. Colon tumor number per mouse, tumor number per tumor bearing mouse in C57BL/6J $APC^{Min/+}$ mice fed diets containing apple pomace, corn starch, apple residue, apple juice, or EtOH extract.[+]

COLON	**Tumor Number / mouse**	**Tumor number/tumor bearing mouse**
Sex		
Female	1.12 ± 0.18 [a]	1.87 ± 0.21
Male	1.65 ± 0.17 [b]	2.08 ± 0.18
Treatment		
Apple pomace (intact)	1.82 ± 0.28	2.42 ± 0.30
Corn starch	1.15 ± 0.27	1.88 ± 0.31
Residue	1.41 ± 0.29	1.94 ± 0.33
Apple juice	1.50 ± 0.28	2.18 ± 0.31
EtOH extract	1.03 ± 0.28	1.44 ± 0.30

[+] Data presented as Least Square Mean ± SEM. Means and standard errors presented are for analysis of unranked values. Statistical comparisons presented for analysis of variance and treatment comparisons using ranked values.

[abc] Means not sharing a common superscript within a column and category are different ($P<0.05$).

Table 9. Average tumor size, total tumor burden, and tumor incidence in the colon of C57BL/6J $APC^{Min/+}$ mice fed diets containing apple pomace, corn starch, apple residue, apple juice, or EtOH extract.[+]

COLON	**Average Tumor Size (mm^3)**	**Total Tumor Burden (mm^3)**	**Incidence (%)**
Sex			
Female	7.57 ± 1.24	12.94 ± 2.43	59.3 ± 6.4 [a]
Male	8.73 ± 1.02	17.38 ± 2.00	79.7 ± 5.1 [b]
Treatment			
Apple pomace (intact)	10.3 ± 1.7	23.3 ± 3.4 [c]	75.0 ± 9.0
Corn starch	4.8 ± 1.8	7.5 ± 3.5 [a]	61.5 ± 9.7
Residue	9.5 ± 1.9	16.9 ± 3.8 [abc]	73.9 ± 9.3
Apple juice	9.0 ± 1.8	18.3 ± 3.5 [bc]	68.0 ± 9.5
EtOH extract	7.2 ± 1.8	9.9 ± 3.4 [ab]	72.0 ± 9.2

[+] Data presented as Least Square Mean ± SEM. Means and standard errors presented are for analysis of unranked values. Statistical comparisons presented for analysis of variance and treatment comparisons using ranked values.

[abc] Means not sharing a common superscript within a column and category are different ($P<0.05$).

Table 10. Cecal tumor number per mouse, tumor number per tumor bearing mouse in C57BL/6J $APC^{Min/+}$ mice fed diets containing apple pomace, corn starch, apple residue, apple juice, or EtOH extract.[+]

CECUM	**Tumor Number/ mouse**	**Tumor number/ tumor bearing mouse**
Sex		
Female	0.52 ± 0.10 [d]	1.23 ± 0.13 [d]
Male	0.81 ± 0.10 [e]	1.54 ± 0.10 [e]
Treatment		
Apple pomace (intact)	0.62 ± 0.16	1.42 ± 0.20
Corn starch	0.84 ± 0.16	1.31 ± 0.15
Residue	0.76 ± 0.17	1.65 ± 0.18
Apple juice	0.53 ± 0.17	1.31 ± 0.20
EtOH extract	0.59 ± 0.16	1.25 ± 0.20

[+] Data presented as Least Square Mean ± SEM. Means and standard errors presented are for analysis of unranked values. Statistical comparisons presented for analysis of variance and treatment comparisons using ranked values.

[abc] Means not sharing a common superscript within a column and category are different ($P<0.05$).

[de] Sex effect trend ($P<0.10$).

Table 11. Average tumor size, total tumor burden, and tumor incidence in the cecum of C57BL/6J $APC^{Min/+}$ mice fed diets containing apple pomace, corn starch, apple residue, apple juice, or EtOH extract. [+]

CECUM	**Average Size (mm^3)**	**Total Burden (mm^3)**	**Incidence (%)**
Sex			
Female	1.66 ± 0.81	2.17 ± 0.97 [a]	40.7 ± 6.4
Male	2.97 ± 0.62	4.19 ± 0.74 [b]	53.1 ± 6.3
Treatment			
Apple pomace (intact)	2.98 ± 1.23 [bc]	3.55 ± 1.48 [bc]	40.0 ± 10.0
Corn starch	2.08 ± 0.90 [bc]	2.74 ± 1.08 [bc]	64.0 ± 9.8
Residue	3.06 ± 1.08 [c]	5.24 ± 1.30 [c]	45.8 ± 10.4
Apple juice	0.70 ± 1.23 [a]	0.79 ± 1.48 [a]	41.7 ± 10.3
EtOH extract	2.75 ± 1.21 [ab]	3.58 ± 1.45 [ab]	44.0 ± 10.1

[+] Data presented as Least Square Mean ± SEM. Means and standard errors presented are for analysis of unranked values. Statistical comparisons presented for analysis of variance and treatment comparisons using ranked values.

[abc] Means not sharing a common superscript within a column and category are different ($P<0.05$).

VI. DISCUSSION

Composition of Apple Fractions

The chemical composition of apple pomace and fractions prepared from apple pomace was assessed by quantifying ethanol-soluble carbohydrates, ash, crude protein and polyphenolic content. Results of these analyses confirmed that ethanol extraction was effective in producing a fraction that was enriched in polyphenolic compounds. The ethanol not only extracted polyphenols from apple pomace but also extracted large concentrations of carbohydrates into the solution. Quantification of the total carbohydrate concentration in the apple juice and apple EtOH extract actually resulted in values greater than 100% of dry matter. Two possible explanations for this observation are 1) inaccuracies in quantification due to different carbohydrate contents of the fractions compared to the standard used (a mixture of glucose, fructose and galactose), and 2) increased yields due to additional water of hydration added when oligosaccharides and polysaccharides are hydrolyzed when concentrated sulfuric acid is added to the samples.

Among the four fraction preparations apple juice had the highest ash content, indicating that much of the mineral content of the apple pomace was in juice fraction. As anticipated, the apple residue had lower phenolic and extractable carbohydrate contents following ethanol extraction. Dietary fiber composition of the fractions has not been analyzed, but it is anticipated that the majority of the unidentified material in the apple pomace and apple residue is comprised by dietary fiber. This has been demonstrated by Kosmala et al. (2011), who found that the fiber content increased after apple pomace was extracted with ethanol or acetone.

Plant polyphenolics have received high interest recently because of their promising properties as chemoprevention agents against cancer and other chronic diseases. Apple pomace, a major waste product of apple processing, previously has been reported to contain relatively large concentrations of procyanidin, chlorogenic acid, epicatechin, and quercetin (Lu and Foo, 2000). However, in the present study we found a low content of chlorogenic acid and have not detected epicatechin or procyanidin in apple pomace. Apple processing may affect polyphenol compounds since the material we used for this experiment was apple pomace after pressing and collecting juice, which is primarily derived from the apple flesh. Moreover, the content and composition of total polyphenols depends on apple variety. Escarpa and Gonzalez (1998) have analyzed phenolic compounds in the peel of four different apple varieties (Golden and Red Delicious, Granny Smith and Green Reineta) and found that the content of chlorogenic acid in each variety was different and ranged from 0.006 - 0.440 mg/g fresh sample. Similarly, we found a low concentration of chlorogenic acid in apple juice and it was not detected in the ethanol extract and apple residue. As anticipated, we observed that the ethanol extract contained the greatest concentrations of rutin, quecertin and other flavonol glycosides among four apple fractions. Considerable research has investigated the impact of these phenolics on colon cancer risk in various models and it has been demonstrated that chlorogenic acid (Kasai et al., 2000) and quecertin (van der Woude et al., 2003) can be protective against carcinogenesis. However, the effects of these compounds *in vivo* may be limited because the potential of these compounds to reach biologically relevant concentrations at the target tissues depends on the food matrix, interactions between compounds, digestion and bioavailability of apple phytochemicals (Boyer and Liu, 2004).

Mouse Body Weights

The male mice gained more weight than females throughout the feeding period. Dietary treatment also significantly influenced mouse body weight. Treatment differences in body weights of male mice were significant after one week of diet administration, whereas treatment effects were less pronounced and did not occur until week four for female mice. The effect of dietary treatment on body weights in females took a longer time to be observed than in males because female mice generally gain weight slower than male mice. Since most studies utilize only male mice, there are no comparable studies demonstrating this effect published in the scientific literature. However, these observations are consistent with previous findings in our laboratory using other dietary interventions.

In this study, we found that apple juice had higher carbohydrate content than apple residue (Table 2) and that apple juice-fed mice weighed more than apple residue-fed mice (Figures 3 and 4). The difference in chemical composition of each apple fraction used to prepare experimental diets can explain the differences in body weight observed in mice consuming the different diets. Mice consuming the diets containing apple juice and apple EtOH extract would have increased simple sugars in their diets compared to those consuming the other diets. This may partially explain the relatively greater body weights observed for mice consuming these diets. Conversely, mice consuming apple residue and apple pomace generally had the lowest body weights throughout the experiment. This observation likely is the result of these mice consuming greater concentrations of dietary fiber during the experiment. After being extracted with ethanol, the total dietary fiber content is increased in apple pomace residue (Kosmala et al., 2011). The soluble fiber fraction of total dietary fiber can form a viscous gel in the stomach and delay the release of chyme from the stomach into the proximal small intestine, which can create

a feeling of postprandial satiety (Groff and Gropper, 1999). One study demonstrated that consuming carbohydrates without plant fiber resulted in higher postprandial hyperglycemia compared to consuming the same amount and type of carbohydrates and plant fiber together. For example, it has been demonstrated that eating whole apples is accompanied by higher satiety and lower serum insulin levels than with the apple juice (Haber et al., 1977). The increased fiber consumption by mice consuming the apple residue diet and, to a lesser extent, the apple pomace-containing diet also would decrease the energy density of these diets and further contribute to the reduced weight gain observed in mice consuming these treatments.

Small Intestinal Tumors

The number of small intestinal tumors was significantly higher in females than males. This difference was observed in the proximal and distal sections as well as the total for the entire small intestine. However, male mice had greater adenoma average size in the small intestine compared to females. The net effect of these observations was that there was no difference between females and males in total small intestinal adenoma burden. The proximal small intestine had the fewest tumors compared to the medial or distal sections, but the average size of the tumors in the proximal small intestine was considerably larger than that observed in the rest of the small intestine. Previous studies examining the effects of apple components on colon cancer typically have used only male rodents (Ohkami et al., 1995; Gosse et al., 2005; Barth et al., 2005 and 2007). The differences observed between both genders in the present study using $APC^{Min/+}$ mice was consistent with results that have been previously seen in our lab.

In this study, dietary treatment significantly affected total small intestinal tumor number. Mice consuming the cloudy apple juice treatment had 29.7% fewer total small intestinal tumors

compared to mice consuming the apple residue, which had numerically the greatest number of small intestinal tumors. This reduction of tumor number by apple juice was also found in the medial and distal small intestinal sections. Other studies have demonstrated that cloudy apple juice can reduce adenoma formation in rodent models of human colon cancer (Gosse et al., 2005, Barth et al., 2005 and 2007). Barth and colleagues (2005) found that cloudy apple juice can inhibit carcinogen-induced DNA damage and epithelial cell proliferation. Apple juice also reduced the number of aberrant crypt foci in rats treated with dimethylhydrazine by 28%. These results were attributed to the high content in procyanidin, an important component of apple juice. In a follow-up study, the same authors suggested that the chemopreventive efficacy of cloudy apple juice was the result of synergy between procyanidin and cloudy particle fractions (Barth et al., 2007). However, in the present study, we did not identify procyanidin in the apple juice pressed from the starting apple pomace. This observation could be due to differences in the starting material (e.g. apple varieties) for our study compared to that of Barth et al. (2007) or different methods for separation and quantification of polyphenolic compounds.

As anticipated, we observed that the ethanol-extracted apple residue contained lower concentrations of polyphenolic compounds compared to the original apple pomace. Although mice consuming the apple residue had numerically the highest number of small intestinal tumors, the numbers were not significantly different than those observed in mice consuming apple pomace or the corn starch (control) diet. Mice consuming apple residue had significantly greater numbers of small intestinal tumors than mice consuming the diets containing apple juice or ethanol extract. We are not aware of any other research examining the effect of apple residue on colon cancer risk. However, one study showed that feeding $APC^{Min/+}$ mice with 20% apple pomace as a source of resistant carbohydrates resulted in increased tumor numbers in the small

intestine (Mandir et al., 2008). Interestingly, mice consuming apple pomace and residue had higher numbers of tumors than those consuming the apple juice treatment, but mice consuming apple pomace and residue had smaller average adenoma size. The mechanism of this effect is unclear, but the increase of adenoma size is commonly associated with increased crypt fission in the intestinal mucosa (Mandir et al., 2008).

We expected that the polyphenol-rich ethanol extract would have the greatest inhibitory effect on adenoma development. Although mice consuming the diet containing ethanol extract had significantly fewer small intestinal tumors than those consuming apple residue, this protective effect was not as pronounced as was observed in mice that consumed apple juice-containing diets. Several studies have examined the potential of apple juice (Kahle et al., 2005; Schaefer et al., 2006; Barth et al., 2005 and 2007) and apple polyphenol extract (Gosse et al., 2005; Liu et al., 2005; Fini et al., 2011) to reduce cancer development in a variety of models, but there have been no previous studies to our knowledge that compared the effects of apple juice and apple polyphenol extract. In the present study, the potential mechanism whereby apple juice administration reduced small intestinal tumorigenesis is unclear. Additional research on relative doses of polyphenols and other constituents in apple juice should be considered to elucidate potential mechanisms for this anti-adenoma activity.

Large Intestinal and Cecal Tumors

Male mice had a significantly greater incidence of colonic adenomas than females. Male mice also had a greater number of colonic tumors per mouse compared to females. This was contrary to the effects observed in the small intestine, wherein female mice had greater adenoma numbers than male mice, but is consistent with other research conducted using $APC^{Min/+}$ mice

in our laboratory. Male mice also tended ($P<0.10$) to have greater numbers of cecal tumors per mouse and tumors per tumor-bearing mouse, but there was no difference in cecal adenoma incidence between males and females.

There were no significant effects of diet on colon tumor incidence, tumor number per tumor bearing mouse, or the average size of colonic adenomas. However, there was a significant diet effect on total tumor burden in the colon. Mice consuming apple pomace had significantly greater colon tumor burden than mice consuming the corn starch (control) diet or the diet containing ethanol extract.

There was no significant diet effect on cecal tumor incidence or on tumor numbers, but there was a significant diet effect on average size and total adenoma burden in the cecum. Mice consuming diets containing apple juice had the smallest size and total burden of adenomas, and those consuming diets containing apple residue had the largest tumor sizes and burdens. These observations tended to parallel those observed for small intestinal adenoma numbers. Although the weight of the cecum and the short chain fatty acid (SCFA) concentration was not measured in this study, some studies have shown that the presence of fiber in the diet can increase cell proliferation, enlarge the cecum, decrease pH and change SCFA production (Lupton and Kurtz, 1993; Aprikian et al., 2003). It is possible that effects such as these that are associated with fiber consumption could be correlated with cecal adenoma development in this model. Further research is necessary to determine the mechanisms whereby cecal tumorigenesis is impacted by diet.

The positive effect of apple juice on adenoma development was not consistent throughout the intestinal tract, as it had no beneficial effects in the colon despite its positive effects in the small intestine and cecum. There has been one published study in ileostomy patients that

demonstrated that apple juice polyphenols were predominantly absorbed from or metabolized in the small intestine, and that approximately 0-33% of ingested polyphenols might reach the colon (Kahle et al., 2005). This may be the reason why apple juice affected small intestinal and cecal tumorigenesis in this study, but had no significant effect in the colon. In another study, a group of scientists in Germany used apple extracts fermented with human colonic microflora to examine the interaction of fecal microflora and ingested polyphenols on human cell lines derived from colon adenoma (LT97) and carcinoma (HT-29). They found that polyphenols were extensively degraded (99.9%) in this system, reducing the effectiveness of apple extract to inhibit cell growth as compared to non-fermented apple polyphenol extract (Veeriah et al., 2007).

Even though apple pomace was rich in polyphenolic compounds compared to other fractions, mice consuming the apple pomace-containing diet had the highest colonic tumor burden in this study. This lack of effect could be due to a number of factors. It is possible that the polyphenols and other constituents in apple pomace such as dietary fiber simply do not inhibit colonic tumor development. Alternatively, degradation of polyphenols during cecal and colonic fermentation may have prevented their potential effects on colonic tumorigenesis. Further study is necessary to assess the effects of these apple fractions on colonic tumorigenesis.

The relationship between colon cancer and dietary fiber intake is controversial. One study showed that rats which had been treated with azoxymethane to induce colon tumors had lower incidence of colon tumors after feeding with an apple pectin-containing diet (Ohkami et al., 1995). Contrary to this result, another study conducted using $APC^{Min/+}$ mice fed a diet supplemented with apple pomace found that mice had increased intestinal epithelial cell proliferation and crypt fission, resulting in higher tumor numbers and larger tumor burdens (Mandir et al., 2008). It also has been hypothesized that fermentation of pectin may produce low

levels of butyrate which are not sufficient to inhibit the enzyme 7α-dehydroxylase, a key enzyme which generates secondary bile acids that can act as tumor promoters (Jacobasch et al., 2008).

Further research is needed to assess mechanisms whereby apple fractions can influence colon cancer risk. Future studies should access fiber characteristics and fiber metabolism to better understand interactions between fiber and colonic microflora on colon cancer risk. Additional research also is necessary to ascertain the effects of ethanol-extractable polyphenols on cancer risk. In this study, the ethanol extract reduced some indices of cancer risk (e.g., smaller tumor burden compared to apple pomace). Nevertheless, the ethanol extract derived from apple pomace used in this experiment did not significantly reduce intestinal tumorigenesis compared to the control diet for any of the parameters studied in this experiment. Hence, we conclude that the ethanol-extracted polyphenolics did not reduce intestinal tumor development in this experiment.

VII. SUMMARY AND CONCLUSIONS

This study demonstrated that intestinal tumor development in $APC^{Min/+}$ mice was influenced by feeding diets that had been supplemented with specific fractions derived from apple pomace. When incorporated into diets, apple pomace and its fractions had somewhat differential effects on intestinal tumor development in the small intestine, cecum and large intestine. Consumption of diets containing apple juice led to the smallest numbers of small intestinal adenomas in both female and male mice, whereas mice consuming diets containing the apple residue had the greatest numbers of tumors. We were unable to describe the mechanism whereby apple juice consumption decreased tumor numbers in small intestine, but speculate that this effect could be associated with the polyphenols or other components in apple juice.

Male mice had a significantly greater incidence of colonic adenomas and greater colonic adenoma numbers compared to female mice. Male mice also had greater tumor burdens and tended to have higher tumor numbers in the cecum compared to female mice. In contrast, female mice had greater numbers of small intestinal adenomas than male mice. These effects are consistent with previous research conducted in our laboratory but are not widely recognized due to the fact that most studies of colon cancer development using animal models utilize only male mice. Despite these sex differences in tumor development, the treatment effects generally were similar for female and male mice and few interactions were detected.

Cecal adenoma incidence was not significantly influenced by sex or by diet, but male mice had significantly greater cecal tumor burdens and tended to have greater numbers of cecal tumors when compared to female mice. Diet also significantly influenced cecal tumor development, with mice consuming diets containing apple juice having the smallest cecal

average adenoma size and lowest overall tumor burden in the cecum when compared to the other treatments. This observation was consistent with the effects of apple juice-containing diets in decreasing small intestinal tumor multiplicity, but did not carry through to colon tumor development.

Collectively, these results indicate that apple juice-containing diets had the greatest effect on intestinal tumor development in $APC^{Min/+}$ mice compared to other treatments. The mechanisms for these effects are unknown, but presumably are related to the composition of polyphenolics and other compounds present in the apple juice. Further research is necessary to investigate the bioavailability of apple juice phytochemicals and the mechanisms whereby apple fractions can influence intestinal tumor development. Replicating these findings in other animal models (e.g., azoxymethane-induced colon carcinogenesis in rats) would be an important first step to confirming the effects observed in this study. It also would be important to further purify the polyphenolics present in the apple fractions used in this experiment and determine the effects of these compounds on cancer cell growth using *in vitro* models such as HT-29 or HCT116 cancer cell lines.

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