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Pectin Does Not Inhibit Intestinal Carcinogenesis in APC-Deficient Min/+ Mice

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APC-germline mutation creates predisposition for intestinal tumorigenesis. $APC^{Min/+}$ mice, developing tumors preferentially in the small intestine and only minimally in the colon, were fed pectin-enriched diets (10% galacturonan; degree of methoxylation = 37.0 and 70.4%) or standard diet. Pectins used in the present study do not inhibit intestinal tumorigenesis and rather accelerate it in $APC^{Min/+}$ mice. Both pectins exhibited prebiotic effects associated with high fermentative formation of acetate but producing low butyrate. The differences of the short-chain fatty acid concentrations between cecum and colon and those between colon and feces were larger than expected and increased with cancer progression, indicating an inhibition of butyrate absorption. Pectins transported more bile acids toward the colon than the standard diet and caused a higher generation of secondary bile acids despite lower pH values. Overexpression of COX-2 resulted in lower antioxidative capacity, thus promoting cancer. Apoptosis increased in hyperplasia but decreased in late adenomas. When biological modular design principles are taken into consideration, it can be expected that pectin also reinforces colorectal tumorigenesis of patients suffering from APC gene defects.

KEYWORDS: Pectin; anticarcinogenic effect; $APC^{Min/+}$ mouse; butyrate absorption; bile acid; Tunica mucosa; antioxidative capacity

INTRODUCTION

Diet-mediated prevention that lowers the incidence of cancer is much more effective and cheaper than the development of new therapeutic strategies. The suppression of colorectal tumor development by intake of food containing fermentable dietary fiber exemplifies this concept. Resistant starch type 3 suppresses 1,2-dimethylhydrazine-induced colon carcinogenesis totally in rats (*1*), whereas other prebiotics, among them pectin, may be less effective (*2–6*).

The specificity of these anticarcinogenic effects results from strong interactions between intestinal microbial activities and the metabolism of the colonocytes (*7*). They are directly related to butyrate, an end product of carbohydrate fermentation in the large bowel. Butyrate is an essential substrate for the energy metabolism of the epithelial cells and mediates effects on the expression pattern of distinct genes (*8*).

The main objective of the present paper was to examine whether a pectin-enriched diet can be an efficient cancer chemoprevention approach for high-risk populations. This was done by feeding $APC^{Min/+}$ mice diets enriched with either low-methoxylated (LM) or high-methoxylated (HM) pectins and comparing the results to those with a standard control diet.

Pectins are most abundant in fruits such as citrus fruits, apples, and berries, vegetables, and other plant materials. Pectin polysaccharides occurring in the primary cell wall of dicotyledonous plants belong to the most complex natural macromolecules. They are unbranched (homogalacturonan), have a linear backbone with side chains (xylogalacturonan, rhamnogalacturonan I, arabinogalactan, arabinan), or are highly branched (rhamnogalacturonans II, arabinogalactan II) and are most likely covalently bound to each other (*9*). Therefore, pectin polysaccharides in the plant cell wall are more or less insoluble, whereas isolated soluble commercial pectin consists mainly of long linear chains of α-(1→4)-glycosidic linked D-galacturonic acid units (homogalacturonan) interrupted by α-(1→2)-linked L-rhamnose units. The carboxyl groups of pectin are partly esterified with methanol. Pectins in fruits are mostly high methoxylated, whereas pectins in vegetables are mostly middle- or low-methoxylated (*10*). Many functional and physiological properties of pectin, such as viscosity, rheological behavior, gel formation, interaction with metal cations and steroids and also its enzymatic or bacterial degradation, depend on the degree of methoxylation. Isolated pectins are broadly applied in the food industry (gelling and thickening agents) and in pharmaceutical preparations (drug-delivering formulations).

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Isolated pectins, modified pectins, and pectic oligosaccharides are used as prebiotic food products that beneficially affect the host.

It has been hypothesized that LM and HM pectins exhibit anticarcinogenic properties by suppression of endotoxin-induced proinflammatory response (*11*) and as antagonist to galectin-3, due to the many galactoside residues of pectins. Galactin-3 mediates cell−cell adhesion and cell−matrix interactions whereby multiple signal cascades will also be activated. It is also a binding component of β-catenin regulating the Wnt/β-catenin signaling pathway, which plays an essential role in tumorigenesis caused by APC gene defects (*12, 13*). Galactin-3 further stimulates angiogenesis, a basic requirement for tumor growth (*14*). Tumor development is usually associated with changes in the pattern of cell surface carbohydrates (*15*), and the adenoma−carcinoma sequence (*16*) appears to operate in the same manner in the small intestine as in the large one. Therefore, $APC^{Min/+}$ mice (multiple intestinal neoplsia), a murine model of familial adenomatous polyposis (FAP) that predisposes to intestinal polyposis, was used.

The used $APC^{Min/+}$ mouse model has several advantages compared to many carcinogen-induced tumor models, because it is a clinically relevant model characterized by a defined hereditary mutation and, therefore, suitable for testing chemopreventive compounds in the early stages of carcinogenesis. "Min" defines a dominant heterozygous nonsense mutation of the murine APC gene converting codon 850 from leucine (TTG) to a stop codon (TAG). This germline mutation causes spontaneous intestinal neoplasia. $APC^{Min/+}$ mice develop more than 50 adenomas throughout the intestinal tract and rarely survive beyond 120 days (*17*). In contrast to humans, in $APC^{Min/+}$ mice, most of the tumors are located in the small intestine. How pectin-enriched food may influence the carcinogenesis is still unknown.

In the present study, the effects of citrus pectin with two different degrees of methoxylation have been investigated with regard to the development of tumors and cancer-related parameters.

MATERIALS AND METHODS

Pectins. LM and HM citrus pectins without additives were obtained from CP Kelco ApS, Lille Skensved, Denmark. The galacturonan content of the pectins was determined by the *m*-hydroxydiphenyl method, and methyl ester groups were analyzed by the chromotropic acid method as described previously (*18*). The intrinsic viscosity [η] was determined in 0.155 M NaCl (HM pectin) or in 0.05 M NaCl/0.005 M sodium oxalate (LM pectin) at 25.0 °C and pH 6.0 using an Ubbelohde viscosimeter as described previously (*18*). The intrinsic viscosity is related empirically to the molecular weight by the Mark–Houwink relation.

The LM pectin had the following characteristics (mean values, $n = 6$): galacturonan, 70.33%; degree of methoxylation, 37.0%; intrinsic viscosity, 441.0 mL/g galacturonan. The HM pectin had the following characteristics: galacturonan, 68.94%; degree of methoxylation, 70.4%; intrinsic viscosity, 782.0 mL/g galacturonan.

Animal Experiment. Female C57/BL/6J-Min/+ (24) and 8 healthy control mice (3 Min+/+ and 5 Balb c mice) (all 5−6 weeks old) (Bomholtgard Breeding and Research Center Ltd., Bomholtgard, Denmark) were used for the experiment. Body weight on arrival was 12.3 ± 1.45 g. All Min/+ mice were tested for heterozygosity of the APC mutation. Animals were divided into healthy control group and three diet groups of Min/+ mice. **Table 1** shows the composition of the used diets. All animals were fed semisynthetic standard diets. The pectin-enriched diets contained 10% galacturonan. The mice were housed in stainless steel cages with splint bedding under a 12/12 h light/dark cycle and had free access to food and water. Mice were fed for 56 days and carefully watched for clinical symptoms. Body weights were registered every three days.

Table 1. Composition of the Diets Used

ingredient	g/kg of diet		
	control diet	LM pectin diet	HM pectin diet
wheat starch[a]	630	488	485
pectin	0	142[b]	145[c]
casein[d]	200	200	200
sunflower oil[e]	50	50	50
microcrystalline cellulose[f]	50	50	50
mineral mixture[g]	50	50	50
vitamin mixture[h]	20	20	20

[a] Waxy maize starch (National Starch and Chemical Co., Bridgewater, NJ). [b] Low-methoxylated (LM) pectin (CP Kelco ApS, Lille Skensved, Denmark) with 70.33 g of galacturonan/100 g; final pectin content = 10 g/100 g of diet. [c] High-methoxylated (LM) pectin (CP Kelco Aps) with 68.94 g of galacturonan/100 g; final pectin content: = 10 g/100 g of diet. [d] Dauermilchwerk Peiting GmbH, Landshut, Germany. [e] Thomy GmbH, Karlsruhe, Germany. [f] Rettenmeier GmbH, Ellwangen, Germany. [g] Altromin GmbH, Lage, Germany. Composition of the mineral mixture (g/kg): Ca, 185; P, 145; K, 140; Na, 88; Cl, 72; S, 34; Mg, 16; Fe, 4; Mn, 2; Zn, 0.6; Cu, 0.16; F, 0.08; I, 0.008; Se, 0.004; Co, 0.002. [h] Altromin GmbH. Composition of vitamin mixture (mg/kg): vitamin A, 225; vitamin E, 8000; vitamin K_3, 10; vitamin B_1, 1000; vitamin B_2, 1000; vitamin B_6, 750; vitamin B_{12}, 1.5; niacin, 2500; panthotenic acid, 2500; folic acid, 500; biotin, 10; choline chloride, 50000; *p*-aminobenzoic acid, 5000; myo-inositol, 5000; vitamin C, 20; methionine, 3500 as well as 500 IU of vitamin D_3.

At the end of the experiment, animals were anesthetized and killed by decapitation, and autopsies was performed. Intestine, cecum, liver, spleen, and kidneys were removed, and weights as well as noticeable features such as pathological behavior of animals as well as macroscopic changes of tissues, organs, and intestinal contents were recorded. Defined specimens of the organs were fixed in 4% paraformaldehyde and prepared for histology and immunohistochemistry as described in ref *1*. The small and large bowels were cut open longitudinally, washed in ice-cold phosphate-buffered saline, fixed flat in 4% paraformaldehyde, and stained with hematoxylin for the evaluation of tumors. Colorectal and selected small intestinal tumors were dissected and processed for histology and immunohistochemistry. Contents of ileum, cecum, and large intestine and feces were collected, prepared for analysis, and immediately stored at −20 °C.

The experimental protocol was performed according to international and national guidelines. All treatments and diets were formally approved by the Senatsverwaltung für Soziales and Gesundheit des Landes Berlin, Berlin, Germany.

Histology, Immunohistochemistry, and Microscopy. Sections (2 μm) were dewaxed in toluol and rehydrated. For histological examination sections were stained with hematoxylin and eosin (Sigma, St. Louis, MO) and mounted in Entellan (Merck, Darmstadt, Germany). Immunohistochemistry was performed as described before (*1*). Antigen unmasking was carried out by heat treatment. Endogenous peroxidase activity and unspecific binding were blocked. Primary antibody rabbit anti-COX-2 [polyclonal antibody SC-1745 COX-2 (C-20), Cayman Chemical Co., Ann Arbor, MI] was applied overnight at 4 °C. A biotin-conjugated goat anti-rabbit IgG followed by a streptavidin–biotin−horseradish peroxidase complex (StreptABComplex/HRP, DakoCytomation, Glostrup, Denmark) and diaminobenzidine (DakoCytomation) was used for visualization. Negative controls were performed by omitting the primary antibody. Under these conditions, no immunoreactivity was detectable. Cell death was detected by Klenow kit (Calbiochem, San Diego, CA) according to the manufacturer's instructions.

Tumor scoring was performed using a stereo microscope (SZH10, Olympus, Hamburg, Germany). All further microscopic investigations and morphometric measurements were done with the aid of a light microscope (Eclipe E1000, Nikon, Düsseldorf, Germany) in combination with a camera (CCD 1300CB, Vosskühler, Osnabrück, Germany) and digital analysis software Lucia Image 4.61 (Nikon).

Analytical Methods. Short-chain fatty acids (SCFA) were analyzed in the intestinal contents and in freshly taken feces by gas chromatography as described previously (*19*).

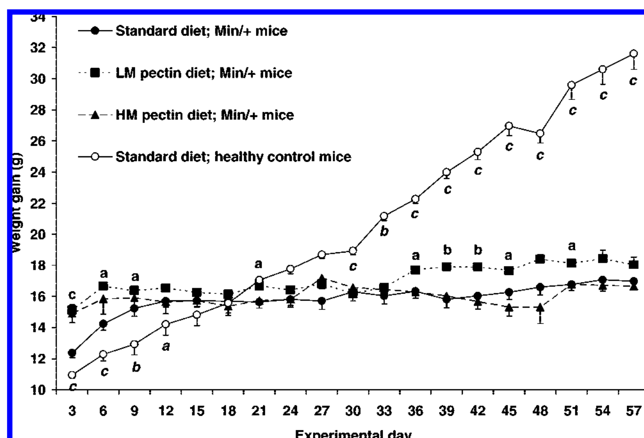

Figure 1. Weight progression of Min/+ mice fed the standard diet or diets containing low-methoxylated (LM) or high-methoxylated (HM) pectin as well as of healthy control mice fed the standard diet for 56 days. Values are means ± SEM; $n = 3$–9. Mean values were significantly different from Min/+ mice fed the standard diet: a, $P < 0.05$; b, $P < 0.005$; c, $P < 0.001$.

The extraction and purification procedures of freeze-dried fecal materials and analysis of bile acids by HPLC using precolumn derivatization with 4-bromomethyl-7-methoxycoumarin and fluorescence detection are given elsewhere (*18*).

To determine galacturonan in feces, 10 mg of lyophilized material was extracted with 1 mL of 50% aqueous ethanol (v/v) for 24 h at 20 °C. After centrifugation (15 min at 6000*g* and 4 °C), the concentration of low molecular weight pectin was estimated colorimetrically in the supernatant using the *m*-hydroxydiphenol method as described previously (*18*).

For reticulocyte enumeration, blood films were prepared, fixed with cold methanol, and stained with brilliant kresyl blue (Sigma, Deisenhofen, Germany). Cells containing stained precipitates were counted. Results are given as percentage of 1000 red blood cells examined (*20*).

A chemiluminescence method was used to determine the antioxidative properties of blood. Ascorbic acid and Trolox were used as calibration standards. The photoinduced chemiluminescence method (LS-50B Luminescence Spectrophotometer Perkin-Elmer, Ueberlingen, Germany) was performed as described earlier (*21*) for water- and lipid-soluble substances.

Statistical Analysis. Statistical analysis was performed using Statistical Package for Social Sciences software SPSS 11.0 (SPSS Inc., Chicago, IL). All values are given as mean values and standard deviation of the means (SEM). Data were analyzed by one-way ANOVA, and differences between the pectin groups and the control group were evaluated by Dunnett's *t* test and Dunnett's T3 test for multiple post hoc comparisons. Differences with $P < 0.05$ were considered to be significant.

RESULTS

Food Intake and Weight Progression. The $APC^{Min/+}$ mice consumed 2.76 ± 0.79 g of LM-pectin-containing diet, 3.44 ± 0.79 g of HM-pectin-containing diet, or 2.44 ± 0.19 g of standard diet per day, respectively. In contrast to the healthy control mice fed the standard diet, food intake of the LM and HM pectin and the standard diet groups of $APC^{Min/+}$ mice decreased continuously in the last 4 weeks of the experimental period (data not shown). The growth of Min/+ mice, independent of the diet applied, was also reduced. Above 16 g of body weight, growth retardation was obvious in Min/+ mice (**Figure 1**). During the 56 experimental days, the weight differences were 3.4 g for $APC^{Min/+}$ mice fed HM pectin, only 1.8 g for the LM pectin group, and 4.3 g for animals fed the standard diet. One LM-pectin-fed mouse died at day 50 by a tumor-caused ileus. Its body weight was 13 g. Body weights of healthy control mice increased in the same period to approximately 30 g, corresponding to a weight gain of 13 g.

Digesta and Tissue Weights, Fecal Output of Pectin. Table 2 summarizes the diet-mediated influence on the intestine, cecum, liver, heart, kidney, and spleen. As expected, both pectins increased intestinal and cecum weights nearly in the same manner, mostly due to their higher contents. The maximal increase was found in cecal contents, where the highest rate of fermentation occurred. Attention is directed to the fact that Min/+ mice of all three diet groups showed increased spleen weights. Liver weights rose only slightly in Min/+ mice. The weights of kidneys and hearts did not differ significantly among all of the tested diet groups.

Feces contained 3.14 ± 0.32 and 3.37 ± 0.32% low molecular weight galacturonan on a dry matter basis at day 3, if the mice were given diets with LM and HM pectin, respectively. These concentrations decreased to 0.83 ± 0.08 and 1.21 ± 0.07% at day 7 and to 0.24 ± 0.02 and 0.31 ± 0.01% at day 14, when the concentration of pectin was significantly lower in the LM pectin group ($P < 0.05$; $n = 4$–12). At the end of the experiment, feces were galacturonan-free. These results show that the Min/+ mice were not able to ferment the applied amount of pectin completely in the initial phase of the experiment. The observed effects may be due to both an adaptation process and a lower food uptake. A reason for this effect is that the key enzyme for the depolymerization of pectin, pectate lyase, prefers low-methoxylated pectins as substrates.

Tumor Data. No polyps were found in animals of the healthy control group, but all Min/+ mice developed tumors. **Figure 2** shows a colon polyp of a Min/+ mouse fed LM pectin. The pictures illustrate (left) the closely arranged vessels in the polyp with a predisposition to bleeding. From the size of the longitudinally cut polyp (right) it is obvious that such a big tumor can cause an ileus. Most of the polyps were located in the small intestine. Less than 2% of tumors occurred in the colon and rectum (**Table 3**). None of the two pectin-enriched diets inhibited intestinal tumor growth in Min/+ mice compared to pectin-free fed APC-deficient animals. In contrast, tumorigenesis started earlier, and colorectal tumors had the tendency to be larger in pectin-fed animals. In the small intestine, tumor sizes were generally smaller. Their diameters varied from 0.30 to 2.70 mm in the small intestine and from 0.40 to 3.15 mm in the large intestine. The number of tumors increased in turn from proximal to distal in both small and large intestine, respectively. In the small intestine, 20% of tumors were located in the duodenum, 47% in the jejunum, and 33% in the ileum. In the large bowel, most of the tumors were identified in the distal part. On average, 80% of tumors occurred in the colon descendens and in the rectum; 20% were identified in the proximal part of the colon. In the pectin-fed animals, all polyps of the large intestine were classified as adenocarcinomas, but in Min/+ mice fed the standard diet, only 80% of the polyps were adenocarcinomas and 20% were adenomas.

Colonic dysplasia pattern did not differ significantly in the three diet groups of the $APC^{Min/+}$ mice; the number of dysplasias were 19.0 ± 3.1 (LM pectin), 16.3 ± 1.2 (HM pectin), and 18.7 ± 2.4 (standard diet). The number of dysplasias was 9−10 in the small and 1−2 in the large intestine. A pectin-enriched diet was not suitable for preventing tumorigenesis in APC gene deficient mice.

Distribution of Lymph Follicles Resembling That of Tumors. Often lymph follicles were located neighboring tumors. In the small intestine, 9.7 ± 0.4 lymph follicles were present

Table 2. Weights of Organs of Min/+ Mice Fed the Standard Diet or Diets Containing Low-Methoxylated (LM) or High-Methoxylated (HM) Pectin at the End of the Experiment (Day 56)

	standard diet		LM pectin diet[a]	HM pectin diet[a]
	control mice	Min/+	Min/+	Min/+
spleen (mg)	57.5 ± 1.3	145.7 ± 28.3	205.2 ± 21.5a	185.0 ± 14.0a
liver (mg)	1011.4 ± 28.8	1062.1 ± 73.7	925.5 ± 48.8	1056.6 ± 44.3
heart (mg)	78.8 ± 2.5	80.0 ± 5.0	71.8 ± 2.3	73.8 ± 2.9
kidneys (mg)	212.5 ± 5.0	212.2 ± 14.6	209.9 ± 4.9	195.8 ± 6.3
total intestine (mg)	1108.9 ± 38.2	1267.1 ± 115.6	2325.1 ± 151.3a	2188.5 ± 139.7a
cecum (mg)	177.3 ± 9.9	180.0 + 22.7	542.2 ± 66.8a	559.0 ± 64.2a

[a] Values are means ± SEM; $n = 3$–10. Mean values were significantly different from Min/+ group fed standard diet: a, $P < 0.001$.

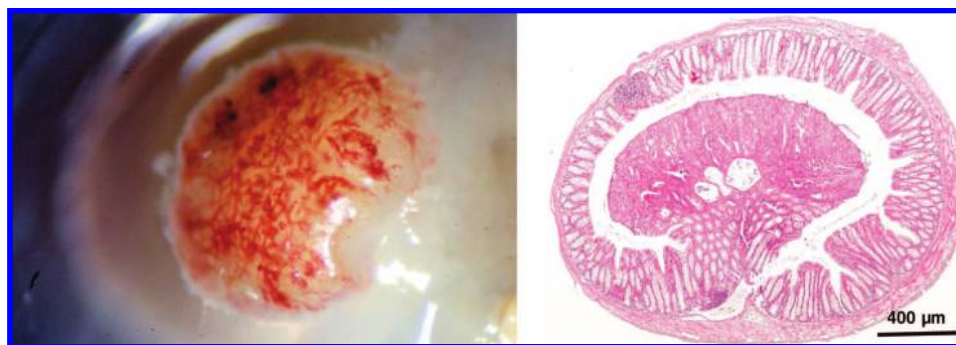

Figure 2. Colon polyps in Min/+ mouse fed the low-methoxylated pectin-containing diet for 56 days: histological picture of a colon polyp with distinct vessel pattern (left); longitudinal cut pedunculated polyp (hematoxylin-eosin staining) (right).

Table 3. Tumor Number and Size in Min/+ Mice Fed the Standard Diet or Diets Containing Low-Methoxylated (LM) or High-Methoxylated (HM) Pectin at the End of the Experiment (Day 56)

	tumor number		tumor size[a] (mm)	
diet	small intestine	colon	small intestine	colon
standard	70.0 ± 11.2	1.17 ± 0.12	1.12 ± 0.41	1.41 ± 0.77
LM pectin	69.5 ± 15.0	1.00 ± 0	1.30 ± 0.40	2.98 ± 0.40
HM pectin	79.6 ± 38.1	0.80 ± 0.61	1.10 ± 0.40	2.14 ± 0.24

[a] In all, the size of 1038 tumors was measured.

Table 4. Effect of Low-Methoxylated (LM) or High-Methoxylated (HM) Pectin on the Antioxidative Capacity in Plasma of Min Mice during Intestinal Carcinogenesis

	nmol of ascorbic acid turnover/L[a]			
	standard diet		LM pectin diet	HM pectin diet
day	control mice	Min/+ mice	Min/+ mice	Min/+ mice
0	178.0 ± 10.9	148.7 ± 9.9	148.6 ± 10.3	151.7 ± 10.6
27	nd	125.9 ± 6.1	nd	nd
34	nd	108.2 ± 3.8	91.9 ± 3.6a	91.5 ± 3.9a
56	151.9 ± 4.5b	57.4 ± 8.4	50.4 ± 3.6	44.7 ± 5.4

[a] Values are means ± SEM; $n = 3$–10. Mean values were significantly different from Min/+ group fed standard diet: a, $P < 0.05$; b, $P < 0.001$; nd, not determined.

in Min/+ mice fed the standard diet, 10.1 ± 0.3 lymph follicles were identified in the HM pectin group, and 11.7 ± 0.6 lymph follicles were found in the LM pectin group ($P = 0.078$ vs Min/+ mice fed the standard diet); most of them were detected in the jejunum. In control mice fed the standard diet, 7.7 ± 0.3 lymph follicles were found in the small intestine ($P < 0.05$ vs Min/+ mice fed the standard diet). In the large bowel, 16.2 ± 6.8 lymph follicles occurred in Min/+ mice fed the standard diet, 12.5 ± 7.6 in the HM pectin group, and 15.0 ± 0.0 in the LM pectin group. Approximately 90% of these lymph follicles occurred in the distal colon and only 10% in the proximal part of the large intestine.

Intestinal Bleeding. With progression of carcinogenesis, bleeding from polyps appeared, coloring the intestinal content dark violet. This finding correlated with the count of reticulocytes in blood and the increased spleen weights (**Table 2**). An enormous increase of red pulpa was detected histologically in spleen sections of Min/+ mice of all diet groups. The number of reticulocytes was 3.24 ± 0.49% in the blood of healthy control mice and increased to 22.4 ± 0.61% in the blood of HM-pectin-fed Min/+ mice, to 25.3 ± 0.80% in LM-pectin-fed animals, and to 24.2 ± 0.7% in the group fed the standard diet. All blood pictures of Min/+ mice were characterized by anisocytosis.

Oxidative Load and Apoptosis. The antioxidative capacity in the plasma was diminished in Min/+ mice fed the pectin-enriched or standard diets (**Table 4**). The antioxidative capacity decreased further in the course of the experimental period due to the increasing oxidative load of the organism with progression of carcinogenesis. The lowest antioxidative capacity with a value of 21.3 nmol of ascorbic acid turnover/L of plasma was detected in the LM-pectin-fed Min mouse suffering from an ileus caused by large polyps.

In tissues from healthy mice, a very low expression of COX-2 was detected. Immunoreactive positive cells were mainly found in the upper crypt region and in the extracellular matrix layer. COX-2 overexpression was demonstrated in $APC^{Min/+}$ mice of all three diet groups in the morphological intact intestinal wall as well as in the neoplasias (**Figure 3A**). Most cells from both the epithelial layer and the underlaying submucosa showed anti-COX-2 staining. Also, many endothelial cells convening the submucosal vessels indicated a positive reaction (**Table 5**).

In unaffected tissues from healthy animals, the typical distribution of apoptotic cells was found on the top of the crypt. In $APC^{Min/+}$ mice fed the HM pectin, the LM pectin, or the standard diet, the apoptotic activity was increased during the stages of early hyperplasia and adenoma. The malignant transformation into late adenomas and carcinomas was accompanied by a loss of apoptotic cells together with a decrease

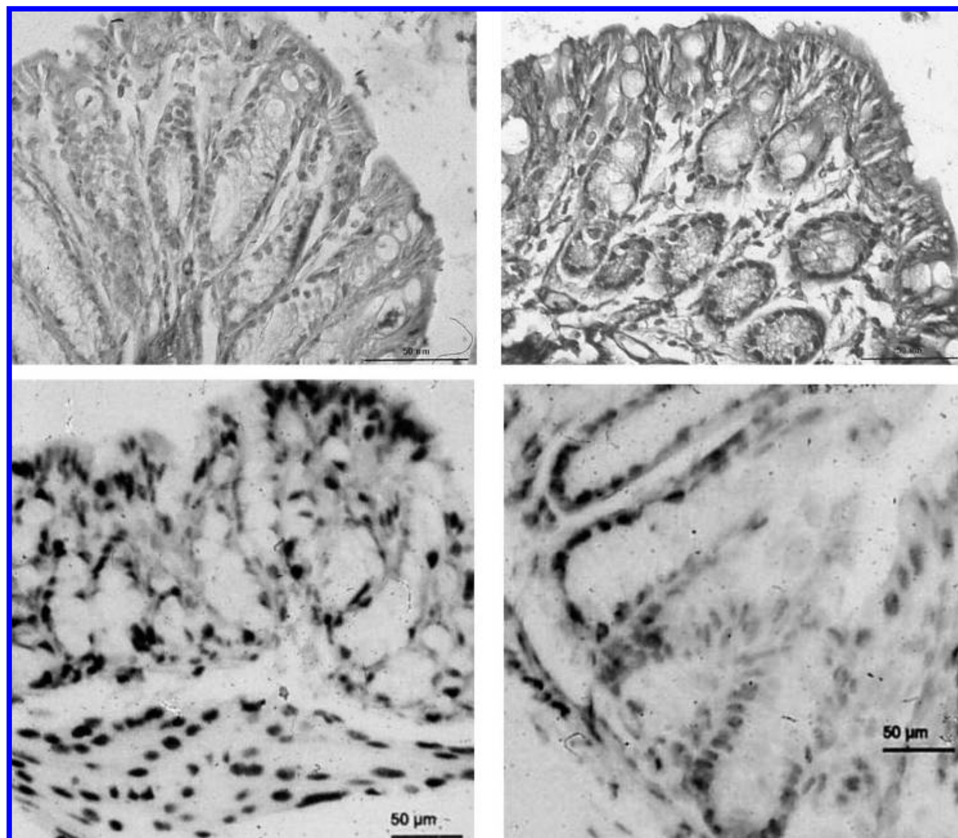

Figure 3. COX-2 immunoreactivity (**A**, top) and apoptotic activity (KLENOW technique) (**B**, bottom) of Min+/+ mouse fed the standard diet (left) and of Min/+ mouse fed the low-methoxylated-pectin-containing diet (right) for 56 days.

Table 5. Semiquantitative Evaluation of Intestinal Immunohistochemical Findings[a]

		apoptosis			COX-2		
mice	diet	M	A	C	M	A	C
healthy control	standard	+	no	no	(+)	no	no
Min/+	standard	+	++	(+)	+	++	+++
Min/+	LM pectin	+	++	(+)	+	++	+++
Min/+	HM pectin	+	++	(+)	+	++	++++

[a] M, morphologically normal mucosa; A, adenoma; C, carcinoma; no, no tumors; (+), <5% positive cells; +, 4–14% positive cells; ++, 15–29% positive cells; +++, 30–50% positive cells; ++++, >50% positive cells.

in epithelial cells during connective tissue migration (**Figure 3B**). The fewer epithelial cells that remained, the lower was the number of Klenow-positive cells. Thereby, no connective tissue cells showed apoptotic signals in this stage of tissue transformation (**Table 5**).

Short-Chain Fatty Acids. Both HM and LM pectins were fermented by the intestinal microbiota with formation of SCFA, causing a decrease of pH in the large bowel contents. Consistently, this effect resulted in an increase of the contents of the large intestine. A maximal increase was found in cecal content corresponding with the largest rate of fermentation (31 μmol of total SCFA and 2 μmol of butyrate/g of wet weight). Minimal fermentation was observed in the ileum (**Figure 4**). Both pectins enhanced particularly the production of acetate and propionate, whereas the ratio of butyrate decreased. Surprisingly, the differences of SCFA concentrations between cecum and colon, respectively, between colon and feces were reduced to a lower extent than expected (**Table 6**). The SCFA accumulation refers to a smaller rate of SCFA absorption. This is not the case in healthy control mice. It should be emphasized here that with tumor progression the rate of colonic butyrate absorption decreased, resulting in higher

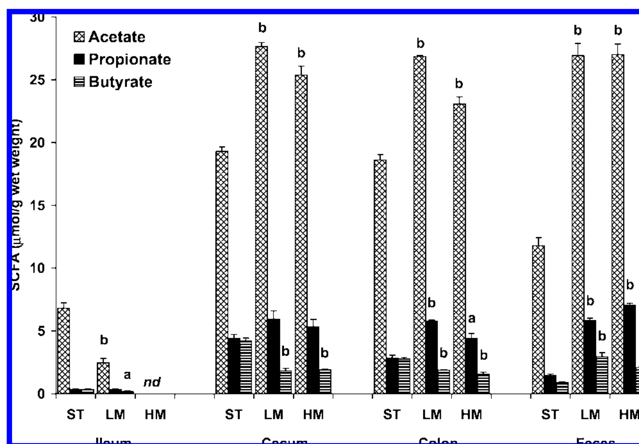

Figure 4. Short-chain fatty acids (SCFA) (micromoles per gram of wet weight) in intestinal contents and feces of Min/+ mice fed the standard diet (ST) or diets containing low-methoxylated (LM) or high-methoxylated (HM) pectin for 56 days. Values are means ± SEM; $n = 4$–9. Mean values were significantly different from Min/+ group fed standard diet: a, $P < 0.05$; b, $P < 0.001$; nd, not determined.

Table 6. Estimation of SCFA Absorption in the Colon

mice	diet	acetate	propionate	butyrate	sum SCFA
		SCFA Difference,[a] Cecum − Colon			
healthy control	standard	0.67	1.55	1.43	3.65
Min/+	LM pectin	0.78	0.15	0.05	0.88
Min/+	HM pectin	2.29	0.92	0.34	3.55
		SCFA Difference,[a] Colon − Feces			
healthy control	standard	6.82	1.37	1.90	10.10
Min/+	LM pectin	−0.006	−0.06	−1.08	−1.20
Min/+	HM pectin	−3.91	−2.63	−0.51	−7.05

[a] Differences are given in micromoles per gram of wet weight. Positive values show the degree of SCFA absorption. Low and negative differences point to an inhibition of SCFA absorption. The SCFA differences cecum − colon characterize the situation in the proximal colon and the differences colon − feces that of the distal colon.

Table 7. Bile Acids in Feces of Min/+ Mice Fed the Standard Diet or Diets Containing Low-Methoxylated (LM) or High-Methoxylated (HM) Pectin as well as of Min+/+ Mice Fed the Standard Diet at Day 50

	nmol/g of wet wt[a]			
	standard diet		LM pectin diet	HM pectin diet
bile acid	control mice	Min/+ mice	Min/+ mice	Min/+ mice
cholic acid	90.0 ± 2.15c	142.2 ± 3.8	129.3 ± 13.4	184.2 ± 11.7ad
deoxycholic acid[b]	114.2 ± 7.96c	360.3 ± 6.1	453.4 ± 20.7a	643.3 ± 13.6cd
7-ketodeoxycholic acid	8.6 ± 0.58	9.4 ± 0.7	11.8 ± 1.7	29.2 ± 1.5cd
12-ketolithocholic acid[b]	54.0 ± 1.32b	96.1 ± 5.3	199.7 ± 8.6c	201.3 ± 7.7c
chenodeoxycholic acid	11.4 ± 0.77	9.4 ± 1.1	16.4 ± 1.2a	30.4 ± 1.7cd
lithocholic acid[b]	132.5 ± 8.47b	237.7 ± 8.7	608.0 ± 14.0c	501.4 ± 13.8cd
α-muricholic acid	88.5 ± 4.8b	138.8 ± 6.4	242.2 ± 8.8c	262.5 ± 8.8c
β-muricholic acid	25.2 ± 1.56	24.4 ± 2.8	56.4 ± 5.5a	79.2 ± 2.0cd
hyocholic acid	26.2 ± 1.51a	37.3 ± 3.5	122.9 ± 7.7c	102.7 ± 8.4b
hyodeoxycholic acid[b]	65.7 ± 5.54	87.3 ± 5.9	182.1 ± 2.4c	228.7 ± 5.9cd
ursodeoxycholic acid	29.0 ± 1.56	36.7 ± 2.7	78.1 ± 10.1a	75.4 ± 1.3c

[a] Values are means ± SEM; $n = 3$. Mean values were significantly different from Min/+ group fed standard diet: a, $P < 0.05$; b, $P < 0.005$; c, $P < 0.001$. Mean values were significantly different from LM pectin group: d, $P < 0.05$. [b] Secondary bile acids.

fecal butyrate concentration. Due to the partial inhibition of butyrate absorption in the colon, the proportion between acetate, propionate, and butyrate shifted to higher butyrate concentrations. The same conclusion can be drawn from the results of SCFA concentrations in feces of Min/+ mice fed the pectin-enriched diets, investigated during the first 24 experimental days and the period between days 28 and 56, respectively. The concentrations of butyrate increased significantly from 3.2 to 9.5% ($P < 0.001$) (data not shown).

Bile Acids. The excreted fecal concentrations of total bile acids indicated that ileal bile acid reabsorption was lowered in Min/+ mice fed the standard diet compared with healthy mice. Pectin supplementation increased much more the concentration and daily excretion of bile acids in comparison to the animals fed the standard diet (**Table 7**; **Figure 5**). Fecal concentration of bile acids increased also with duration of the experiment. Despite the lower pH values, resulting from proton dissociation of the high SCFA concentrations, the portion of secondary bile acids was enhanced in the large intestine in the groups fed the pectin-enriched diet compared to the standard diet group (**Figure 5**). The concentration of the potentially oncogenic bile acid deoxycholic acid was nearly doubled. The concentrations of lithocholic acid, hyodeoxycholic acid, and 12-lithocholic acid were also higher in animals of the pectin groups, whereas the concentrations of the primary bile acid cholic acid remained relatively constant (**Table 6**).

The concentration of fecal primary, secondary, and total bile acids increased in all Min/+ mice groups with experimental time (**Figure 5**). This effect was significantly larger in both groups fed the pectin-containing diets compared to Min/+ mice receiving the standard diet.

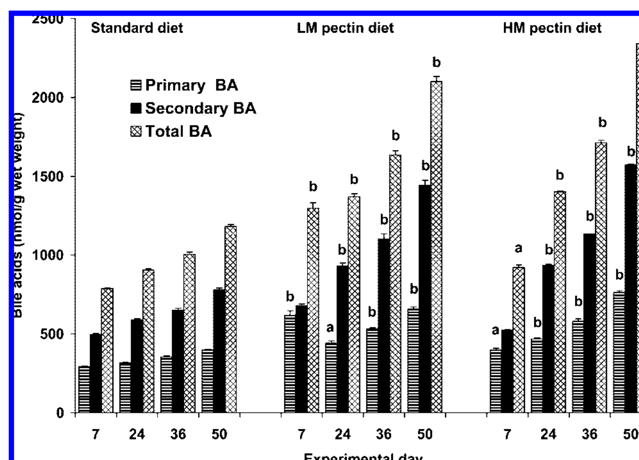

Figure 5. Bile acids (BA) (micromoles per gram of wet weight) in feces of Min/+ mice fed the standard diet or diets containing low-methoxylated (LM) or high-methoxylated (HM) pectin for 56 days. Values are means ± SEM; $n = 3$. Mean values were significantly different from Min/+ group fed standard diet: a, $P < 0.005$; b, $P < 0.001$.

Thickness of Intestinal Wall Layers. Figure 6 shows the investigated layers of the intestinal walls. There were distinct differences in the thickness of the small intestinal wall between the animals fed the pectin-enriched diets or standard diet (**Table 8**). Both pectins stimulated the expansion of the Tunica mucosa, where the absorptive cells are located. These effects were more strongly induced in the proximal than in the distal small intestine. LM pectin increased the thickness of the Tunica mucosa more evidently than HM pectin. The thickness of the

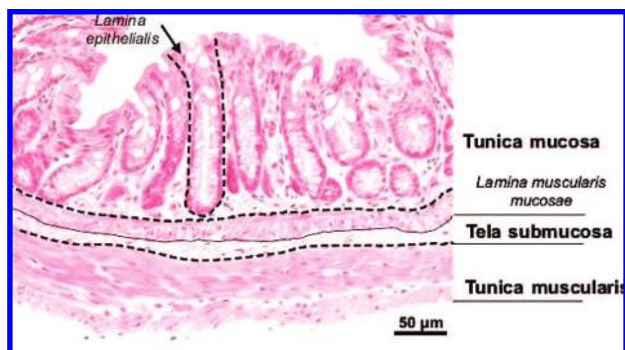

Figure 6. Measured layers of colonic wall (hematoxylin-eosin staining).

Tela submucosa was, however, slightly diminished in the proximal small intestine of mice fed the pectin-containing diets. The thickness of the Tela submucosa was unchanged in the distal part. The Tunica mucosa of the colon was thicker than that in the small intestine. Neither in the proximal nor in the distal colon did pectin change the diameter of the Tunica mucosa or Tela submucosa. The thickness of the Tunica muscularis increased continuously from the proximal small intestine downward to the distal part and further to the proximal and distal colon, but pectin did not change this layer of the wall. There were also no diet-mediated effects on the thickness of the Lamina epitheliales or the Lamina muscularis mucosae.

DISCUSSION

The intestinal mucosa regulates absorption of nutrients and ions while excluding the passage of antigens and other toxic agents. The architecture of the intestinal epithelium differs in the distinct intestinal parts corresponding to their special biological functions. Tumors generally arise from stem cells. This process is accompanied by numerous genetic and epigenetic alterations. Nutritional factors can modulate the dedifferentiation in the small and large intestine either positively or negatively. The strongest association between nutrition and carcinogenesis exists in the large bowel (*1, 7*). The colonocytes need the end products of carbohydrate degradation, the SCFA, especially butyrate. In the distal colon, butyrate activates the stem cells, which are located near the base of the crypts. In the mid-crypt zone, butyrate regulates the differentiation to mucous-secreting goblet cells and absorptive cells by stopping the dividing of the epithelial cells. The epithelial cells at the top of the crypt undergo apoptosis. This process is activated only by butyrate in tumor cells (*22*).

Fermentable carbohydrates and their prebiotic effects are therefore of great importance to maintain the balance of this continuous process of normal mucosa self-renewal, but not all carbohydrates entering the large bowel produce the same health-promoting effects. This is not surprising because dietary fibers differ significantly in their chemical and physical properties. In $APC^{Min/+}$ mice, pectins did not inhibit tumorigenesis independent of their degree of methoxylation. They seem even to accelerate carcinogenesis in the colon. All polyps found in the pectin-fed animals were large adenocarcinomas, but only 80% were large adenocarcinomas in APC-deficient mice fed the standard diet. These results do not agree with some published data on anticarcinogenic actions of pectins found in animal experiments using carcinogen (azoxymethane or 1,2-dimethylhydrazine) induced colorectal cancer models (*2, 3, 9, 12, 23–26*). These carcinogens need to be activated in the liver and in the colon, and these reactions are influenced by pectins (*3–5, 24*). In agreement with the results described in this paper, no anticarcinogenic effects of pectins were found with direct-acting carcinogens, such as *N*-nitrosomethylurea (*27, 28*).

Basic requirements for colorectal anticarcinogenic effects are two conditions: a sufficiently high fermentative butyrate production and adequate butyrate absorption. However, fermentation of pectin delivered only a low portion of butyrate. The study of Duncan et al. (*29*) using [1-^{13}C]acetate indicated that most of the butyrate-C derived from external acetate requires carbohydrates as a source of reducing power for butyrate synthesis from acetyl-CoA. The butyrate kinase expressed in distinct strains is responsible for energy recovery as ATP when butyryl-CoA is converted into butyrate via butyryl phosphate. An analogous bacterial metabolism of butyrate production may also be assumed in mice. Pectin fermentation is not capable of transforming external acetate into butyrate; therefore, the portion of acetate is very high among the end products, whereas butyrate output is consequently very low.

For the first time, a continuously decreasing absorption of butyrate into colonocytes of $APC^{Min/+}$ mice was shown in this study. These data agree with the progression of tumorigenesis. Absorption of SCFA is catalyzed by the sodium-coupled monocarboxylate transporter 1 (SMCT1), which has also the function of a tumor suppressor (*30*). SMCT1 is down-regulated in a very early stage of cell dedifferentiation detectable in over 50% of colonic aberrant crypt foci and adenomas (*31*). Consequently, the stimulation of Na^+, Cl^-, and water absorption decreases and a cellular butyrate lack occurs in colonocytes. The insufficient butyrate supply leads to a deficient energy metabolism and an ineffective function of butyrate as a promoter of normal cell differentiation and inducer of apoptosis in tumor cells.

The promoter function of butyrate is related to its ability to inhibit histone deacetylases and thereby to influence gene expression (*32*). Butyrate can induce cell cycle arrest and may prevent normal proliferation through p21, an inhibitor of cyclin-dependent kinases, as well as p21-independent mechanisms, as stabilization of p53 and tumor cell sensitization to Fas-mediated cytotoxicity (*33, 34*). Butyrate may also inhibit overexpression of COX-2 in stimulated tumor cells (*35*). None of these butyrate-mediated protective effects were found in the colon mucosa of $APC^{Min/+}$ mice fed the pectin-enriched diets. COX-2 was up-regulated and apoptosis increased in early adenomas, but was inhibited with tumor progression. These are all characteristic features of carcinogenesis.

Pectins are extensively degraded in the gastrointestinal tract and are fermented. Correspondingly, the luminal pH values dropped strongly in the cecum (pH 6.6) and in the proximal colon (pH 6.3), but only to a lesser degree in the distal colon (pH 6.8) (*22*). These findings are in agreement with the enhanced volume of the cecum content in pectin-fed mice. As was shown earlier, pectin does not increase excretion of feces (*22*).

The physical and chemical characteristics of pectin, including its ability to bind primary bile acids in the small intestine, may additionally contribute to accelerate tumorigenesis in the colon. Primary bile acids are substrates for the generation of secondary bile acids in the large bowel by bacterial dehydroxylation. The fecal concentrations of bile acids indicate that the relative high pH and the low luminal butyrate concentration are not sufficient to inhibit the activity of the key enzyme 7α-dehydroxylase in the distal colon, as is possible with a resistant starch type 3 supplemented diet (*36*). Deoxycholic acid and lithocholic acid are the dominant secondary bile acids in pectin-fed animals. Both are particularly implicated in the promotion of colon tumorigenesis (*37*). Nonconjugated secondary bile acids produce also duodenal tumorigenesis in $APC^{Min/+}$ mice by activation

Table 8. Thickness (Micrometers) of Tunica Mucosa, Tela Submucosa, Tunica Muscularis, Lamina Epithelialis, Lamina Muscularis Mucosae, and Total Walls in Proximal and Distal Parts of Small Intestine and Colon of Min/+ Mice Fed the Standard Diet or Diets Containing Low-Methoxylated (LM) or High-Methoxylated (HM) Pectin at the End of the Experiment (Day 56)[a]

diet	tunica mucosa	tela submucosa	tunica muscularis	lamina epithelialis	lamina muscularis mucosae	total wall
Small Intestine Proximal						
standard	187.2 ± 10.8	19.8 ± 0.3	49.8 ± 2.8	6.2 ± 0.7	3.6 ± 0.3	249.9 ± 17.6
LM pectin	469.9 ± 16.5c	12.9 ± 1.5a	36.0 ± 2.6a	9.0 ± 0.3b	<1	528.8 ± 13.6c
HM pectin	367.2 ± 42.5a	13.4 ± 1.1b	37.6 ± 3.2a	8.8 ± 0.7	5.0 ± 0.6	449.5 ± 43.0a
Small Intestine Distal						
standard	199.8 ± 18.5	10.5 ± 1.2	43.1 ± 8.7	6.3 ± 1.0	3.3 ± 0.5	265.9 ± 9.3
LM pectin	354.0 ± 13.2c	12.7 ± 1.2	61.6 ± 4.8	8.1 ± 0.5	3.1 ± 0.9	444.0 ± 18.2c
HM pectin	245.9 ± 5.6a	12.7 ± 2.0	37.4 ± 2.7	8.3 ± 0.3a	4.5 ± 0.6	309.9 ± 8.2a
Colon Proximal						
standard	150.5 ± 15.3	24.0 ± 4.5	60.3 ± 3.1	6.2 ± 0.7	6.5 ± 0.9	273.2 ± 7.7
LM pectin	109.7 ± 8.4a	17.2 ± 2.3	106.9 ± 14.9a	8.5 ± 0.8	5.1 ± 1.3	248.1 ± 10.1
HM pectin	78.2 ± 3.7a	29.6 ± 2.8	62.1 ± 6.5	8.1 ± 0.7	7.1 ± 1.5	186.5 ± 5.0c
Colon Distal						
standard	165.1 ± 10.1	26.7 ± 2.7	71.7 ± 4.0	7.1 ± 0.6	10.2 ± 1.5	280.2 ± 10.7
LM pectin	166.6 ± 9.7	21.6 ± 3.7	80.5 ± 6.4	8.8 ± 1.0	8.5 ± 0.9	285.3 ± 10.3
HM pectin	202.6 ± 3.0	30.0 ± 0.6	78.3 ± 16.1	8.8 ± 0.2	14.0 ± 1.4	328.5 ± 13.7

[a] Values are means ± SEM; $n = 3$–7 (20 different areas per mouse and intestinal segment were measured). Mean values were significantly different from Min/+ group fed standard diet: a, $P < 0.05$; b, $P < 0.005$; c, $P < 0.001$.

of COX-2 expression resulting in enhanced formation of prostaglandin E_2, higher concentrations of β-catenin, and a lower rate of apoptosis (*38, 39*). The potentially oncogenic acting deoxycholic acid was highest in $APC^{Min/+}$ mice fed HM pectin. HM-pectin-enriched diet caused also the highest total bile acid excretion. This result points to a stronger binding of primary bile acids to pectin with increasing degree of methoxylation.

Dark violet intestinal contents reflected bleeding from adenomas and carcinomas answered by an increased rate of erythropoiesis in spleen and the appearance of younger red cell populations in the blood. The observed spleenomegaly not only was a compensatory response to blood loss but also was due to an enhanced megakaryopoiesis. In mice with germline-mutated APC, pectins are not able to stop intestinal tumorigenesis. This may be associated with β-catenin overexpression typical of abnormalities in several proliferative tissues in $APC^{Min/+}$ mice (*17*). Pectins did not suppress this pathological proliferative state.

Among the molecular abnormalities identified in adenomatous polyposis is the elevated expression of COX-2, a key enzyme in the arachidonic acid pathway, generating multifunctional prostanoids, which plays a crucial role in the pathogenesis of tumor progression. Overexpression of COX-2 is induced by cytokines, growth factors, tumor promoters, oncogenes, and carcinogens and contributes to higher generation of nitric oxide (*40*). COX-2 promotes aberrant proliferation, inhibits apoptosis, and favors metastasis by enhancing cell mobility and adhesion. The continuous decrease of the antioxidative capacity in the blood plasma of $APC^{Min/+}$ mice fed standard or pectin-containing diets illustrated this stress state.

In the small intestine, polyposis development is not associated with butyrate-mediated effects, and bacterial butyrate formation was still minimal in the ileum. Inhibition of carcinogenesis may be realized only after absorption of nutritional compounds that inhibit uncontrolled cell division in epithelial cells, such as quercetin (*41*).

Viscous pectin films spreading on the mucosa surface may impair nutrient absorption and as a result cause an expansion of the Tunica mucosa characterized by longer villus height and crypt length (*42, 43*). Stark et al. (*44*) described also a small increase of the Tunica mucosa and Tunica muscularis areaa in the ileum and in the colon of rats fed 15% pectin. We did not find differences in the thickness of either layer in the colon of mice that received 10% pectin.

In summary, a reduction of tumorigenesis was not achieved by pectins with either high or low degree of methoxylation in the small or large intestine. Neither pectin suppressed COX-2, an enzyme inhibiting apoptosis.

The genetic predisposition for intestinal neoplasia becomes obvious in $APC^{Min/+}$ mice by 3 weeks of age, when tumor growth is initiated by the loss of expression of the wild-type APC allele (*45*). From this time therapeutic strategies other than supplementation of prebiotics are necessary to inhibit colorectal tumorigenesis. Pectins are ineffective for that objective.

ABBREVIATIONS USED

CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; FAP, familial adenomatous polyposis; HCA, hyocholic acid; HDCA, hyodeoxycholic acid; HM, high methoxylated; HPR, hydroxyperoxidase; KDCA, 7-ketodeoxycholic acid; KLCA, 12-ketolithocholic acid; LCA, lithocholic acid; LM, low methoxylated; MCA, muricholic acid; Min, multiple intestinal neoplasia; SCFA, short-chain fatty acid; UDCA, ursodeoxycholic acid.

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