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The Influence of Fermentable Fibers on Intestinal Growth and Tumorigenesis in Min^+ Mice

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Masters degree in Science

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THE INFLUENCE OF FERMENTABLE FIBERS ON INTESTINAL GROWTH AND TUMORIGENESIS IN *Min*$^{+}$ MICE

By

Elizabeth Ann Rondini

A THESIS

Submitted to
Michigan State University
In partial fulfillment of the requirements
For the degree of

MASTER OF SCIENCE

Department of Food Science and Human Nutrition

2000

ABSTRACT

THE INFLUENCE OF FERMENTABLE FIBERS ON INTESTINAL GROWTH AND TUMORIGENESIS IN *Min*$^+$ MICE

BY

ELIZABETH ANN RONDINI

Fermentation of dietary fiber and starch in the colon produces short-chain fatty acids (SCFA), namely acetate, propionate, and butyrate. Butyrate has anti-neoplastic effects *in vitro.* This study examined the effects of the fermentable dietary fibers fructooligosaccharides (FOS), modified starch, and gum arabic on intestinal tumors and tissue parameters in *Min*$^+$ mice, a murine model of familial adenomous polyposis (FAP) in humans. *Min*$^+$ mice (4 weeks old) were separated into one of five treatment groups and fed a powdered AIN-93G diet with 15% fat and 6% of a dietary fiber or an inert bulk source. The dietary fiber/bulk sources used were 1) cellulose, 2) FOS, 3) modified starch, 4) gum arabic, and 5) kaolin. After 10 weeks of treatment, the animals were sacrificed and the colon and small intestine (SI) were removed, measured, and then fixed. The cecum was emptied and then fixed and weighed. The number and size of adenomas in each tissue were recorded, and short chain fatty acids (SCFA) were quantified in cecal contents. The highly fermentable fibers (groups 2, 3, and 4) produced significantly greater cecum weights and SI and colon lengths, and had significantly greater concentrations of SCFA compared to the less fermentable cellulose and kaolin. There were no significant differences ($P < 0.05$) between treatment groups for small intestinal (71/mouse), colon (1/mouse), or cecal (0/mouse) tumors. It was concluded that fermentable fibers are trophic to the small and large intestines and cecum through SCFA production, but do not influence tumorigenesis in *Min*$^+$ mice.

ACKNOWLEDGEMENTS

I would like to thank my major advisor, Dr. Bennink, for his help, support, and guidance. I would also like to extend my gratitude to my committee members. To Dr. Chenoweth for her continued support of my academic achievements, and Dr. Bourquin, for giving me the opportunity to do this experiment.

I would also like to thank my family, especially my parents, for their love, faith, and encouragement. Also for their support, both emotionally and financially, which has made my education both possible and meaningful.

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I. INTRODUCTION

Colon cancer is currently the third leading cause of cancer-related deaths in the US. Burkitt proposed in the late 1960s that fiber is protective against colon cancer and a range of gastrointestinal disorders common to Westernized populations (Burkitt, 1971). This hypothesis stimulated numerous studies over the past 30 years. The results of this research, however are conflicting and inconclusive. In recent years there have been many developments that have led to a better understanding of the genetic mechanisms of colon cancer.

Colon cancer develops through a sequence of mutations (Vogelstein et al, 1988). The *APC* gene is mutated in the inherited condition familial adenomatous polyposis (FAP) in humans and is also one of the earliest, and most frequent, mutations thought to occur in the adenoma-carcinoma sequence. The protein encoded by the *APC* gene is important for regulation of cell proliferation. *Min*$^+$ (multiple intestinal neoplasia) mice, the murine homologue of FAP in humans, have a fully penetrant germline mutation in the *APC* gene and develop numerous benign tumors throughout the intestine (Su et al, 1992). Therefore, the *Min*$^+$ mouse may be a reasonable *in vivo* model to study the effects of metabolites that can possibly alter gene transcription and influence the promotion stage of colon cancer development.

Bacterial fermentation of dietary fiber in the colon results in the production of short-chain fatty acids (SCFA), primarily acetate, propionate, and butyrate. Butyrate has been shown to induce differentiation in various colon cancer cell lines rendering the cells non-cancerous. The exact mechanism whereby butyrate induces differentiation is not fully known, but may involve histone hyperacetylation and p21 up-regulation (McBain et

al, 1997; Hague et al, 1993). In contrast to their possible anti-carcinogenic effects *in vitro*, SCFA are also trophic to the small intestine and colon. Butyrate is the primary energy source for colonocytes and locally stimulates cellular proliferation. SCFA, after absorption in the colon, have also been shown to be trophic to the small intestine, likely through a systemically-mediated mechanism. Because SCFA stimulate proliferation in normal tissue, it has been suggested that SCFA may also enhance growth of neoplasms (Jacobs, 1987). Therefore, this study was designed to determine the influence of fermentation on intestinal tumorigenesis in *Min*$^{+}$mice.

II. REVIEW OF LITERATURE

A. EPIDEMIOLOGY OF COLON CANCER

A.1 Incidence of colon cancer

Cancer is currently the second leading cause of death in the United States (Greenlee et al, 2000). Of the different types of cancer, colorectal cancer accounts for 11% of all cancer deaths, with an expected incidence of 93,800 new cases in the US in 2000 (Greenlee et al, 2000). Approximately 5% of colon cancer cases have a well-defined genetic pattern whereas 80-90% of cases occur sporadically (Burt, 1995). Environmental factors identified in the etiology of colon cancer include diet, inadequate physical activity, obesity, and ulcerative colitis (Shike, 1999).

A.2 Diet and colon cancer

Dietary and environmental factors are thought to contribute to 80-90% of all cancers (Drasar and Hill, 1972). High intakes of dietary fat, especially from animals, and low intakes of vegetable and cereal fibers, vitamin D, and calcium are associated with an increased risk of colon cancer development and may account for much of the worldwide variation in colon cancer incidence (Macrae, 1999). Burkitt proposed in the late 1960s that the high fiber diets common in African populations are associated with lower colon cancer incidences (Burkitt, 1971). Epidemiological studies examining dietary fiber intake and colon cancer incidence, however, have not been conclusive (reviewed in Bingham, 1997). Case-control studies consistently show an inverse association between vegetable and fruit intake and colon cancer risk. The inverse relationship between

vegetable fiber intake and colon cancer risk is strongest for raw, green, and cruciferous vegetables (Potter, 1999). Bingham summarized thirty case-control studies that examined the relationship between dietary fiber intake and colon cancer risk from 1969-89 (Bingham, 1990). Eleven of 22 studies showed a reduced risk associated with increased intakes of fiber, 12/19 for vegetable fiber intake, and 3/13 for cereal fiber consumption (Bingham, 1990). Recently published case-control studies examining the effect of wheat bran supplementation on either colon cancer risk or adenoma recurrence have also been inconclusive. Two showed no effect on adenoma recurrence, one showed no effect on adenoma recurrence but a decrease in adenoma size with wheat bran, and one showed wheat bran was protective only for rectal polyp development (Alberts et al, 2000; Schatzkin et al, 2000; Macrae, 1999). In short, no strong conclusions between dietary fiber intake and colon cancer can be made from epidemiological studies.

B. MOLECULAR BASIS OF COLON CANCER:

B.1. Physiology of the Colon:

The human colon is approximately 150 cm (5 ft.) in length with an overall surface area of 1300 cm^2 (Eastwood, 1983; Cummings and Macfarlane, 1991). Histologically, the colon contains multiple invaginations that extend into the mucosa layer to form crypts (Kinzler and Vogelstein, 1998). The mucosa is divided into three portions, namely the epithelium, lamina propria, and muscularis mucosae (Eastwood, 1983). The epithelium lines the mucosa and is maintained by immature stem cells located in the middle crypt of the proximal colon and at the base of the crypts in the distal region (Karam, 1999). Proliferation occurs in the lower 2/3 of the crypts and as cells migrate towards the lumen,

they differentiate into absorptive, goblet, or enteroendocrine cell types (Eastwood, 1993). After approximately 3-6 days differentiated cells at the top of the crypt are shed into the lumen (Lipkin et al, 1963).

B.2 Genetic alterations in colon cancer

A balance between cell division and death is important for maintaining the epithelial lining. The earliest biomarker for colon cancer development is a hyperproliferative state of the mucosa associated with an expansion of the proliferation zone to the upper part of the crypt and a concomitant decrease in differentiation (Winawer et al, 1991). Neoplastic progression in colon cancer as well as other cancer types involves genetic mutations resulting in the cumulative loss of functional tumor suppressor genes and/or dominant activation of proto-oncogenes (Fearon and Vogelstein, 1990). Vogelstein et al (1988) have examined the frequency of genetic mutations in various tumors and proposed a sequence of events important for the development of colon cancer. These include, in order, mutations in adenomatous polyposis coli (*APC*), *k-ras*, deleted in colon cancer (*DCC*), and *p53* genes (Figure 1).

APC, located on chromosome 5q21 in humans, encodes a protein with tumor suppressive properties (Kinzler et al, 1991). As discussed later, inactivation of both alleles of the gene is permissive for adenoma formation, suggesting APC acts as a "gatekeeper" of colonic epithelial proliferation (Powell et al, 1992; Kinzler and Vogelstein, 1996). The *ras* proto-oncogenes encode proteins with intrinsic GTPase activity and are located on chromosome 12p (Kinzler and Vogelstein, 1998). K-*ras*

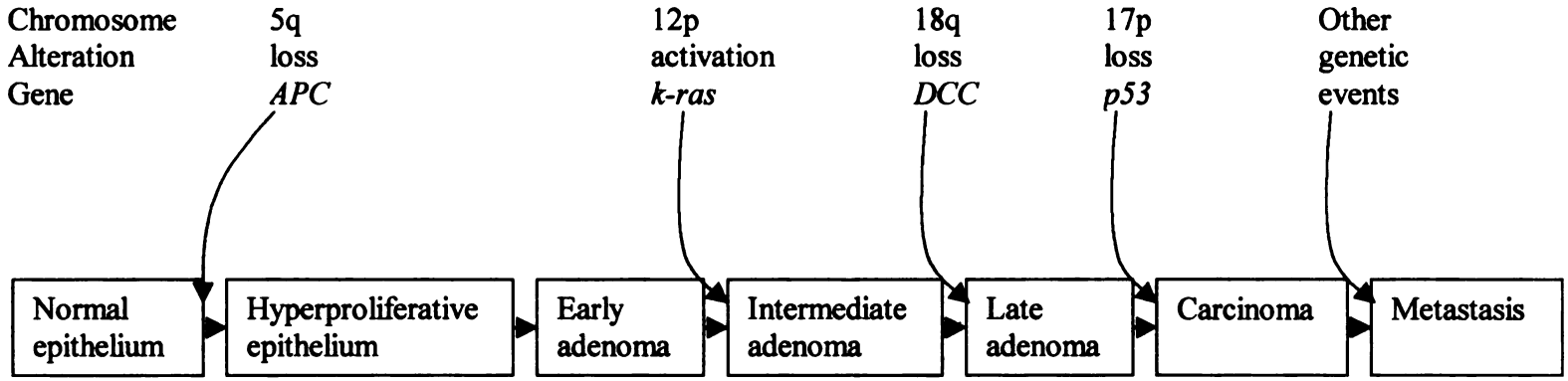

Figure 1. Genetic alterations in colon cancer. *Adapted from:* Vogelstein et al, 1988.

mutations generally occur later in adenoma growth and have been detected in 50% of colorectal adenomas larger than 1 cm (Vogelstein et al, 1988). *DCC* is a tumor suppressor gene located on chromosome 18q (Kinzler and Vogelstein, 1998). *DCC* mutations generally occur late in tumor progression, and are found in 47% of advanced adenomas and 73% of carcinomas (Vogelstein et al, 1988). Mutations in the *p53* gene occur late in tumor progression, most likely in the adenoma-carcinoma transition (Vogelstein et al, 1988). In normal cells, the p53 protein is upregulated in response to genomic damage, and is associated with cell cycle arrest, either in G1 and/or G2. This ensures repair of damage and/or leads to apoptosis of rapidly proliferating cells.

B.3 Inherited Predispositions to Colon Cancer

Inherited predispositions to colon cancer are responsible for 5-10% of colon cancer cases and have led to a better understanding of genetic events which occur in tumor formation (Rudy and Zdon, 2000). Familial adenomatous polyposis (FAP) is an autosomal dominantly inherited condition affecting approximately 1 in 7,000 people (Kinzler and Vogelstein, 1996). Affected individuals inherit a germline mutation in one of the alleles of the *APC* gene, increasing their susceptibility to colon cancer development (Nishisho et al, 1991). The initiating event in tumor formation involves a somatic mutation in the remaining wild type (WT) allele of the *APC* gene (Groden et al, 1991). Individuals with FAP will commonly develop hundreds to thousands of benign adenomas throughout the colon, usually by 20-30 years of age (Su et al, 1992). If left untreated, colon cancer will develop by the age of 40 (Rudy and Zdon, 2000).

Mutations in both alleles of the *APC* gene occur in approximately 60-80% of sporatic colon tumors, and has been proposed to be the initiating event in the pathway that leads to colon cancer (Vogelstein et al, 1988; Fearon and Vogelstein, 1990). The following section will discuss the functional domains of APC and potential tumor suppressive properties.

B.4 Cellular functions of APC

A schematic diagram of the functional domains of the WT APC protein is shown in Figure 2 (Polakis, 1997). *APC* is a 2843 base pair gene encoding a multi-functional protein found in a variety of cell types (Shoemaker, 1997). Along the colonic crypt there exists differences in *APC* expression, increasing as cells migrate towards the lumen (Smith et al, 1993). The central region of the protein contains two binding sites for β-catenin, consisting of a 15 amino acid (aa) repeat region and a 20 aa repeat region, the latter of which contains phosphorylation sites necessary for β-catenin binding and its down regulation (Shoemaker et al, 1997; Polakis, 1997; Ilyas and Tomlinson, 1997). Analysis of regions commonly mutated in sporatic colon cancer as well as germ-line mutations indicate that mutations occur most frequently in codons 1286-1513, named the mutation cluster region (MCR) (Miyaki et al, 1994, Shoemaker et al, 1997). More than two-thirds of mutations result in frameshift, and one-third involve nonsense point mutations, both which result in premature truncation of the APC protein (Polakis, 1997).

In the human condition FAP, the severity of colon tumors as well as the presence of extra-colonic manifestations varies with the location of the mutation (Shoemaker et al,

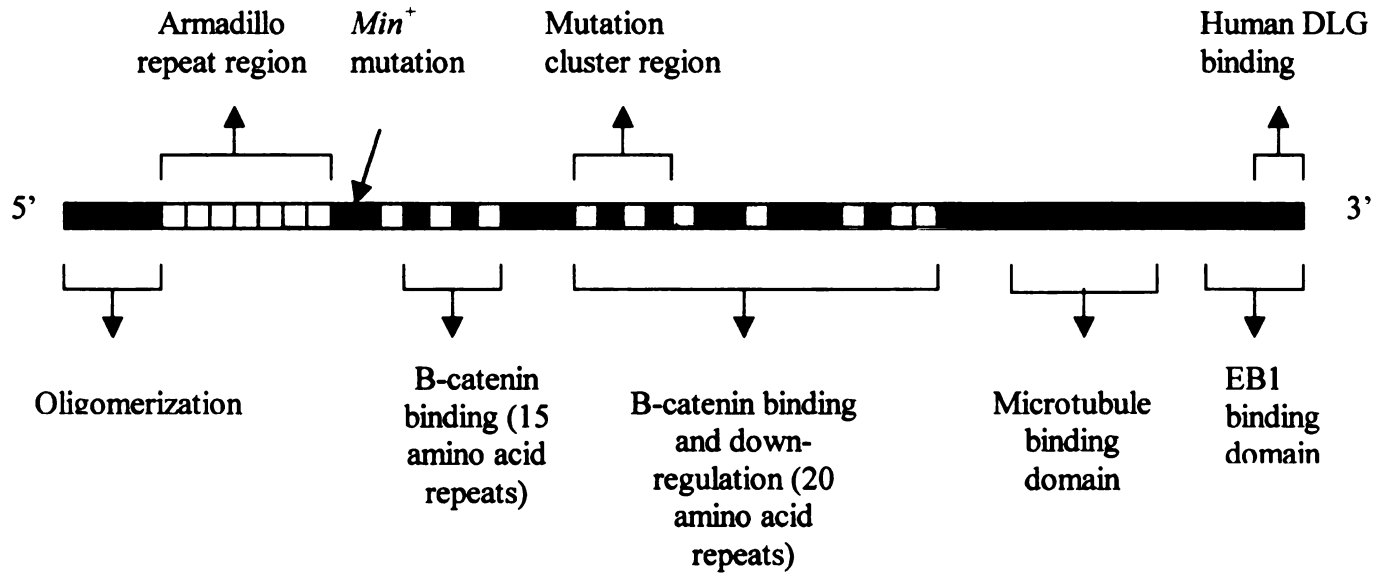

Figure 2. **Potential functional domains of *APC*.** Modified from Polakis, 1997.
Abbreviations: *Min*$^+$, multiple intestinal neoplasia; DLG, discs large protein.

1997). Congenital hypertrophy of the retinal pigment epithelium occurs in FAP patients with mutations after codon 500 (Dobbie et al, 1996). Mutations in *APC* occurring after codon 1400 cause an increased frequency of osteomas, epidermoid cysts, and desmoid tumors, but decreased frequency of colonic tumors (Dobbie et al, 1996; Fodde et al, 1999; Olschwang et al, 1993; Shoemaker et al, 1997). Because both germline and somatic mutations occur in the 5′ region of *APC*, the functional regions for β-catenin degradation and binding of microtubules, EB1, and DLG may all be involved in the tumor suppressive properties of APC. However, as discussed above, mutations occurring after the MCR are associated with decreased incidence in colon tumors, and a higher frequency of extra-colonic manifestations (Wallis and Macdonald, 1996). Mutations occurring prior to the MCR often cause higher tumor multiplicity as seen in germline mutations. Therefore, the β-catenin binding and degradation sites appear to be most important for inhibition of colon tumor development.

B.4.1 Interaction between E-cadherins, β-catenin, and APC

Cell adhesion and cell-cell communication is important in regulating contact inhibition between cells, tissue homeostasis, and morphogenesis (Miller and Moon, 1996). This process is mediated by cellular adhesion molecules (CAMs), notably immunoglobulin-type, adherins, and cadherins (Miller and Moon, 1996). E-cadherins, which are involved in adheren junctions in epithelial cells, are calcium-dependent transmembrane proteins which homotypically bind adjacent cells (Ilyas et al, 1997). The interaction of these proteins between cells forms tight cell-cell junctions important in establishing cell polarity, shape changes, and migration (Peifer, 1997). The intracellular,

carboxyterminus domain of E-cadherin binds the 92 kDa protein β-catenin (*Drosophilia* homologue Armadillo), which connects E-cadherin to α-catenin, attaching the complex to the actin cytoskeleton (Yamada and Geiger, 1997; Ilyas et al, 1997). This attachment is necessary for cadherin function, and serves an architectural role for the cell.

Dissociation of the cadherin complex occurs through phosphorylation of the amino-terminal binding domain of β-catenin, which displaces it from the cadherin complex (Ilyas et al, 1997). Several tyrosine kinase receptors have been identified and shown to interact directly with membrane-bound β-catenin (Morin et al, 1999). Two growth factors, epidermal (EGF) and hepatocyte growth factors (HGF) act via the receptors to stimulate dissociation of the β-catenin/E-cadherin complex (Ilyas et al, 1997). Loss of cadherin function either through tyrosine phosphorylation of β-catenin, mutations in the binding site of the cadherins, or mutations in the N-terminal domain of β-catenin loosens cell-cell junctions and allows cellular migration. This is an important process in tissue healing, angiogenesis, and in oncogenic transformation (Ilyas et al, 1997).

Aside from the role in adherens junctions, β-catenin exists in a monomeric (free) form in the cytoplasm which, upon stimulation, functions in a signal transduction pathway. Under normal conditions, the cytosolic level of β-catenin is tightly controlled by APC. Aberrant increases in the cytosolic level of β-catenin induces the activity of glycogen synthetase kinase 3β (GSK 3β), a serine/threonine kinase which phosphorylates both β-catenin and APC (Orford et al, 1997; Polakis, 1997). Phosphorylation results in an APC/β-catenin/GSK-3B complex which acts to degrade β-catenin by the ubiquitin-

proteosome pathway (Orford et al, 1997). Another protein, axin, serves as a scaffold for the complex and is necessary for the activity of GSK-3β (Hedgepeth et al, 1999).

In steady-state conditions, β-catenin is thought to be in equilibrium with APC and cadherins, so low cytoplasmic levels are maintained. Initiation of the Wnt/wingless signal transduction pathway, which ultimately inhibits GSK 3β, or mutations in the β-catenin binding site on APC results in stabilization of cytosolic β-catenin (Morin, 1999). When this occurs, β-catenin can freely translocate into the nucleus, where it interacts with a family of high mobility group-box (HMG) containing transcription factors (TCF-1, TCF-2, TCF-4, LEF-1) (Yokoya et al, 1999; Kuhl and Wedlich, 1997). Association with these proteins forms a ternary complex important for transcription to occur (Morin et al, 1999). A few of the target genes have been found to be those involved in cell cycle control and apoptosis, including *c-Myc*, cyclin D1, *c-Jun*, and *Fra-1* (Mann et al, 1999; Mayer et al, 1999). Cadherins also possess LEF promoters on their transcription domain.

Although β-catenin signaling is important in embroygenesis, there is compelling evidence that the nuclear localization of β-catenin is also a common event in colon cancer. Because APC negatively regulates the free cytosolic form of β-catenin, and this protein is mutated in 60-80% of colon tumors, it has been suggested that its tumor suppressive activity is through regulation of β-catenin levels in the cell. Supporting this hypothesis, restoration of wild-type APC to cells deficient in this protein results in recruitment of β-catenin from the nucleus (Korinek et al, 1997). Also, overexpression of E-cadherin in cancer cells results in increased cytosolic and membrane-bound β-catenin and restoration of cell adhesion (Orsulic et al, 1999). In addition, there appears to be a strong inverse relationship between cancer cell progression and nuclear localization of β-

catenin (Hao et al, 1997). Cancer cells located at the invasion front of tumors posses more nuclear staining of β-catenin compared to cells in the tumor center which retain a more membranous expression, similar to normal epithelium (Brabletz et al, 1998). Although the regulation of cytosolic β-catenin is likely controlled by several proteins, a functional APC protein appears to be among the most important components in the cytosol.

B.5 Animal Model of Colon Cancer:

In any animal study, the biological plausibility, or ability to extrapolate findings to human conditions, is an important consideration. Relevant to colon cancer, the *Min*$^+$ (multiple intestinal neoplasia) mouse shares many similarities to the human condition FAP. *Min*$^+$ mice exhibit a fully penetrant germline mutation on codon 850 in the murine homolog of the adenomous polyposis coli (*APC*) gene (Figure 2) (Su et al, 1992). As a result, mice carrying the mutation develop, on average, 50+ adenomas throughout the intestinal tract. (Su et al, 1992, Moser et al, 1990). Studies examining intestinal tumor formation in *Min*$^+$ mice suggest that tumors are initiated in the first few weeks of life, and tumor multiplicity is established by 67 days of age (Shoemaker et al, 1995). The initiation of intestinal tumor development is thought to preceed somatic loss of the remaining wild type *APC* allele (Oshima et al, 1995). The lifespan of *Min*$^+$ mice is relatively short, 119±31 days, and the cause of death is commonly severe anemia due to intestinal bleeding (Moser et al, 1990).

There are many similarities between *Min*$^+$ mice and FAP patients both at a genetic level and phenotypically. The murine and human *APC* genes share 86% and 90%

similarities at the nucleotide and amino acid levels, respectively (Su et al, 1992). The *Min*$^+$ mutation is the result of a TTG-TAG transversion, causing premature truncation of the APC protein, which is common to mutations observed in humans (Su et al, 1992). Extracolonic features, including desmoid tumors and epidermoid cysts, which are seen in some forms of FAP have also been reported in *Min*$^+$ mice (Shoemaker et al, 1995). The main difference between the *Min*$^+$ mutation compared to human FAP is the distribution of tumors. In humans, most of the tumors develop in the large intestine, whereas in *Min*$^+$ mice, the majority of tumors are found in the small intestine. Notwithstanding this, because of the mutational similarities in the *APC* gene and the impact on tumor development, the *Min*$^+$ mouse model may provide insight into chemopreventative agents for intestinal carcinogenesis.

C. DIETARY FIBER

Dietary fiber, as initially defined by Trowell in 1972, is the "skeletal remains of plant cell walls incapable of digestion by human alimentary enzymes" (Trowell, 1976). This definition has been expanded to include a broad range of polysaccharides (storage polysaccharides and plant gums and mucilages) and lignin (Chesson, 1995). For purposes of this paper, resistant starches and indigestible oligosaccharides will also be included in the traditional definition as dietary fiber components. The recommended intake of dietary fiber is currently 25-30 g/day, however in populations consuming Westernized diets, intake is 11-14 g/day (Greenwald and Lanza, 1986). The main sources of dietary fiber, components, and food types are shown in Table 1.

Table 1. Dietary fiber polysaccharides. *Adapted from* Phillips G.O., 1998.

Main Source	Soluble in Water	Major Groups	Components	Foods
Storage material	Partly	Starch	Amylose Amylopectin	Fruits, seeds, tubers
Structural material	No	Cellulose		All cell walls
	Yes	Non-cellulose	Pectic substances	Mainly fruits and vegetables
	Slightly		Hemicelluloses	Cereals Fruits and vegetables
Non-structural material	Yes Yes	Mucilages Gums		Algal seaweed, exudates, seeds and fruits

C.1 Components of dietary fiber:

The types of polysaccharides found in plant cell walls vary with the plant origin and its maturity (Selvendran and Verne, 1988). A major portion of dietary fiber present in the human diet comes from parenchymous tissues in fruits and vegetables, whereas wheat bran and other cereal products contribute more of the lignified tissues (Selvedran and Verne, 1988). Analytical methods used for determining dietary fiber commonly involve removal of soluble sugars, lipophillic materials, protein, and starch and then separation with either ethanol or centrifugation to yield soluble and insoluble fractions (reviewed by Asp, 1995). The sugar moieties vary with the polymer present. Hydrolysis of cellulose yields glucose, hemicellulose yields xylose and glucose (in dichots), and

pectic substances yield uronic acid, galactose, and arabinose (Selvedran and Verne, 1988).

C.2 Non-fermentable Fibers

Insoluble, non- or poorly fermentable fibers present in the diet include lignins, cellulose, and some hemicelluloses (Johnson and Southgate, 1994). Lignins are polyphenolic compounds found mainly in mature plants and cereal fibers (Johnson and Southgate, 1994). Cellulose is a high molecular weight compound composed of linear polymers of glucose linked by β 1-4 glycosidic bonds (Johnson and Southgate, 1994). Hydrogen bonding tightly packs cellulose chains into fibrils, making them relatively inaccessible to digestion (Johnson and Southgate, 1994). *In vitro* indigestibility with human fecal flora for cellulose (Solka Floc) and wheat bran have been previously reported to be 97-99% and 58-63%, respectively (Ehle et al, 1982).

C.3 Fermentable Fibers:

C.3.1 Resistant Starches

Starches consumed in the diet are hydrolyzed in the small intestine by α-amylase. However, some starches, termed resistant starches, may escape digestion and become substrates for fermentation in the colon (Cummings and Macfarlane, 1991). Resistant starch (RS) is classified into three categories, namely RSI, RSII, and RSIII (Cummings et al, 1987) Starches in the RSI category are resistant to digestion due to limited amylase accessibility to the starch molecules because of physical constraints. Examples include starches from whole or coarsely ground grains and seeds. In native (raw) starch

granules (RSII), amylose and amylopectin chains are packed into a crystalline structure, making them less susceptible to enzymatic degradation (Gallant, 1995). Because cooking disrupts (gelatinizes) the crystalline structures, only foods consumed in the raw form and containing RSII are nutritionally significant (Englyst and Cummings, 1986). The third class (RSIII) form as a result of food processing (Cummings and Macfarlane, 1991). Slow, prolonged cooling of cooked starches allow some of the amylose chains to crystallize, forming "retrograded starch." If these crystalline, retrograded starch structures are not gelatinized during the reheating process that typically occurs before consumption, the retrograded starch will not be digested (McWilliams, 1993). Approximately 8-40 g/day of resistant starches become available for fermentation in the colon (Cummings and Macfarlane, 1991).

C.3.2 Oligosaccharides

Approximately 2-8 g of oligosaccharides are consumed per day and pass to the colon for fermentation in populations consuming Western diets (Cummings and Macfarlane, 1991). One class of oligosaccharides, oligofructoses, are low molecular weight polymers composed of β-1-2-D fructose units (Roberfroid, 1999). Inulin, (degree of polymerization (DP) between 10-60) and fructo-oligosaccharides (DP < 10) are found in large quantities in chicory roots, garlic, leeks, and Jerusalem artichokes (Roberfroid et al, 1993; Roberfroid, 1999). Oligofructoses, unlike gums, do not appear to reduce cholesterol or bile acid absorption in the small intestine, but are rapidly fermented in the colon (Andersson et al, 1999). Fructo-oligosaccharides (FOS) also have been shown *in vitro* to selectively stimulate the growth of Bifidobacterium at the expense of bacteriodes,

clostridia, and coliforms (Gibson and Roberfroid, 1995). Due to the perceived benefits of Bifidobacteria, FOS is currently the only oligosaccharide classified as a "prebiotic" food (Gibson and Wang, 1994).

C.3.3 Gums

Plant gums are a diverse group of heteroglycans produced by plants under conditions of stress (Stephen, 1995). They are composed of a variety of large molecular weight compounds used by the food industry as thickeners and stabilizers (Stephen, 1995). The sugar composition varies between the different types of gums, although most contain a high proportion of galactose residues (McWilliams, 1993b). Variations in sugar residues dictate the degree of solubility in water, viscosity, and ability to form gels in food systems (Stephen, 1995). Among the different types of gums, gum arabic is a large molecular weight (MW 850,000) polysaccharide composed primarily of rhamnose, arabinose, glucuronic acid, and galactose residues (Phillips, 1998). Gum arabic is not digested in the small intestine, but is fermented in the colon. Ross et al (1984) reported that gum arabic was completely fermented, primarily in the cecum of rats (Ross et al, 1984).

D. FERMENTATION OF DIETARY FIBER

Short chain fatty acids (SCFA) are the primary end products of fermentation by anaerobic bacteria residing in the colon. Relative proportions of the SCFA produced are estimated to be 60:25:10 for acetate, propionate, and butyrate respectively (Scheppach et al, 1995). Dietary fiber is the main substrate for fermentation, with smaller contributions

from protein, mucin, and epithelial cells (Cummings and Macfarlane, 1991). In recent years it has become evident that the quantity and relative proportions of SCFA can be manipulated by varying the diet. The significance of this and the recognition that SCFA are the primary metabolic fuels for the colonocyte has led to research on SCFA utilization in a variety of pathological conditions (Roediger, 1990). Of particular interest is that butyrate is a differentiating agent in various colon cancer cell lines, leading some to believe that the fermentation of fiber can potentially alter colon cancer risk. The following will review what is known regarding SCFA production and absorption, and the possible role of fermentation in colon cancer prevention.

D.1 Products of anaerobic fermentation in the colon

There are over 400 species of bacteria residing in the colon, with total colonic bacteria numbering $10^{11\text{-}12}$/g dry feces (Cummings, 1981). Most bacteria are saccrolytic, and use the Embden-Meyerhof glycolytic pathway for hexose degradation (Cummings, 1981). As shown in Figure 3, the major product of this pathway is pyruvate, which is further metabolized to produce the three main SCFA, acetate, propionate, and butyrate (Hill, 1986). Lactate, hydrogen, carbon dioxide, formate, and methane are also produced (Miller and Wolin, 1979). The overall equation for the reaction derived from rumen metabolism is:

$$58\ (CH_2O)_6 \rightarrow 62\ CH_3COOH + 22\ CH_3CH_2COOH + 16\ CH_3(CH2)_2COOH +$$
$$60.5\ CO_2 + 33.5\ CH_4 + 27\ H_2O \text{ (Miller and Wolin, 1979).}$$

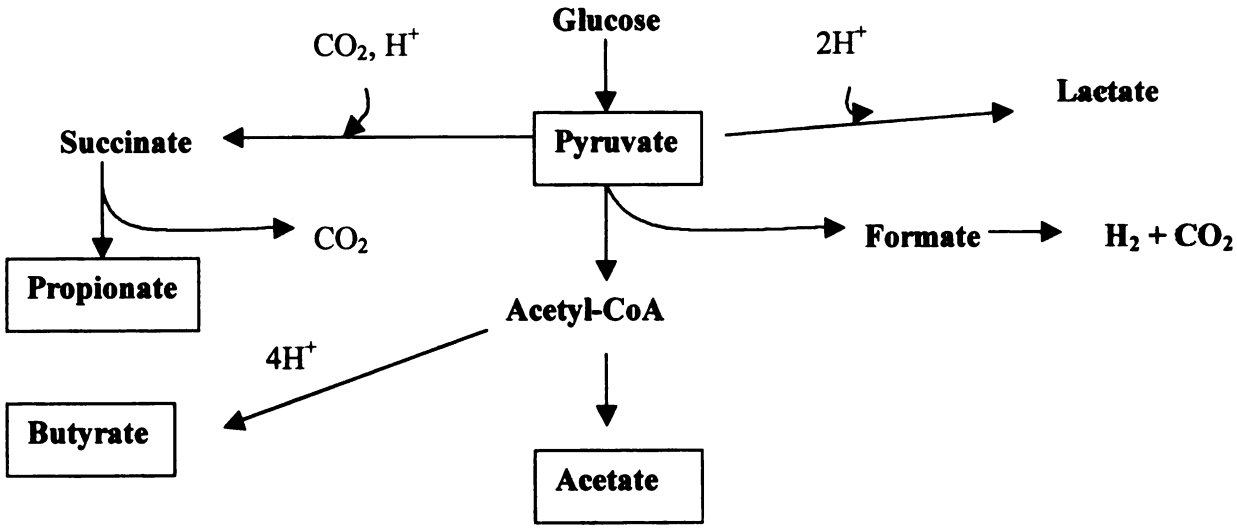

Figure 3. End products of anaerobic fermentation in the colon. *Adapted from:* Hill, 1995.

D.1.1 Gas Production

Gases (hydrogen, methane, carbon dioxide) produced during fermentation are rapidly absorbed and expelled through the lungs or excreted as flatus (Cummings and Macfarlane, 1991). Fermentation is the only significant source of hydrogen gas in the body, so breath hydrogen measurements are sometimes used as indices of fermentation. Hydrogen that is not excreted is further metabolized by methanogenic, acetogenic, and sulphate-reducing bacteria (Cummings and Macfarlane, 1991). Approximately 30-60% of healthy humans favor methane production over H_2 excretion and methane levels can also rise in pathological conditions such as when polyps and colon cancer occur (Cummings and Macfarlane, 1991; Haines et al, 1977). Lactate production is usually low in humans, but concentrations increase in malabsorptive conditions. Rapid fermentation of readily available sugars causes a significant decrease in luminal pH, which inhibits lactate-utilizing enzymes and increases lactate concentrations (Cummings, 1981).

D.1.2 Short Chain Fatty Acids:

As mentioned previously, the primary substrates for fermentation in the colon are starches that escape digestion in the small intestine and dietary fibers (Cummings and Macfarlane, 1991). The SCFA produced in the highest quantity is acetate, followed by propionate and butyrate (Cummings and Macfarlane, 1991). Although fermentation favors acetate production, propionate and butyrate yields are sensitive to changes in the diet. Overall, fermentation patterns are influenced by the composition of the substrate, transit time, and fermentability (Edwards and Eastwood, 1994; Bergreen et al, 1993).

Substrates which favor acetate production include pectins and fibers with low fermentabilities (cellulose and oat husk) (Bergreen et al, 1993; Ehle et al, 1982). Shifts to propionate production occur with fermentation of gums and resistant starches from wheat and maize (Bergreen et al, 1993; Cummings et al, 1996). Butyrate concentrations tend to increase with the amount of starch available. Sheppach et al (1988) showed that administrating arcarbose, a drug that inhibits starch digestion in the small intestine, to humans resulted in an increase of fecal butyrate concentrations compared to controls. Fructo-oligosaccharides, oligosaccharides, and potato starch have also been shown to favor butyrate production (Campbell et al, 1997; Fergusen et al, 2000).

D.2 SCFA Absorption and utilization

More than 95% of SCFA produced are efficiently absorbed by humans, and absorption is thought to be both concentration-dependent and pH-independent (Scheppach, 1994; Rechkemmer and von Engelhardt, 1988). The pKa values for acetate, propionate, and butyrate are 4.75, 4.87, and 4.81 respectively, and therefore at physiological pH (6.0-7.0) are predominately in the anionic form (Rechkemmer and von Engelhardt, 1988). SCFA absorption is associated with an increase in bicarbonate secretion into the lumen and Na^+ and Cl^+ absorption (Binder and Mehta, 1989). Although there may be marked species differences, the following model for SCFA absorption in the guinea-pig colon has been proposed by von Engelhardt and Rechkemmer (1992), von Engelhardt et al (1994), and extended by von Engelhardt (1995) (Figure 4).

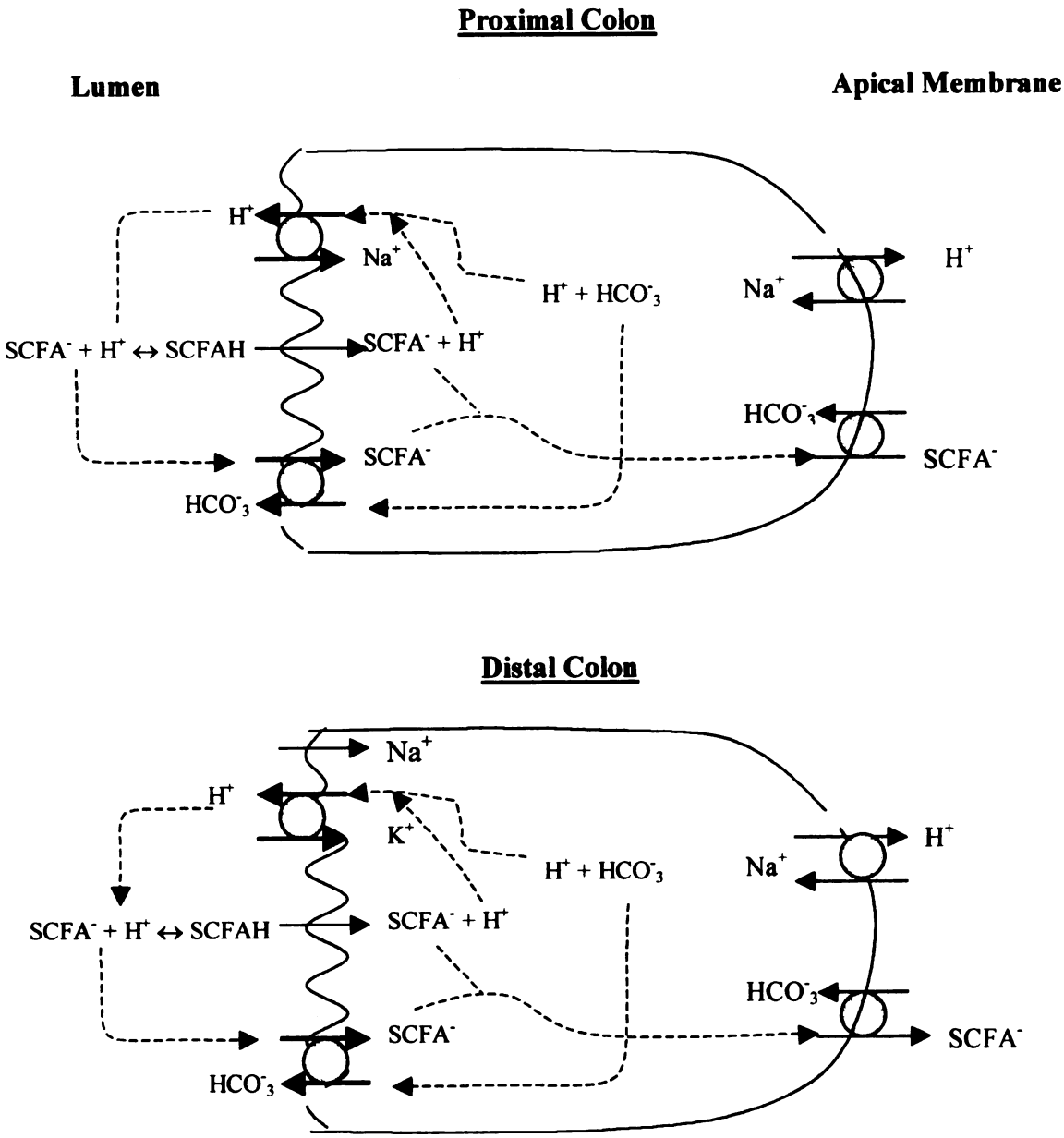

Figure 4. SCFA absorption from the proximal and distal colon. *Modified from* von Engelhardt, 1995.

In the proximal colon, approximately 30-50% of SCFA are absorbed in the lipid soluble form and H^+ ions are derived from an active Na^+-H^+ exchange in the apical membrane. The remainder of the ionized fatty acids are absorbed by a $SCFA^-$-HCO^-_3 exchange mechanism. Intracellular carbonic anhydrase provides both H^+ and HCO^-_3 ions necessary for exchanges to occur through the membrane. In the distal colon, lipid solubility and chain length are thought to be more important than in the proximal colon for SCFA absorption (Rechkemmer and von Engelhardt, 1988). In the apical membrane, K^+-H^+ ATPase provides hydrogen ions necessary for luminal protonation of the acids and for non-ionic diffusion to occur. Ionized forms of SCFA are exchanged for HCO^-_3 by a SCFA-HCO^-_3 transport mechanism. The intracellular pH in both regions of the colon is thought to be regulated by a Na^+-H^+ exchange located on the basolateral membrane, however SCFA transport through the basolateral membrane has not been fully elucidated (von Engelhardt, 1995).

After absorption, butyrate is primarily used by the colonocyte for β-oxidation (Roediger, 1982). Utilization of SCFA by the colonocytes is in the order butyrate>propionate>acetate. Studies in sudden death victims have shown propionate and acetate travel though the blood stream to the liver, although some may also be used locally in the colon (Cummings et al, 1987). Propionate acts as a substrate for gluconeogenesis via its metabolism to succinate. Studies done on the utilization of propionate *in vivo* have also suggested that it has cholesterol-lowering effects after its metabolism in the liver, possibly by inhibiting HMG-reductase. Acetate is likewise absorbed and used for hepatic ketogenesis and synthesis of phospholipids, cholesterol,

and long-chain fatty acids. Acetate is the only SCFA found in significant amounts in peripheral blood and can be used by skeletal muscles and the heart as an energy source (Cummings et al, 1987).

D.3 Clinical significance of SCFA

SCFA are the main energy source for colonocytes (Roediger, 1982). Deficiencies in the production and/or utilization of SCFA have been implicated in a range of clinical conditions, emphasizing the importance of these acids for colonic health. Lack of luminal nutrition, as occurs in famines and starvation, decreases energy provision to the small intestine and colon (Roediger, 1990). Although systemic circulation supplies alternate fuel supplies to the colon, long-term starvation results in decreased oxidative capacity, decreases in sodium and water absorption, and mucosal atrophy (Roediger, 1990). This is commonly seen in patients on long-term total parenteral nutrition (TPN) and has been demonstrated with colonic bypasses in the rat. Diverting the fecal stream away from the colon causes mucosal inflammation that is histologically similar to ulcerative colitis (UC), and which can be partially alleviated with butyrate enemas (Harig et al, 1989). Long-term TPN also causes mucosal atrophy to both the small intestine and colon. Incorporation of SCFA into TPN solutions has been shown to prevent some of the mucosal atrophy in the small intestine, but not the colon, and may lead to a better prognosis in patients with short bowel syndrome (Koruda et al, 1990).

D.4 TROPHIC EFFECTS OF FIBER AND SCFA ON GUT MORPHOLOGY

D.4.1 Trophic effects of SCFA in the cecum and colon

SCFA are known trophic agents for epithelial cell growth in the small intestine, cecum, and colon, however the mechanism differs between tissues (Sakata, 1987; Lupton and Kurtz, 1993). Fermentation of fiber in the cecum and colon both decrease luminal pH and increase SCFA concentrations. Lupton et al (1993) showed that a decrease in pH was highly correlated to epithelial proliferation in the cecum. This resulted in a greater cecal surface area, and an increase in crypt depth, circumference, and cellularity (Lupton and Kurtz, 1993; Lupton et al, 1988). In the colon, SCFA act locally to stimulate epithelial cell growth. Infusion studies strongly suggest that SCFA are the main trophic agents for proliferation. Sakata et al (1982) reported that SCFA infused into the colon of fasted rats rapidly increased epithelial mitotic and labeling indices, possibly by shortening the G2 phase of the cell cycle. Butyrate produces the greatest hyperproliferative response, followed by propionate and acetate (Sakata, 1987). The effect on colonic cell growth is also dose-dependent (Kripke et al, 1989; Sakata, 1987). Proliferation is confined to the lower 2/3 of the crypt and is associated with an increase in crypt depth and cellularity (Velaquez et al, 1996; Lupton and Kurtz, 1993). SCFA also stimulate colonic and hepatic blood flow. In perfused dog colons, SCFA infusions increased both blood flow and oxygen uptake to the colon with acetate being the most effective (Kvietys and Granger, 1981).

D.4.2 Trophic effects of SCFA in the small intestine

Luminal nutrition, pancreato-biliary secretions, and hormonal stimuli all maintain the epithelial lining of the small intestine (Dowling, 1978). SCFA also are known trophic agents for epithelial growth in the small intestine, most likely through a systemic mechanism (Thompson et al, 1996). Infusional studies suggest that, unlike in the colon, acetate and propionate are more trophic to the small intestine (Koruda et al, 1988). Although the mechanism by which this occurs has not been fully elucidated, possible SCFA-mediated agents include stimulation of the autonomous nervous system and release of the hormones gastrin and enteroglucagon (Frankel et al, 1988).

E. FERMENTATION AND COLON CANCER

Fermentable fibers, if protective, most likely modulate colon carcinogenesis through end-products of fermentation. Aside from the possible benefits of stimulating the growth of certain strains of bacteria, the production of SCFA lowers the pH of intestinal contents. This can alter luminal metabolism, including the formation of secondary bile acids and ammonia (Gibson and Roberfroid, 1995; Fergusen and Harris, 1996). Butyrate, and to a lesser extent propionate, may also act independently to protect the mucosa against malignant transformation, although these effects have been primarily studied *in vitro*.

E.1 Alterations in bile acid metabolism

Populations at high risk for colon cancer development excrete higher levels of bile acids in the stool (Hill et al, 1971). Approximately 2-5% of bile acids escape

reabsorption in the small intestine and are metabolized by bacteria in the colon (Nagengast et al, 1995). The bacterial enzyme β-glucuronidase hydrolyzes the glucuronide derivative in the cecum and colon, and bacterial 7-α dehydroxylase is responsible for converting primary bile acids into secondary acids, namely lithocholic and deoxycholic acid (DCA) (Nagengast et al, 1995). The enzyme 7-α dehydroxylase becomes increasingly unstable at pH values higher or lower than 7.0, and is inhibited at pH values below 6.0, resulting in decreased production of secondary bile acids (Fergusen and Harris, 1996).

Rafter et al (1986) showed in perfused rat colons that 5 mM deoxycholic acid is damaging to the colonic epithelium, resulting in a more cuboidal morphology and erosion of cells from the lamina propria. Decreasing the pH of the perfusate from 7.9 to 5.5 or increasing the concentration of calcium from 0 to 4 mM lessened the damage to the colonic surface. Rectal infusion of deoxycholic acid has also been shown to enhance experimentally induced colon carcinogenesis and is associated with an increase in proliferating cells in the upper part of the colonic crypt (Velaquez et al, 1996). Fermentation lowers luminal pH, and could therefore decrease the activity of 7-α dehydroxylase, resulting in an increased excretion of primary with a subsequent decrease in secondary bile acids in the stool. Precipitation of bile acids out of the aqueous phase of the stool or a decreased concentration of secondary bile acids would decrease exposure and cytotoxicity to the colonocytes.

There is some evidence in humans that addition of dietary fiber can alter bile acid metabolism. Addition of 13-15 g of wheat bran/day for 8 weeks in humans decreased concentrations of secondary bile acids in the stool and caused a significant reduction in

fecal β-glucuronidase and 7-α dehydroxylase activities (Reddy, 1992). In another study, adding 45 g native amylomaize (resistant) starch/day to the diet of healthy volunteers resulted in a significant increase in primary bile acid excretion and a trend towards lower excretions of secondary bile acids (Van Munster et al, 1994). Although the stool pH did not change significantly, total soluble bile acids decreased in the aqueous phase of the stool. Proliferation of colonic crypts also significantly decreased during the amylomaize feeding compared to the control period.

E.2 Alterations in ammonia metabolism

Another mechanism by which SCFA may protect against colon carcinogenesis is by altering luminal metabolism of ammonia. Based on studies in ileostomy patients, approximately 2 g/day of nitrogen-containing compounds enter the large intestine, primarily from endogenous substrates. Levels can also increase with dietary protein consumption (Cummings and Macfarlane, 1991). Urea from systemic circulation also supplies ammonia nitrogen after metabolism in the bowel by bacterial urease (Royall et al). Protein is the major source of nitrogen, and *Bacteroides spp, Propionibacterium spp, Streptococcus, and Clostridium* present in the colon all exhibit proteolytic activity (Cummings et al, 1987). Proteolysis results in accumulation of SCFA, ammonia, and phenolic acids *in vitro* (Cummings et al, 1987).

Ammonia is cytotoxic to colonocytes and stimulates colonocyte proliferation (Lin and Visek, 1991). Lowering the luminal pH through fermentation has been proposed to increase the proportion of the less soluble ammonium ion, which can then be used locally for bacterial synthesis (Visek, 1978). This would result in a decreased exposure of

colonocytes to ammonia. Birkett et al (1996) reported decreased fecal ammonia and phenol metabolites in the feces of humans fed resistant starch. Scheppach et al (1988) also showed that administration of acarbose increased fecal nitrogen excretion in humans, which was associated with an increase in bacterial mass. Thus, fermentation may protect against colon carcinogenesis by altering luminal metabolism, decreasing ammonia and phenol metabolites and attenuating the cytotoxic effects of these nitrogen by-products in the lumen.

E.3 Direct actions of butyrate

The possible direct link between fermentation of dietary fibers and colon cancer prevention has been demonstrated most extensively *in vitro,* and more consistently for butyrate than acetate or propionate. Butyrate, at physiological concentrations of 1-5 mM, has been shown to induce differentiation and apoptosis, modify gene expression, and alter chromatin ultrastructure (Smith, 1986; Barnard and Warwick, 1993; Whitehead et al, 1986). This has been shown to occur in various colon cancer cell lines and other cell types, including white blood cells and breast cancer cells. There is, however, limited *in vivo* evidence to support a direct effect of butyrate on colon carcinogenesis.

In the colon, cells near the upper third of the crypt lose the ability to proliferate and gain cell-specific differentiation markers which dictates the phenotype of progeny cells. A characteristic of many colon cancer cell types is the loss of cell cycle control, resulting in uncontrolled proliferation and a decrease in differentiation. Butyrate induces cell cycle arrest early in the G1 phase of the cell cycle (Barnard and Warwick, 1993). Barnard et al (1993) showed that growth arrest was rapidly reversible and associated with

a decrease in *c-myc* protoncogene expression and an increased expression of alkaline phosphatase. Whitehead et al (1986) showed in LIM1215 colon cancer cells that butyrate treatment resulted in an increase in doubling time from 26 to 72 hours and suppression of cloning efficiency. The effects on cell growth were associated with an increase in differentiation markers, as butyrate-treated cells had increased activities of alkaline phosphatase, CEA, and dipeptidyl amino peptidase over controls.

Inhibition of apoptosis is thought to occur early in colon carcinogenesis, and is associated with loss of APC protein functioning (Morin et al, 1996). Butyrate has been shown in several colon cancer cell lines to induce apoptosis. Heerdt et al (1997) showed that butyrate, but not isobutyrate or heptofluorobutyric acid derivatives, induced apoptosis in the carcinoma cell lines HT29 and SW260. Hague et al (1995) confirmed these results in both adenoma and carcinoma colon cancer cell lines. However, 2 of the 4 carcinoma cell lines were shown to be more resistant to butyrate-induced apoptosis.

The mechanisms by which butyrate can induce apoptosis and/or differentiation are not fully known. Histone hyperacetylation, which alters DNA accessibility to endonucleases and repair proteins, can be altered with butyrate treatment *in vitro* (Smith et al, 1986). The mechanism appears to involve direct inhibition of deacetylase enzymes, primarily through a histone H4 acetylation (McBain et al, 1997). McBain et al (1997) showed that the time required for butyrate-induced apoptotic death correlated to the time required for tertracetylation of histone H4. They also reported that carboxylic acids 3-5 carbons in length were more effective in inducing hyperacetylation. Other possible mechanisms involving butyrate-induced apoptosis include upregulation of the p21 cell

cycle inhibitor and downregulation of the epidermal growth factor receptor (EGF) (Hague et al, 1993; Archer et al, 1998).

F. Fermentation and experimental colon carcinogenesis

Colon cancer is induced in rodent models by administration of carcinogens (DMH or AOM) either subcutaneously, intraperitoneally, or by gavage. Depending on the time of carcinogen exposure, one can examine the effect of diet during initiation (before carcinogen) or promotion (after carcinogen exposure). Differences in dosage, type, and route of administration, however, cause many inconsistencies between study designs (Whiteley and Klurfeld, 2000). End-point measurements used to assess the effect of diet on colon cancer development in this model include abberant crypt foci (ACF) and/or number of colon tumors. Another model for colon cancer studies is the *Min*$^{+}$ mouse model, as discussed previously.

There is limited evidence to suggest that fermentable fibers are protective against experimentally induced colon carcinogenesis. However, proposed mechanisms involved in chemoprevention vary widely. In carcinogen-induced colon cancer, oat bran, corn bran, and guar gum do not appear to influence tumor development, whereas wheat bran is more consistently associated with a protective effect (Zoran et al, 1997; Compher et al, 1999; McIntyre et al, 1993). In one of these studies, there was a negative association found between intraluminal butyrate concentrations and tumor mass regardless of the dietary treatment (McIntyre et al, 1993). Reddy et al (1997) reported that the chicory fructans, oligofructose and inulin, when fed at 10% of the diet, inhibited ACF precancerous lesions and crypt multiplicity in AOM-induced carcinogenesis. Both potato

and resistant starches also have been shown to inhibit the development of ACF in rats when fed during the promotion phase of tumorigenesis. The protective effect of resistant starch was associated with an increase in intraluminal butyrate concentrations over controls (Thorup et al, 1995; Cassand et al, 1997). Using *Min*$^{+}$ mice as a model, one study reported that FOS (6% of diet) inhibited colon tumor incidence and was associated with an increase in gut associated lymphoid tissue development in this group (Pierre et al, 1997). However, in two studies using resistant starch and one study using wheat bran, no protective effect on colon tumor incidence was reported (Pierre et al, 1997). Thus, inulin, FOS, and wheat bran appear to consistently protect against colon carcinogenesis, whereas there are inconsistencies between models for the effect of resistant starches on colon carcinogenesis.

III. RATIONALE:

The addition of fiber to the diet can influence fermentation patterns in the colon, and the concentration of butyrate is particularly sensitive to the type and amount of fiber in the diet (Rombeau and Kripke, 1990). Dietary fiber includes a heterogenous group of compounds with varying physiochemical properties. Therefore, it becomes increasingly difficult to correlate fiber *per se* with colon cancer risk. Examination of fermentation patterns, at the site of production, allows more quantitative associations to be made and can give insight into possible mechanistic roles of fermentation metabolites in colon cancer prevention. The types of fermentable fibers used in the present study were fructo-oligosaccharides (FOS), modified starch, and gum arabic. FOS yields high amounts of butyrate, and resistant starch yields either high amounts of butyrate or propionate (Campbell et al, 1997; Cummings et al, 1996; Berggren et al, 1993). Gum arabic, a plant extrudate, yields higher propionate at the expense of acetate (Annison et al, 1995; Titgemeyer et al, 1991). The controls used in this study include cellulose, which is minimally fermentable in the cecum and colon, and kaolin, an inert bulking agent that is non-fermentable. The two bulk sources were included in the diet to minimize muscular atrophy in the small and large intestines.

Min$^+$ mice were chosen as the model in this study because, similar to humans with FAP, they have a genetic predisposition to colon cancer. However, colon cancer rarely occurs in *Min*$^+$ mice due to their short life span (120 ± 31 days), making it necessary to study dietary effects at earlier stages of adenomatous growth. In humans, about 70-90% of colon cancers arise from adenomatous polyps, and the larger the polyp,

the more likely it is for cancer to develop (Rudy and Zdon, 2000). Therefore, interventions that result in reduced tumor burden (decreased number and/or reduced polyp size) are more likely to delay the progression to cancer.

IV. OBJECTIVES

The primary objective in this study was to determine if the concentrations and patterns of SCFA in the cecum of mice are alterable by varying the fiber content in the diet. The second objective was to determine whether fermentation metabolites decrease intestinal tumor incidence and size in *Min*$^+$ mice. Certain fiber sources and SCFA produced by fermentation in the large bowel have previously shown trophic effects throughout the intestinal tract. Therefore, the third objective was to differentiate trophic effects of SCFA from unfermented bulk by measuring intestine lengths and cecal weights.

V. NULL HYPOTHESES

The null hypotheses to be tested are: 1) fermentation does not protect against tumor development in the intestinal tract of *Min*$^+$ mice; 2) SCFA amounts or types in the cecum do not differ among treatment groups; and 3) fermentable and non-fermentable fiber sources are not trophic to the small intestine, cecum, or colon.

VI. MATERIALS AND METHODS

Animal Care: C57BL/6J *Min*$^{+}$ and *Min*$^{-}$ mice, aged 3-4 weeks, were obtained from a colony maintained at Michigan State University. Mice were not genotyped for the *APC* mutation prior to the experiment, but were divided into groups under the assumption that half the offspring would be *Min*$^{+}$. The animals (n=128) were grouped by litter, sex, and weight and randomly assigned to treatment groups. Mice were housed in plastic cages (2-3 mice/cage) in temperature (23°C ± 2°) and humidity (40-60%) controlled rooms with a 12 hour off/on light cycle. All mice had continual access to food and water and were observed daily for health status. This study was approved by the Michigan State University All-University Committee on Animal Use and Care.

Diets: Mice were fed a powdered version of the AIN-93G diet with slight modifications, however, the nutrient:energy ratio was similar for all diets and comparable to the AIN-93G diet (Reeves, 1993). All diets contained 15% fat (wt/wt) as soybean oil and 6% (wt/wt) of either a fiber source or inert bulk (Table 2). LoDex 15 was used as the cornstarch source to minimize resistant starch reaching the colon for fermentation. Cellulose (Solka Floc) and kaolin, an inert bulk, were used as the controls.

Experimental Design: Mice began nutritional intervention at 4 weeks of age. Due to the relatively short life span of these mice (120 ± 31 days), they were fed experimental diets for a total of 10 weeks. At 10 weeks post-treatment, the animals were

Table 2. Dietary composition of the modified AIN-93G diets. The compositions are presented as g/100 g diet.

Ingredient	Modified AIN-93G (Control)	FOS	Modified Starch	Gum Arabic	Kaolin
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
LoDex 15 (Dextrinized Cornstarch)	41.29	41.29	41.29	41.29	41.29
Sucrose	10.00	10.00	10.00	10.00	10.00
AIN-93G-MX (Mineral Mix)	3.87	3.87	3.87	3.87	3.87
AIN-93G-VX (Vitamin Mix)	1.06	1.06	1.06	1.06	1.06
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)	0.0025	0.0025	0.0025	0.0025	0.0025
Cellulose (Solka-Floc)	6.00				
Fructo-oligosaccharides (FOS)		6.00			
Modified Starch			6.00		
Gum Arabic				6.00	
Kaolin					6.00
Total:	100.0	100.0	100.0	100.0	100.0

sacrificed by CO_2 inhalation and exsangunatation around the same time of day (6-10 a.m.) to control for diurnal variation in epithelial cell kinetics. The small intestine and colon were excised and rinsed with lukewarm tap water. Small intestinal and colon lengths were measured to the nearest mm under fixed tension (8 g). The small intestines were then cut into thirds, opened, and rinsed with tap water. After excision of the cecum, the contents were immediately removed, weighed, and kept at -80° C until analysis of SCFA could be performed. All tissues were placed in a phosphate-buffered saline solution (PBS, pH 7.4) immediately following excision, pinned flat on cardboard, and then fixed in a 10% neutral-buffered formalin solution (pH 7.4) overnight (20-24 h). After fixation, all tissues were stored in 1% neutral buffered formalin and refrigerated (4 °C) until counting was completed. Cecums were subsequently blot-dried with paper towels and weighed to the nearest hundredth of a gram.

Scoring of Adenomas: To avoid variation between researchers, all counting was performed by one person who was blinded to treatments. Tissues were stained in a 0.2% methylene blue phosphate-buffered solution (pH 7.4), and viewed with a stereo microscope to detect adenomas. Carriers (*Min* $^{+}$) were determined by presence of adenomas. When carrier status was ambiguous, DNA was extracted from a piece of liver, obtained at sacrifice, and polymerase chain reaction (PCR) was performed as previously described (Luongo et al, 1994). Intestinal adenomas and average diameter for each adenoma were recorded. Colonic and cecal tumors were recorded as the total number per tissue, and a three dimensional measurement (mm^3) was recorded. The three dimensional measurements for colonic tumors were then converted to spheric volumes ($4\pi lwh/3$).

SCFA Analysis: Cecal contents were thawed and hydrated with 3 or 4 volumes of double-distilled water (ddH_2O). Individual pHs were obtained after dilution using a Fischer pH probe. Meta-phosphoric acid (25% wt/wt) was added to the samples in a 1:1:4 ratio (m-phosphoric acid:sample wt:ddH_2O), and samples were centrifuged at 15,000 x g for 5 minutes. The supernatant (4uL) was injected into a gas chromatograph (column: 10% SP1200/1% H_3PO_4 on 80-100 Chromosorb, glass column 3.2 mm I.D. by 2 m long). Nitrogen was the carrier gas. The injection and detector temperatures were 150 °C, and the column oven was temperature-programmed (initial temperature: 110 °C for 5 minutes, increased by 3 °C/min until final temperature of 127 °C). SCFA were detected by flame ionization and quantified by comparison to known standards of acetate, propionate, isobutyrate, butyrate, valerate, and isovalerate. The mean of two sample injections differing by < 5% was used to quantify the SCFA.

Statistical Analysis: Statistical analyses were performed using SAS statistical software (SAS Institute, Inc. Cary, NC, Version 7.0). All statistical differences were detected using a critical value of $P < 0.05$ with the F statistic. When significant treatment, sex, and carrier status effects were detected, appropriate least squares means (LSM) were compared by the least significant difference method. The total number of tumors for the small intestine, colon, and cecum were totaled, ranked, and analyzed by the Kruskal-Wallis test. Small intestine and colon lengths and cecum wet weights were adjusted for metabolic body size ($kg^{3/4}$). After adjustment, the tissue parameters and SCFA concentrations were analyzed as a 2X5X2 factorial design (sex, treatment, and carrier status). Body weights were analyzed by repeated measures analysis of variance of body weight/ sex/carrier status/time. All data are reported as mean ± SEM.

VII. RESULTS

Mouse Body Weights: Dietary treatment did not significantly influence body weight at any time point in the study. As expected, males weighed significantly more than females ($P < 0.0001$). Carriers (*Min*$^{+}$) weighed significantly less than non-carriers ($P < 0.001$). This difference was significant between male carriers and non-carriers at 7 weeks of treatment ($P < 0.002$) and for females, this difference was significant by week 8 ($P < 0.04$) (Figures 5 and 6, respectively).

Tumor numbers: There was no significant effect of treatment on adenoma numbers in the small intestine or polyp numbers in the colon or cecum (Table 3).

Tissue Parameters: Small intestinal and colonic tissue lengths (mm) and the cecum wet weights (g) are shown in Table 4. After adjustments were made for metabolic body size, there was a significant treatment effect for small intestinal lengths ($P < 0.0001$), colon lengths ($P < 0.0015$), and cecum wet weights ($P < 0.0001$) with the fermentable fibers (FOS, resistant starch, and gum arabic) having heavier cecums, and the FOS and gum arabic groups producing longer small intestines and colons than the controls (cellulose and kaolin). Females had significantly greater cecum weights and small intestinal and colon lengths than males. Non-carriers (*Min*$^{-}$) had significantly shorter small intestines and colons than carriers.

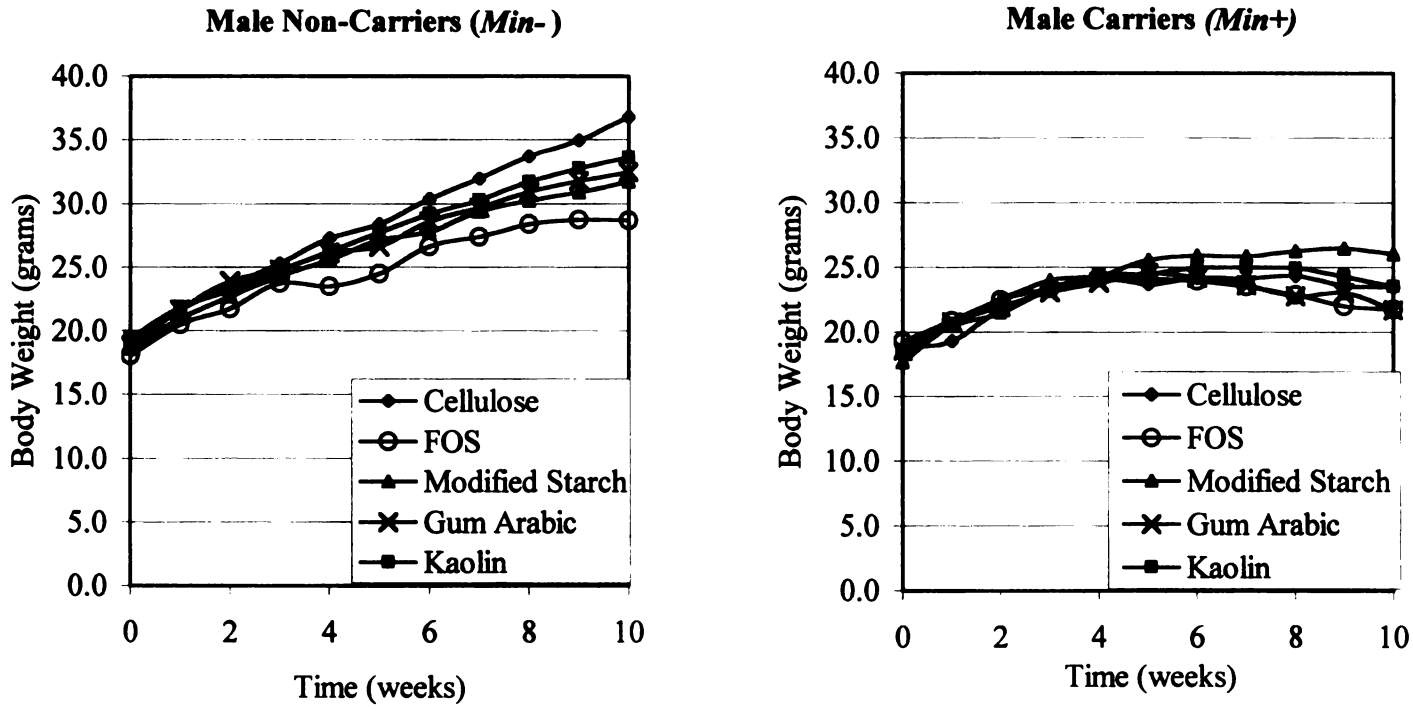

Figure 5. Body weight gain of male Min^+ and Min^- mice during dietary intervention. There were no significant treatment differences ($P < 0.05$) at any time point. There was a significant effect due to carrier status ($Min^+ > Min^-$), which was significant at 6 weeks of treatment ($P < 0.05$).

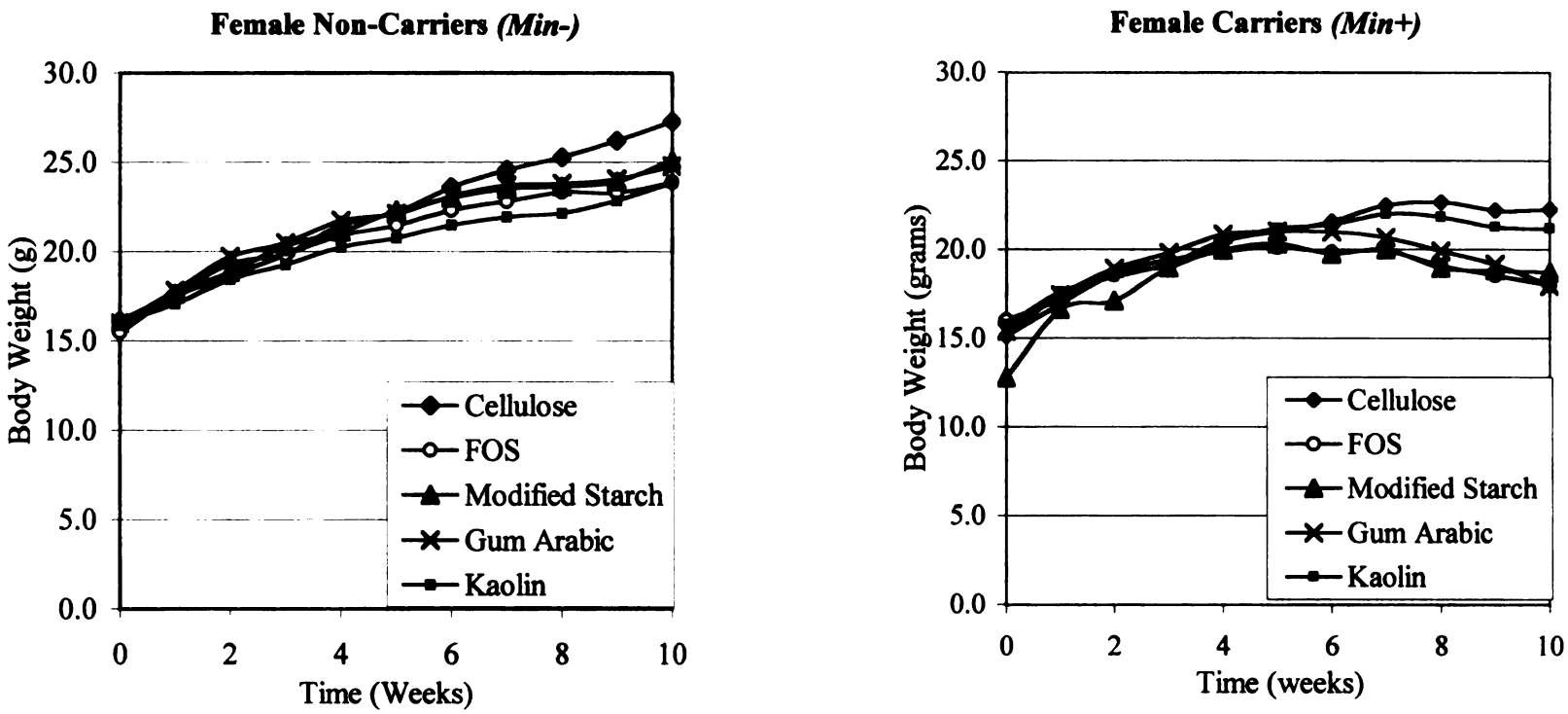

Figure 6. Body weight gain of female Min^+ and Min^- mice during dietary intervention. There were no significant treatment differences ($P < 0.05$) at any time point. There was a significant effect due to carrier status ($Min^+ > Min^-$), which was significant at 8 weeks of treatment ($P < 0.05$).

Table 3. Small intestinal, colonic, and cecal tumor numbers in *Min*$^+$ mice fed either a fiber or inert bulk source at (6 g/100 g) of diet[1].

Treatment	SI Tumor Numbers	Colon Tumor Incidence	Total Colon Tumor Numbers	Solid Colon Tumor Numbers	Colon Tumor Size (mm^3)	Cecal Tumor Numbers
Cellulose (n=13)	79 ± 10	62%	1.0 ± 0.3	1.0 ± 0.3	34 ± 12	0.3 ± 0.2
FOS (n=14)	72 ± 10	57%	0.7 ± 0.3	0.7 ± 0.3	14 ± 12	0.6 ± 0.2
Modified Starch (n=5)	89 ± 17	40%	0.8 ± 0.5	0.8 ± 0.5	7 ± 20	0.5 ± 0.3
Gum Arabic (n=16)	65 ± 9	69%	1.6 ± 0.3	1.6 ± 0.3	42 ± 11	0.1 ± 0.1
Kaolin (n=14)	66 ± 10	43%	0.7 ± 0.3	0.7 ± 0.3	39 ± 12	0.1 ± 0.2

[1] Data were analyzed with Kruskal-Wallis test for ranked data, results are presented as mean ± SEM, and n = number of animals/treatment. Small intestinal tumors were the total of three sections (proximal, middle, and distal). Total colon tumors are the sum of flat and raised tumors. Solid colon and cecum tumors represent tumors where a three-dimensional measurement could be taken. There were no significant differences among treatments.

Table 4. Mean tissue lengths (mm) in the small intestine and colon and cecal wet weights (g) in *Min*$^{+}$ and *Min*$^{-}$ mice fed different sources of fiber or an inert bulk source (6 g/100 g diet)[1,2].

		Small Intestine Tissue Lengths (mm)[3]	Colon Tissue Lengths (mm)[3]	Cecum Wet Weights (mg)[3]
Treatment	Cellulose (n=26)	5.7 ± 0.1^{a}	1.39 ± 0.03^{a}	1.1 ± 0.06^{ab}
	FOS (n=26)	6.9 ± 0.1^{c}	1.55 ± 0.03^{c}	2.1 ± 0.06^{c}
	Modified Starch (n=24)	6.2 ± 0.2^{b}	1.43 ± 0.04^{b}	1.5 ± 0.07^{b}
	Gum Arabic (n=26)	6.4 ± 0.1^{b}	1.51 ± 0.03^{bc}	1.9 ± 0.06^{c}
	Kaolin (n=26)	6.0 ± 0.1^{ab}	1.39 ± 0.03^{a}	1.2 ± 0.06^{a}
Sex	Male (n=66)	5.8 ± 0.08^{a}	1.4 ± 0.02^{a}	1.4 ± 0.04^{a}
	Female (n=62)	6.7 ± 0.08^{b}	1.5 ± 0.02^{b}	1.7 ± 0.04^{b}
Carrier Status	Non-Carriers (n=66)	5.5 ± 0.08^{b}	1.3 ± 0.02^{b}	49.1 ± 17
	Carriers (n=62)	7.0 ± 0.09^{a}	1.6 ± 0.02^{a}	49.2 ± 16

[1] Tissue lengths were taken immediately following excision and measured under fixed tension (8g). Data were analyzed using ANOVA, results are presented as mean ± SEM, and n = number of animals/treatment.

[2] Superscripts a, b, c, d denote differences ($P < 0.05$) per treatment, sex, or carrier status.

[3] Adjusted for metabolic body size ($kg^{3/4}$). Expressed as mm or mg/g of metabolic body size

SCFA Analysis: The millimolal (m*m)* concentrations of SCFA (umole/g cecal contents) are shown in Table 5. Dietary treatments affected both total SCFA and individual SCFA concentrations. For all three fermentable fibers, there were significantly greater concentrations of SCFA in cecal contents than for either the cellulose or kaolin treatments ($P < 0.0001$). The cecal contents from mice fed kaolin had the smallest concentration of acetate followed by cellulose. Propionate concentration was highest in mice fed the FOS and modified starch, whereas mice fed FOS and gum arabic had greater amounts of butyrate in the cecal contents. The concentration of valerate was highest in mice fed modified starch, and within this treatment, females produced significantly more valerate than males ($P < 0.0001$). Carrier status also affected both individual and total SCFA concentrations. For both acetate ($P < 0.01$) and total SCFA ($P < 0.03$), *Min*$^-$ produced higher concentrations than *Min*$^+$ mice. Carrier status also affected the branch-chain fatty acids isobutyrate ($P < 0.0001$) and isovalerate ($P < 0.0001$) with carriers producing higher amounts than non-carriers.

Table 6 shows the pH of cecal contents and molal proportions of individual SCFA for each dietary treatment. The FOS treatment yielded both higher propionate and butyrate levels. The lower total m*m* SCFA among all carriers (Table 5) was most likely due to a decrease in acetate production. The FOS and modified starch groups had significantly lower molal proportions of acetate than the other treatment groups. In the FOS group, there was a shift towards propionate and butyrate production, and towards propionate and valerate production in the modified starch group. The three fermentable fiber groups also had lower cecal pH values compared to controls (Table 6).

Table 5. Millimolal (umole/g) concentrations of individual SCFA per gram cecal contents in *Min*$^+$ and *Min*$^-$ mice fed different sources of fiber or an inert bulk (6g/100g diet)[1,2].

		mm						
		Acetate	**Propionate**	**Butyrate**	**Isobutyrate**	**Isovalerate**	**Valerate**	**Total**
Treatment	Cellulose (n=26)	37 ± 2.4^{ab}	6.3 ± 0.8^{a}	8.5 ± 0.9^{b}	1.6 ± 0.07^{b}	1.6 ± 0.05^{a}	1.7 ± 0.1^{a}	57 ± 3.7^{a}
	FOS (n=26)	43 ± 2.3^{bc}	13 ± 0.3^{b}	12 ± 0.9^{c}	1.7 ± 0.07^{b}	1.8 ± 0.05^{b}	1.8 ± 0.09^{ab}	74 ± 3.6^{b}
	Mod. Starch (n=24)	43 ± 3.0^{bc}	12 ± 1.0^{b}	8.6 ± 1.1^{b}	1.6 ± 0.09^{b}	1.7 ± 0.06^{b}	3.0 ± 0.1^{c}	70 ± 4.6^{b}
	Gum Arabic (n=26)	46 ± 2.4^{bc}	7.7 ± 0.8^{a}	12 ± 0.9^{c}	1.3 ± 0.07^{a}	1.6 ± 0.05^{a}	2.0 ± 0.1^{b}	70 ± 3.7^{b}
	Kaolin (n=26)	32 ± 2.3^{a}	7.0 ± 0.7^{a}	5.5 ± 0.8^{a}	1.6 ± 0.07^{b}	1.7 ± 0.05^{ab}	1.8 ± 0.09^{a}	49 ± 3.5^{a}
Sex	Male (n=66)	41 ± 1.5	9.6 ± 0.5	9.7 ± 0.6	1.7 ± 0.05^{b}	1.7 ± 0.03^{b}	1.9 ± 0.06^{a}	66 ± 2.3
	Female (n=62)	39 ± 1.7	8.9 ± 0.5	8.8 ± 0.6	1.4 ± 0.05^{a}	1.5 ± 0.04^{a}	2.2 ± 0.07^{b}	62 ± 2.5
Carrier Status	Non-Carrier (n=66)	43 ± 1.5^{b}	9.9 ± 0.5	9.6 ± 0.6	1.5 ± 0.05^{a}	1.6 ± 0.03^{a}	2.1 ± 0.06	68 ± 2.3^{b}
	Carrier (n=62)	37 ± 1.7^{a}	8.6 ± 0.5	8.9 ± 0.6	1.7 ± 0.05^{b}	1.8 ± 0.4^{b}	2.0 ± 0.07	60 ± 2.5^{a}

[1] Statistical analyzes were performed using ANOVA, results are presented as mean ± SEM, and n = number of animals per treatment. Data were considered significant at $P < 0.05$. SCFA were measured from cecal contents as described in the Materials and Methods. There was a significant ($P < 0.01$ and $P < 0.03$) carrier effect for acetate and total SCFA (*Min*$^-$ > *Min*$^+$), and for isobutyrate and isovalerate (*Min*$^+$ > *Min*$^-$, $P < 0.006$, $P < 0001$). Males had higher concentrations of isobutyrate and isovalerate ($P < 0.0001$, $p<0.0006$), whereas females produced more valerate in the modified starch group ($P < 0.0001$).

[2] Superscripts a, b, c, d denote differences ($P < 0.05$) per treatment, sex, or carrier status.

Table 6. pH of cecal contents and molar proportions of SCFA in *Min*$^+$ and *Min*$^-$ mice fed different sources of fiber or an inert bulk (6g/100g diet)[1,2].

			Molal %					
		pH of cecal contents	**Acetate**	**Propionate**	**Butyrate**	**Isobutyrate**	**Isovalerate**	**Valerate**
Treatment	Cellulose (n=26)	8.1^c	65 ± 0.9^b	11 ± 0.5^a	15 ± 0.8^b	2.9 ± 0.2^b	2.9 ± 0.2^a	3.1 ± 0.2^b
	FOS (n=26)	7.6^b	59 ± 0.8^a	18 ± 0.5^c	16 ± 0.8^b	2.4 ± 0.2^b	2.5 ± 0.2^a	2.5 ± 0.2^a
	Mod. Starch (n=24)	7.4^a	61 ± 1.1^a	17 ± 0.7^c	12 ± 1.0^a	2.4 ± 0.2^b	2.6 ± 0.2^a	4.7 ± 0.3^c
	Gum Arabic (n=26)	7.2^a	65 ± 0.9^b	11 ± 0.5^a	16 ± 0.8^b	2.1 ± 0.2^a	2.6 ± 0.2^a	3.1 ± 0.2^b
	Kaolin (n=26)	8.2^c	64 ± 0.8^b	15 ± 0.5^b	11 ± 0.8^a	3.4 ± 0.2^c	3.5 ± 0.2^b	3.7 ± 0.2^b
Sex	Male (n=66)	7.8^b	62 ± 0.6	15 ± 0.3	14 ± 0.5	2.9 ± 0.1^b	3.0 ± 0.1^b	3.2 ± 0.1
	Female (n=62)	7.7^a	63 ± 0.6	14 ± 0.4	14 ± 0.6	2.4 ± 0.1^a	2.7 ± 0.1^a	3.6 ± 0.2
Carrier Status	Non-Carriers (n=66)	7.8	64 ± 0.5^b	14 ± 0.3	14 ± 0.5	2.3 ± 0.1^a	2.4 ± 0.1^a	3.3 ± 0.1
	Carriers (n=62)	7.7	62 ± 0.6^a	15 ± 0.4	14 ± 0.6	3.0 ± 0.1^b	3.2 ± 0.1^a	3.5 ± 0.2

[1] Statistical analyzes were performed using ANOVA, results are presented as mean ± SEM, and n = number of animals per treatment. Data were considered significant at $P < 0.05$. There was a significant ($P < 0.01$) carrier effect for % acetate (*Min*$^-$ > *Min*$^+$), and for isobutyrate and isovalerate (*Min*$^-$ < *Min*$^+$, $P < 0001$). Males had higher molar % of isobutyrate and isovalerate ($P < 0.003$, $P < 0.04$), and females in the modified starch group had higher molar proportions of valerate than males ($P < 0.0001$).

[2] Superscripts a, b, c, d denote differences ($P < 0.05$) per treatment, sex, or carrier status.

VIII. DISCUSSION

In this study, the effect of three highly fermentable fiber sources on tumor development, SCFA production, and tissue parameters were examined in mice with a genetic predisposition to colon cancer. It has been previously reported that resistant starch and FOS are protective against colon carcinogenesis in some animal models (Pierre, 1997; Thorup, 1995). Pierre et al (1997) showed a lower number of total and small colon tumor numbers in *Min*$^+$ mice fed a 6% FOS diet. This effect, however, was significant only when compared to a 2% fiber control (Pierre, 1997). In the present study, there was no effect of dietary treatment on tumor development in either the small intestine, cecum, or colon. Differences between this study and that of Pierre et al (1997) may have been due both to the level of fat in the diets and the length of dietary treatment. Wasan et al (1997) showed that increasing the level of fat from 3 to 15% of the diet significantly increased tumor numbers in both the small and large intestine in *Min*$^+$ mice. In this study, a 15% (wt/wt) fat diet was used compared to an 8% fat diet in the study by Pierre et al (1997). Also, in the present study, mice were fed for a total of 10 weeks and were sacrificed at 98 days of age, compared to 42 and 84 days, respectively, in the study by Pierre et al (1997). Although tumor multiplicity is thought to be established by about 70 days of age in *Min*$^+$ mice, the size of detectable tumors, especially in the colon, could have been influenced by age (Shoemaker et al, 1995). The length of the study may have also confounded possible treatment effects due to weight loss, which was significant for males in this study at 7 weeks and 8 weeks of treatment for females.

There has been some concern that soluble fibers, through fermentation to SCFA, may enhance colon carcinogenesis (Jacobs, 1987). Both a decrease in luminal pH and an increase in SCFA production stimulate cellular proliferation in both the cecum and colon, with butyrate being highly correlated to site-specific proliferation indices in the distal colon (Lupton and Kurtz, 1993). The effect of SCFA on epithelial growth in the normal colon, however is confined to the lower crypt compartments (Velaquez et al, 1996). In contrast, factors which stimulate hyperproliferation in the upper crypt, such as secondary bile acids, promote colon carcinogenesis. Although cellular proliferation was not measured in the present study, the fermentable fibers all significantly increased the molal concentrations of SCFA in the cecal contents of *Min*$^{+}$ mice. However, increased concentrations of SCFA were not associated with an increase in polyp incidence or size in either the cecum or colon, the primary sites of fermentation.

The effect of fermentable fibers on tissue parameters and SCFA concentrations is noteworthy. Mice fed the fermentable fibers had significantly longer SI and colon lengths and heavier cecum wet weights than controls. Small intestinal growth is stimulated both by the presence of viscous fibers in the small intestine and by SCFA-mediated systemic effects. Gee et al (1996) examined the effect of viscosity and fermentation on small intestinal mucosal cell proliferation in rats. Compared to both cellulose and lactulose controls, the more viscous polysaccharides (hydroxypropylmethylcellulose and guar gum) significantly increased small intestinal lengths in rats in a manner which was independent of their fermentation (Gee et al, 1996). Goodland et al (1995) examined trophic effects of fiber in the small intestine, stomach, and colon in both germ-free and conventional rats. They reported that, compared to

fiber-free controls, fiber was trophic to both the small intestinal muscle and gastric epithelium. In contrast, fiber stimulated crypt cell production in both the ileum and colon only in conventional rats. Because fermentation was necessary, it was suggested by the authors that this effect was due to SCFA production (Goodland et al, 1995).

In this study, the three fermentable fibers used were not highly viscous, and cellulose and kaolin were included as poorly or non-fermentable bulk sources. After adjusting for metabolic body size, tissue lengths in all three fermentable fiber groups was different than controls. Because this effect was seen only in the fermentable fiber treatment groups, this suggests that SCFA likely are more trophic to the small intestine than a bulk source alone. There were also significant differences in colon lengths between the fermentable fiber groups and controls. Although the SCFA concentrations were not measured throughout the colon in this study, infusion studies show that SCFA are locally-acting trophic agents to the colonic epithelium (Sakata, 1982). Although the three fermentable fiber sources used in this study are thought to be rapidly fermented, the trophic effect on colon length is suggestive that the luminal SCFA were present distally from the cecum. The weight of empty cecums were also significantly heavier in the fermentable fiber groups than in controls. This effect has been reported in both rats and mice fed fermentable fiber sources and is likely due to an increase in proliferation or increase in cecum size, both from a decrease in pH and an increase in SCFA concentrations (Lupton and Kurtz, 1993).

The increased millimolal concentrations of total and individual SCFA in cecal contents of mice fed fermentable fibers supports the view that fermentation patterns are alterable by dietary modifications. Kaolin, the non-fermentable bulking agent used as a

control in this study resulted in the lowest overall SCFA concentrations in the cecum. The most significant differences were in the major SCFA. FOS yielded greater amounts of both propionate and butyrate at the expense of acetate. This pattern is in agreement with findings from Bergeen et al (1993).

Fermentation of resistant starch and gum arabic resulted in higher propionate and butyrate concentrations, respectively, than controls. Starches have been shown to yield relatively large butyrate concentrations *in vitro* (Cummings, 1991). However, several studies suggest that the type of resistant starch may influence the proportions of propionate and butyrate produced (Bergeen, 1993, Cummings et al, 1986). The starch source used in this study was chemically modified to be resistant to amylases in the small intestine and the precise composition was not known because of propriety reasons. However, based on the molal proportions of SCFA in this study, the modified starch source used is consistent with fermentation patterns previously observed from wheat and maize starch (Bergreen, 1993; Cummings et al, 1996). Valerate production was also significantly higher in the modified starch group, but the significance of this is not known.

Carrier status also influenced fermentation patterns. There were smaller concentrations of acetate and total SCFA and a significant increase in the concentrations of isovalerate and isobutyrate. Production of the branch-chain fatty acids isobutyrate and isovalerate have been previously shown to increase with protein fermentation (Macfarlane, 1992). Severe anemia due to gastrointestinal bleeding is common in *Min*$^{+}$ mice, and is likely to have increased endogenous protein sources to the colon accounting for these differences.

IX. SUMMARY AND CONCLUSIONS

This study demonstrated that fermentation patterns in mice are influenced by differences in dietary fibers and that fermentation has trophic effects to the small and large intestines and to the cecum. The increase in cecum weight was most likely due to increased concentrations of SCFA in the cecal contents. The increase in SI lengths may be due to systemic events stimulated by SCFA. Since the differences in butyrate concentrations occurred in the cecum whereas the majority of neoplasms occurred in the SI and distal colon, this study could not adequately evaluate the presumptive anti-tumor effect of butyrate on intestinal tumorigenesis. However, fermentation did not alter tumor multiplicity or size in *Min*$^{+}$ mice.

X. RECOMMENDATIONS FOR FUTURE RESEARCH

The *Min*$^{+}$ mouse model was used in this study because genetically, it is similar to the human condition of FAP. Tumors occur spontaneously and there is no need to administer carcinogens. Thus, this genetic model of human colon cancer eliminates the variability associated with the type, amount, and method of administration of chemical carcinogens required to induce colon cancer in normal rodents. Although polyp formation in the colon (approximately 1/mouse) was observed in this study, the majority of adenomas were distributed throughout the small intestine. The proposed method of chemoprevention was through short chain fatty acid production, which occurs primarily in the cecum and colon. Although there were clearly systemic trophic effects in the small intestine with fermentable fibers, short chain fatty acids are not found in high concentrations in portal or systemic blood, and would not likely act by this mechanism to inhibit tumor formation in the small intestine. SCFA, if protective most likely act from direct luminal exposure in the colon. Therefore, with a lower colon tumor multiplicity per animal and the relatively small number of carriers per treatment in this study, it may have been difficult to detect differences, if any, in colon tumor multiplicity and size. Future studies specifically examining fermentation and colon cancer with this model should consider more carriers per treatment and attempt to suppress tumor growth in the small intestine, possibly by reducing the level of fat in the diet.

Although we showed that the type of fiber fed to the mice in this study resulted in both different concentrations and proportions of SCFA in the cecum, it was unclear as to whether this effect continued throughout the large intestine. FOS, resistant starch, and

gum arabic are all highly fermentable and could have been completely fermented before reaching the distal colon, where most polyps developed. A combination of fiber sources that delay fermentation to the distal bowel should be considered in future studies.

It is still unresolved as to whether SCFA, in particular butyrate, are protective against colon carcinogenesis *in vivo*. Although some evidence supports a protective role for fermentable fibers that yield high butyrate concentrations, other studies including ours did not find an effect (Pierre et al, 1997). Thus, it also remains possible that higher levels of dietary fiber, continual luminal exposure to butyrate, or butyrate concentrations above which can be achieved through dietary treatment may be needed for butyrate to be an effective chemopreventive agent in the colon. Future studies should be designed with these limitations in mind.

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