

Inhibition of Azoxymethane-Induced Preneoplastic Lesions in the Rat Colon by a Cooked Stearic Acid Complexed High-Amylose Cornstarch

Yinsheng Zhao,^{†,‡} Jovin Hasjim,[†] Li Li,^{†,‡} Jay-Lin Jane,[†] Suzanne Hendrich,[†] and Diane F. Birt^{*,†,‡}

[†]Department of Food Science and Human Nutrition, 220 MacKay Hall, Iowa State University, Ames, Iowa 50011-1123, United States

[‡]Interdepartmental Graduate Program in Genetics, 2102 Molecular Biology, Iowa State University, Ames, Iowa 50011-3260 United States

ABSTRACT: This study evaluated a novel stearic acid complexed high-amylose cornstarch (SAC) for the prevention of preneoplastic lesions in the colon of azoxymethane (AOM)-treated Fisher 344 rats fed resistant starches at 50–55% of the diet for 8 weeks. Uncooked SAC (r-SAC) diet was compared with raw normal-cornstarch diet (r-CS) or raw high-amylose cornstarch diet (r-HA), and water-boiled CS (w-CS) was compared with w-HA and w-SAC, respectively. w-SAC markedly reduced mucin-depleted foci (MDF) numbers compared with w-HA or w-CS. r-HA significantly decreased aberrant crypt foci (ACF) numbers compared with r-CS or r-SAC. Increased cecum weight and decreased cecum pH were observed in the SAC or HA groups. The highest amounts of total or individual short-chain fatty acids (SCFAs) in cecum and of butyrate or propionate in feces were observed in the AOM-treated w-SAC group. This study revealed the effectiveness of a novel resistant starch in inhibiting colonic preneoplastic lesions and the importance of high-moisture cooking on the suppression of colon carcinogenesis by this resistant starch.

KEYWORDS: stearic acid complexed high-amylose cornstarch, resistant starch, cooking, aberrant crypt foci, mucin-depleted foci, short-chain fatty acids

INTRODUCTION

Colorectal cancer (CRC) is one of the most prevalent cancers in Western countries and is rapidly increasing in developing countries. In the United States, CRC is the third most common cancer in both men and women. In 2009, CRC accounted for almost 9% of all cancer deaths.¹ It has been commonly believed that dietary factors play an important role in preventing or enhancing colon cancer development. Previous studies showed evidence that the intake of dietary sucrose or highly digestible starches would increase the development of aberrant crypt foci (ACF) in rodents and was associated with colon cancer in humans.^{2–5} Other studies showed evidence of an inhibitory effect on colonic preneoplastic lesions or colon cancer development in rodents fed raw cornstarch or potato starch compared with rapidly digestible starches.^{6–8} The impact of resistant starch intake on colon cancer, however, remains controversial because some studies did not find protective effects of these carbohydrates against colon carcinogenesis.^{9–11} The differences could be related to issues in the method used for the diet preparation, such as whether the starch was cooked prior to addition to the diet.

Resistant starch (RS) is a portion of dietary starch that is not digested and absorbed in the small intestine of healthy individuals. RS was classified into four classes: type 1 RS, physically inaccessible starch, such as coarsely ground whole grains and legumes; type 2 RS, crystalline (uncooked) starch granules with B- or some C-type crystalline structure, such as raw potato, banana, and high-amylose maize starch; type 3 RS, retrograded amylose, which can be found in cooked and chilled potatoes; and type 4 RS, chemically modified starch.¹² A newly developed type 5 RS consisting of an amylose–lipid complex has been introduced recently,¹³ and the effects of this starch on colon cancer prevention have not been previously reported.

RS cannot be digested in the small intestine, and it enters the large intestine, where it is fermented by the anaerobic microflora to produce short-chain fatty acids (SCFAs). Thus, RS can increase cecal and large intestinal contents, alter microbial populations, and increase large intestinal SCFAs.^{14,15} These physiological properties produced by RS have been proposed to protect against colorectal cancer development.¹⁵

There is a large and diverse bacterial population in the human cecum and colon. The number of bacteria can reach 10^{10} – 10^{11} cfu/g wet wt.¹⁶ More than 400 species of bacteria were identified in human feces, comprising about 50% of the dry weight of feces. Gut bacteria can hydrolyze resistant starch that is not digested in the small intestine and makes it into the cecum and colon, and these bacteria can then ferment this starch to SCFAs. Of the three SCFAs (butyrate, acetate, and propionate), butyrate has been extensively studied and is considered to be the most potent for protection against colon carcinogenesis.^{15–17}

The research reported here aimed to test the hypothesis that an amylose–lipid complex (RSS) could inhibit preneoplastic lesions (colon cancer precursors) in the colon of rats treated with a chemical carcinogen. In this study, we investigated the inhibitory effect of a stearic acid complexed high-amylose maize starch (SAC) on preneoplastic lesions, that is, aberrant crypt foci (ACF) and mucin-depleted foci (MDF), in the rat colon induced by injection of azoxymethane (AOM). AOM is a genotoxic agent frequently used in rodent animal models to induce colon carcinogenesis. It is a metabolite of 1,2-dimethylhydrazine (DMH),

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Table 1. Diet Ingredients for the Raw Starch Diet and Water-Boiled Starch Diet Experiments^a

diet ingredient	raw starch diet	water-boiled starch diet
starch	50.0%	55.0%
casein	20.0%	20.0%
dextrose	15.0%	15.0%
cellulose (insoluble fiber)	5.0%	0.0%
mineral mix (AIN-93)	3.5%	3.5%
choline	0.2%	0.2%
methionine	0.3%	0.3%
vitamin mix (AIN-93)	1.0%	1.0%
corn oil	5.0%	5.0%

^a Starches were purchased from Cargill Inc., Minneapolis, MN, and used as obtained or processed and/or cooked as described in the text. All non-starch diet ingredients in both experiments were purchased from Harland Teklad (Madison, WI) or Sigma-Aldrich (St. Louis, MO).

but it can offer better potency and stability than DMH.^{18,19} Because starchy food in the human diet is generally consumed after cooking, diets prepared with cooked starch will be more meaningful for application in human colon cancer prevention. Meanwhile, the preparation of SAC involved heating, so the cooking of all the starches made for a more precise comparison between SAC and other starches. In addition, to understand the effect of cooking on the inhibitory effects of different starches, we prepared the diets with and without water boiling of the starch. Impacts of various starches on SCFAs and cecal fermentation (cecum pH and weight) were assessed, which provides information on changes in the colon of rats eating different starches that might relate to colon cancer formation and growth.

MATERIALS AND METHODS

Diets. Three starches were evaluated: CS (Cargill Gel 03420; Cargill Inc., Minneapolis, MN), HA (AmyloGel 03003; Cargill Inc.), and SAC (processed using HA in the Department of Food Science and Human Nutrition, Iowa State University).¹³ CS and HA were used as negative and positive controls, respectively.

Two successive experiments were conducted using diets formulated on the basis of the standard diet recommended by the American Society for Nutritional Sciences for mature rats (AIN-93M)²⁰ (Table 1). In the two experiments, the three starches were prepared by different methods. In the first experiment, the raw starch was added at 50% by dry weight into each diet group. In the second experiment, the starches were cooked by boiling in water, and the cooked starch was added into each diet at 55%. The 5% cellulose in AIN-93M was removed in the second experiment to allow for a higher dietary content of the starches under investigation. To make water-boiled starch, the starches were mixed with water gradually during cooking, and the starch–water mixture was slowly stirred throughout the process until a paste was formed. The paste was then cooled for 20 min to room temperature. The water-boiled starch pastes were then mixed with the powder containing the rest of the AIN-93M diet ingredients and made into diets. Diets were prepared every 2 days and immediately fed fresh to the rats. Three samples of each diet were collected and tested for their resistant starch content on day 0. The resistant content of the diets in the raw starch diet experiment was measured according to Megazyme/AOAC method 2002.02,²¹ and the resistant content of the diets in the cooked diet experiment was assessed by using AOAC method 991.43.²² The water content of these samples was determined by drying the diet in an oven at 105 °C for 3 h. The water content was calculated on a dry weight basis.

Animals and Housing. Five-week-old male Fischer 344 (F344) rats were obtained from Charles River Laboratory (Wilmington, MA). Fifty-four F344 rats were obtained for the raw starch diet experiment, and 45 F344 rats were obtained for the water-boiled diet experiment. The animals were housed individually in stainless steel, wire-mesh cages. A stainless steel wire floor was placed on the bottom of each cage to prevent the rats from eating their own feces. During the study the temperature was maintained at 22 ± 1 °C and the relative humidity at 60 ± 5%, and fluorescent lights were on from 6 a.m. to 6 p.m. Diet and water were provided ad libitum. The animal studies were performed in compliance with the guidelines of The Institutional Animal Care and Use Committee (IACUC).

Carcinogen Treatment. AOM was purchased from Midwest Research Institute (Kansas City, MO), and F344 rats were injected at 7 weeks of age. In the raw starch diet experiment, AOM was injected at the dosage of 15 mg AOM/kg rat body weight. In the water-boiled starch diet experiment, AOM was injected at the dosage of 20 mg AOM/kg rat body weight. In both experiments, the rats were dosed by intraperitoneal injection, and the AOM injection dosages for each experiment were decided by dosage studies that assessed the yield of lesions. The dosage of AOM used in each experiment was the highest dose that yielded the most lesions and did not show toxicity in the rats.

Cecal Weight and pH. Five-week-old F344 rats were fed control cornstarch diet (r-CS or w-CS for the respective experiments) for 2 weeks. Then two AOM or saline injections were administered to the rats 1 week apart. During the injection period and 3 days after the second injection, the rats were fed r-CS or w-CS for the raw and water-boiled starch experiments, respectively. Then the rats were divided randomly into three diet groups with 18 rats in each diet group (10 AOM-injected rats and 8 saline-injected rats) for the raw starch diet experiment or 15 rats (10 AOM-injected rats and 5 saline-injected rats) in each diet group for the water-boiled starch experiment. Two rats died in the water-boiled starch experiment (one in the saline-injected w-HA group and one in the AOM-injected w-HA group). The three starch diets were fed to the diet groups respectively for 8 weeks. Body weight was measured weekly. Fresh food was provided every 2 days in a preweighed amount. The food remaining at the end of the 2 day feeding period was weighed, and food disappearance was calculated. The rats were then killed by decapitation, and the cecum was collected, weighed as cecal weight with contents, and cut open. The cecal contents were scraped off from the cecal wall, and the pH and weight of the cecal contents were measured. Then the cecum tissue was rinsed in phosphate-buffered saline, quickly dried with a paper towel, and weighed in the water-boiled starch experiment. The liver was removed and weighed.

Aberrant Crypt Foci and Mucin-Depleted Foci. The rinsed colons were cut open longitudinally, laid flat, and flushed with saline. The distal 75 mm was trimmed and fixed in 10% formalin for 24 h before they were made into specimens. Colons were stained with 1% alcian blue in acetic acid (pH 2.5) and then counterstained with 1% neutral red to view ACF and MDF. ACF were defined as crypts that (i) have altered luminal openings, (ii) exhibit thickened epithelia, and (iii) are larger than adjacent normal crypts. MDF are characterized by the absence or very limited production of mucins. Moreover, MDF are focal lesions (i.e., there is a clear distinction between normal surrounding crypts and the MDF). MDF could also frequently be observed with an elevation above colon surface and a multiplicity (crypts/foci) >3.²³

Short-Chain Fatty Acids. SCFAs were measured in the water-boiled starch experiment only. Fresh fecal samples were collected 2 days before rats were sacrificed. Cecal and colonic contents were collected during the autopsy at the end of the study. All of the samples were kept at −50 °C until analysis. SCFAs, including acetate, propionate, and butyrate, were extracted and analyzed with gas chromatography as described.²⁴ Briefly, specimens were homogenized in 10 volumes of distilled water and centrifuged at 3000g for 10 min; 1 mL of supernatant

Table 2. Resistant Starch Contents of the Three Diets Prepared by Two Methods (Percent Dry Feed Basis)

starch group	resistant starch content of diets ^a (%)	
	raw starch experiment	water-boiled starch experiment
control normal cornstarch diet	0.4 ± 0.6 b	1.9 ± 1.5 c
high-amylose cornstarch diet	13.2 ± 0.8 a	14.3 ± 3.0 b
stearic acid complexed high-amylose cornstarch diet	12.2 ± 0.7 a	25.8 ± 5.0 a

^aData are expressed as the mean ± SD. Resistant starch content of diets in raw starch experiment was measured by Megazyme/AOAC method 2002.02, and resistant starch content of diets in the water-boiled experiment was measured by AOAC method 991.43. Values in each column with different letters are significantly different at $P < 0.05$.

was mixed with 100 μmol of 2-ethylbutyric acid (Sigma-Aldrich, St. Louis, MO) as internal standard. Hydrochloric acid was added to protonize SCFAs, followed by diethyl ether (2 mL) extraction and derivatization with *N*-(*tert*-butyldimethylsilyl)-*N*-(methyltrifluoro)acetamide (Sigma-Aldrich) at 80 °C for 20 min. SCFA silyl derivatives (1 μL) were injected in a split mode into a gas chromatograph (model HP 6890N) (Hewlett-Packard, Roseville, CA) equipped with a flame ionization detector and an SPB5 capillary column (30 m \times 0.25 mm i.d., 1 μm film; Supelco, Inc.). Helium was used as carrier gas. The initial oven temperature was held at 70 °C for 4 min, increased at 7 °C/min to 160 °C, and retained at this temperature for 5 min. The injector temperature was 200 °C, and the detector temperature was 220 °C. A standard SCFA mixture containing acetate, propionate, and butyrate (Sigma-Aldrich) was used for calculation: acetate, $y = 16.191x - 0.0825$ ($R^2 = 0.9996$); propionate, $y = 9.725x - 0.0132$ ($R^2 = 0.9996$); butyrate, $y = 6.7347x - 0.0135$ ($R^2 = 0.9999$), respectively. The concentration of SCFAs was expressed as mol/g wet weight. Digesta SCFA pools were calculated as the product of concentration ($\mu\text{mol/g}$) of individual acids or their sum (total SCFAs) and grams of the cecal content weight.

Statistical Analysis. Body weight, food disappearance, cecal content pH, cecal weight, ACF and MDF numbers, and total amount and concentration of SCFAs were analyzed using a two-way analysis of variance test and with a *t* test post hoc test when statistically significant main effects or interactions were observed. All values were reported as the mean \pm standard deviation. All statistical analyses were performed using SAS software (SAS Institute), and $p < 0.05$ was considered to be significant.

RESULTS

Resistant Content of the Experimental Diets. Resistant contents of the three diets used in the raw starch experiments were 0.4, 13.2, and 12.2% for r-CS, r-HA, and r-SAC, respectively, whereas resistant content rose dramatically from 1.9 to 14.3 and 25.8% for w-CS, w-HA, and w-SAC, respectively (Table 2). The proportion of the resistant content in each diet was based on the dry diet weight after it was mixed with other ingredients. The assessments revealed that the resistant content of w-SAC diet or w-HA diet was substantially greater compared with that of the w-CS diet. For the raw starch diet experiment, the resistant contents of r-HA and r-SAC were not different statistically, but they were significantly higher than that of r-CS. The water-boiled cooking process significantly increased the differences in resistant content between SAC and control starch diets. The Megazyme/AOAC method 2002.02 does not include the process of cooking the diets and is proper for assessing the resistant content of the raw starch diet. AOAC method 991.43 contains a boiling step. For the water-boiled diets, the diet preparation procedure already contained the water-boiling step, so AOAC method 991.43 reflected their resistant content.

Body Weight, Food Disappearance, and Liver Weight. No significant difference was seen in the body weight gain or food disappearance over the period of 8 weeks in either the raw starch diet experiment or the water-boiled diet experiment. A body weight gain of 98 ± 12 g was observed with the rats fed the diets in the raw starch diet study by comparing the ending and beginning body weights; a body weight gain of 101 ± 15 g was seen in the rats fed water-boiled starch diet. In the raw starch diet experiment, the average daily food disappearance of rats fed the raw starch diet was 18.9 ± 1.4 and 15.4 ± 1.8 g by wet weight and dry weight, respectively. In the water-boiled diet experiment, the average daily food disappearance of rats was 14.4 ± 1.2 and 11.3 ± 2.1 g by wet weight and dry weight, respectively. No effects of diet or AOM were observed on liver weight of the rats at the end of either experiment.

Cecum Weight and pH. In both the raw starch diet experiment and the water-boiled diet experiment, total cecal weights with contents (in the raw starch diet experiment) and cecal content weight/cecal tissue weight (in the water-boiled starch diet experiment) were significantly elevated in rats fed SAC and HA (Figures 1A and 2A,B).

Cecal content pH was significantly decreased in rats fed SAC and HA ($p < 0.05$) (Figures 1B and 2C).

Aberrant Crypt Foci and Mucin-Depleted Foci. In the raw starch diet experiment, only ACF numbers were counted because there were too few MDF, and ACF were seen only in AOM-treated rats. There was a significant decrease in the ACF number in rats fed r-HA compared with rats fed r-CS or r-SAC ($p < 0.05$), but the ACF number in rats fed r-SAC did not differ statistically from that of rats fed r-CS diet (Figure 1C).

In the water-boiled diet experiment, MDF were only seen in the AOM-treated rats, and a significant reduction was seen in the rats fed w-SAC and w-HA diets. Although there was a trend toward a decrease of ACF with rats fed w-SAC and w-HA diets, there was no significant difference between these groups (Figure 2D,E).

The numbers of large ACF (crypts/foci ≥ 4) were also counted and compared. A similar trend was observed as in the total ACF numbers. In the raw starch diet experiment, 16.8 ± 4.3 , 6.9 ± 4.4 , and 14.7 ± 8.1 large ACF were observed in rats fed the r-CS, r-HA, and r-SAC diets, respectively. A significant decrease was seen in the large ACF number in rats fed the r-HA diet compared with rats fed the r-CS or r-SAC diet ($p < 0.05$). In the water-boiled diet experiment, 10.5 ± 8.1 , 10.1 ± 6.7 , and 7.9 ± 7.6 large ACF were counted in rats fed the w-CS, w-HA and w-SAC diets, respectively. No significant difference was observed between these groups.

None of the above data were compared between the raw starch experiment and water-boiled starch experiment because the two experiments were not conducted at the same time.

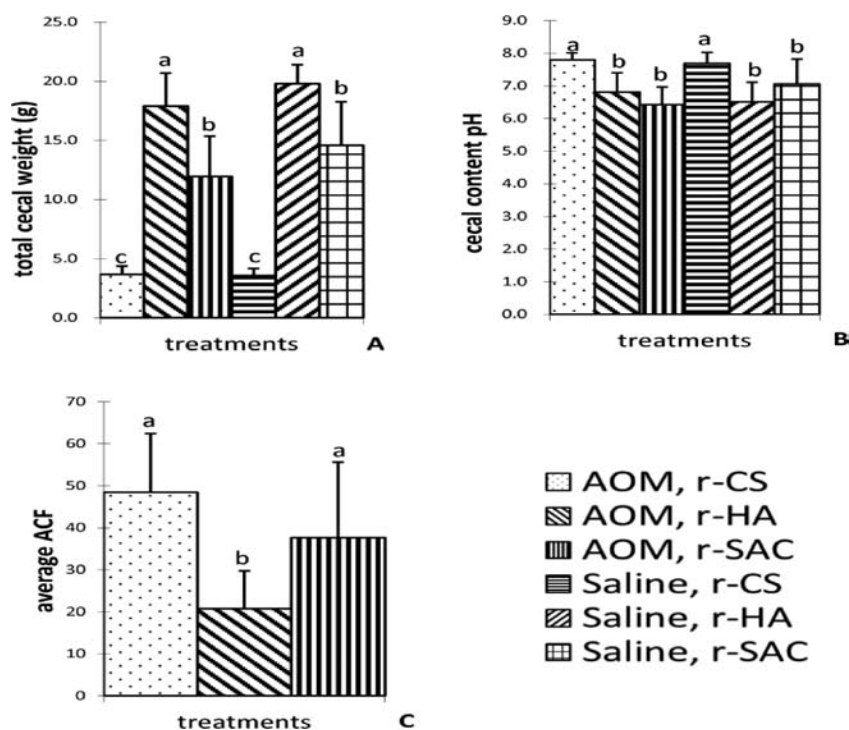


Figure 1. End points collected after rats fed raw starch diets were killed (9 weeks after the first AOM injection): (A) cecal weight (with content) (g); (B) cecal content pH; (C) average ACF number. ACF were measured in the distal 75 mm of the colon. ACF were seen only in AOM-treated rats. Values are the mean \pm SD. Bars with different letters in each panel are significantly different, $p < 0.05$.

Short-Chain Fatty Acids in the Water-Boiled Starch Diet Study. Total amounts of pooled acetate, propionate, and butyrate in the cecum (μmol) were significantly higher in rats fed w-HA and w-SAC compared with rats fed w-CS in both saline- and AOM-treated groups (Table 3). There was no difference in the amount of individual SCFAs in the cecum contents between w-HA and w-SAC diet groups in both saline and AOM treatments. However, in rats treated with AOM, total cecum SCFAs was highest in w-SAC, followed by w-HA and then w-CS.

In all groups, acetate was the most abundant SCFA detected in cecum, colon, and feces. Propionate was the second most abundant SCFA in cecum contents and feces, and the least abundant was butyrate. SCFA concentration was highest in cecum, followed by colon, and then feces. In general, no difference was observed in the individual or total SCFA concentration in the residue of cecum and colon between groups. However, in rats treated with AOM, fecal propionate and butyrate concentrations were significantly increased in rats fed w-HA or w-SAC compared with rats fed w-CS, whereas in animals treated with saline, w-HA significantly increased fecal butyrate concentration compared with w-CS and w-SAC groups.

DISCUSSION

Types 1, 2, and 3 resistant starches are widely present in foods such as navy beans, raw banana, and cooked-and-chilled potato. In this study, a type 5 resistant starch was prepared by complexing a high-amylose cornstarch with stearic acid. w-SAC inhibited MDF after being fed to Fisher 344 rats for 8 weeks compared with w-CS or w-HA. The r-SAC did not inhibit these lesions when fed to rats in comparison to diets containing r-HA or r-CS. To our knowledge, the inhibition of colon carcinogenesis has not been previously attempted using water-boiled normal starch

compared with similarly cooked high-amylose or processed high-amylose cornstarches. SAC also increased cecum weight and decreased cecum pH after rat feeding compared with all CS groups and some HA groups. These changes are expected to be due to an enhanced ability of SAC to avoid hydrolysis in the small intestine and thus increase the fermentation in the large intestine.^{10,13,22,25,26}

An inhibition of MDF preneoplastic lesions by w-SAC at the promotion stage in male Fisher 344 rats was observed, compared with rats fed w-CS or w-HA. In this study, the ACF number tended to decrease in parallel with the effects seen on MDF, although the decrease was not statistically significant. In the raw starch diet experiment, the average ACF number decreased significantly in the rats fed r-HA, but the same trend was not seen in the rats fed r-SAC in comparison with the r-CS. The large ACF multiplicity (crypts ≥ 4) has been suggested to be a better predictor of tumor incidence because large ACFs have much greater potential to progress into cancer.²⁷ In our study the numbers of large ACF in different diet groups reflected the same trend as was observed with the total ACF numbers. These results indicated that after boiling of the starches, an inhibitory effect of w-SAC on colon carcinogenicity was shown compared with the w-HA or w-CS diet; yet compared with the r-HA or r-CS diets, the r-SAC did not inhibit colon preneoplasia. Comparing the resistant content of the diets in the raw starch diet experiment and that of the water-boiled starch diet experiment, we observed that the water-boiling process resulted in a dramatic difference in the resistant contents of w-CS, w-HA, and w-SAC diets. Yet in the raw starch diet experiment, the resistant contents of r-HA and r-SAC were not different. Because r-SAC was a heat-treated product of r-HA, comparison of either resistant content or protective effects of colon carcinogenesis between the cooked starches

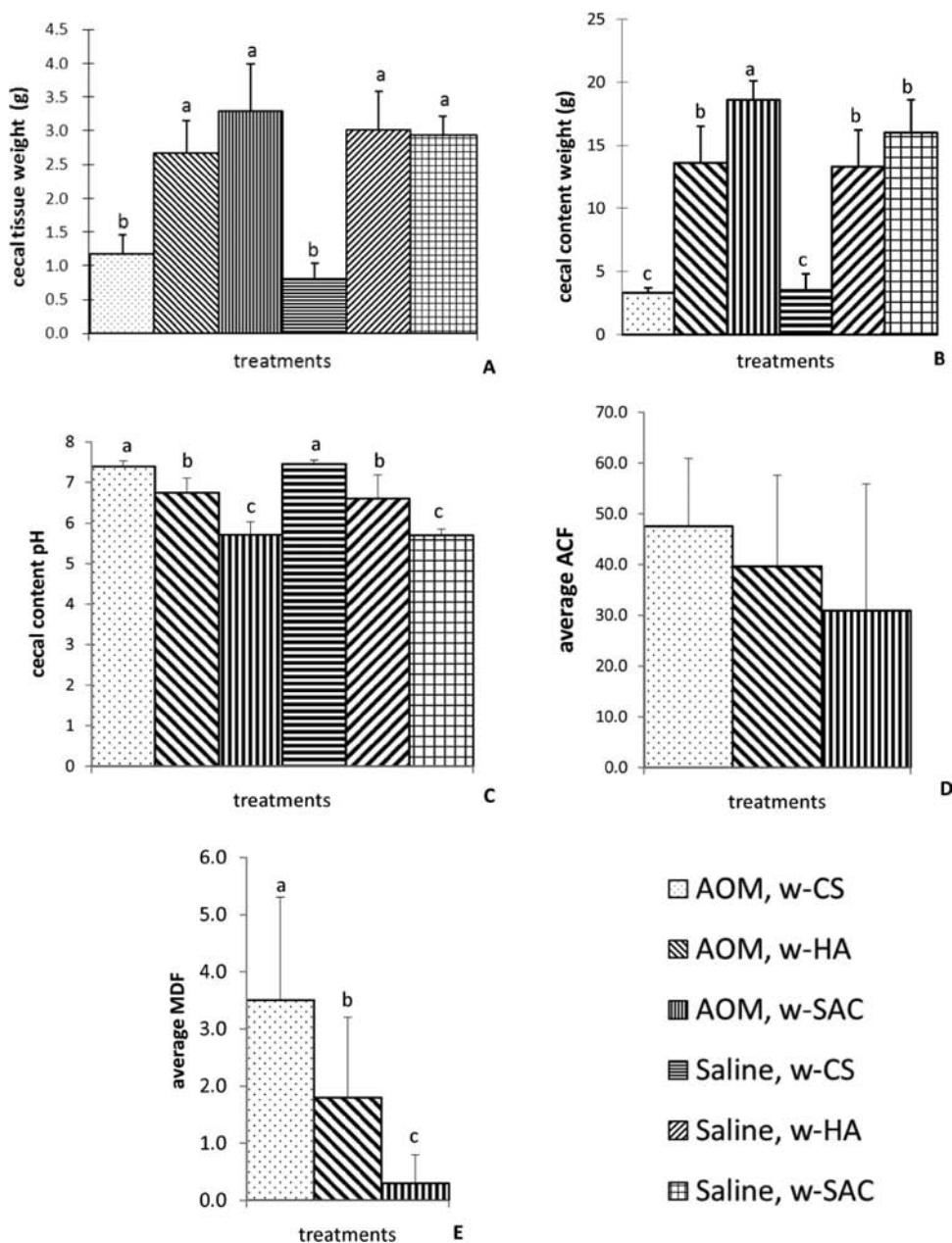


Figure 2. End points collected after rats fed water-boiled starch diets were killed (9 weeks after the first AOM injection): (A) cecal tissue weight (g); (B) cecal content weight (g); (C) cecal content pH; (D) average ACF number; (E) average MDF number. ACF and MDF were measured in the distal 75 mm of the colon. ACF were seen only in AOM-treated rats. Values are the mean \pm SD. Bars with different letters in each panel are significantly different, $p < 0.05$.

was more appropriate than the comparisons between uncooked starches. For CS or HA, heating was expected to cause the starch granule to swell and amylose to leach out from the granule. However, for SAC, we hypothesize that the lipid and amylose formed a complex, which prevented the amylose from leaching and starch granules from swelling. Thus, the resistant content of CS or HA decreased significantly compared with that of SAC after boiling in water. Such a feature of SAC to keep an intact granule could result in improved digestive enzyme resistance. The impact of cooking on starch is important because humans generally consume cooked cornstarch.

ACF have been defined as a putative biomarker for colon cancer, yet recent literature suggested that MDF could be a more

sensitive predictor of colon cancer than ACF. Bird reported ACF as a biomarker for colon cancer in 1987.²⁸ Femia et al. demonstrated that the number of MDF, their multiplicity, and the number of large MDF (crypts > 12) were significantly enhanced by cholic acid, a promoter of colon carcinogenesis, or decreased by piroxicam, a colon cancer-inhibiting drug.²⁹ Research by Pretlow et al. also supported MDF as an effective cancer predictor in a study in F344 rats treated with AOM and phytate.³⁰

Previous studies showed controversial results on the impact of dietary resistant starch on colon carcinogenesis. Bauer-Marinovic et al. showed that hydrothermally treated Novelose 330, a commercially developed RS3, prevented colon tumors in

Table 3. Effect of Experimental Starch Diets on Cecal, Colonic, and Fecal SCFA Concentrations in Fisher 344 Rats Fed the Diets in the Raw Starch Diet Experiment^a

group	saline, w-CS	saline, w-HA	saline, w-SAC	AOM, w-CS	AOM, w-HA	AOM, w-SAC
Cecum (μmol)						
total SCFA	131.6 \pm 28.9 c	653.2 \pm 78.8 ab	643.9 \pm 158.1 ab	177.2 \pm 67.0 c	569.7 \pm 217.6 b	890.5 \pm 260.9 a
acetate	85.8 \pm 23.8 b	417.5 \pm 63.4 a	342.4 \pm 116.4 a	118.3 \pm 47.1 b	371.0 \pm 160.5 a	544.9 \pm 179.2 a
propionate	29.1 \pm 5.6 b	165.9 \pm 88.1 a	188.9 \pm 47.1 a	37.0 \pm 13.4 b	138.1 \pm 52.6 a	217.4 \pm 113.1 a
<i>n</i> -butyrate	16.6 \pm 4.2 b	69.7 \pm 37.8 a	112.6 \pm 69.7 a	21.9 \pm 7.7 b	60.6 \pm 21.9 a	128.2 \pm 55.9 a
Cecum ($\mu\text{mol/g wet wt}$)						
total SCFA	41.1 \pm 12.6	49.0 \pm 6.5	34.2 \pm 6.3	61.5 \pm 23.3	43.1 \pm 16.1	55.8 \pm 14.0
acetate	26.8 \pm 9.3 ab	31.5 \pm 6.5 ab	18.2 \pm 5.5 b	41.1 \pm 16.6 a	28.2 \pm 12.5 ab	34.5 \pm 10.0 ab
propionate	9.1 \pm 2.7	11.8 \pm 4.4	10.0 \pm 1.8	12.9 \pm 4.9	10.3 \pm 3.2	13.6 \pm 7.3
<i>n</i> -butyrate	5.2 \pm 1.8	5.8 \pm 4.0	6.0 \pm 3.5	7.5 \pm 2.2	4.6 \pm 1.5	7.8 \pm 2.7
Colon ($\mu\text{mol/g wet wt}$)						
total SCFA	22.4 \pm 6.6 ab	30.2 \pm 8.0 ab	20.2 \pm 5.5 ab	29.3 \pm 13.8 ab	17.6 \pm 6.8 b	30.9 \pm 10.9 a
acetate	16.4 \pm 4.0	21.0 \pm 4.2	11.8 \pm 3.7	20.8 \pm 8.9	13.6 \pm 5.1	21.1 \pm 6.8
propionate	3.0 \pm 1.4	4.9 \pm 3.5	4.8 \pm 1.4	4.2 \pm 2.4	2.7 \pm 1.3	5.6 \pm 4.2
<i>n</i> -butyrate	3.0 \pm 1.4	4.3 \pm 3.5	3.5 \pm 2.9	4.4 \pm 2.7	1.3 \pm 0.9	4.2 \pm 3.1
Feces ($\mu\text{mol/g wet wt}$)						
total SCFA	16.8 \pm 6.6	16.2 \pm 1.0	13.4 \pm 3.5	10.7 \pm 3.3	16.1 \pm 6.4	16.6 \pm 6.0
acetate	14.9 \pm 5.5	10.3 \pm 5.6	7.7 \pm 1.6	9.6 \pm 2.9	12.7 \pm 5.1	11.3 \pm 3.9
propionate	1.5 \pm 1.7 ab	3.6 \pm 3.1 a	4.4 \pm 1.2 a	0.9 \pm 0.4 b	2.4 \pm 1.3 a	3.5 \pm 1.8 a
<i>n</i> -butyrate	0.5 \pm 0.6 bc	2.2 \pm 2.7 a	1.4 \pm 1.4 ab	0.2 \pm 0.2 c	1.0 \pm 0.5 ab	1.8 \pm 1.3 a

^a Data are expressed as the mean \pm SD. Values in each row without a common letter differ, $p < 0.05$.

1,2-dimethylhydrazine (DMH)-treated Sprague–Dawley rats when incorporated into the diet.³¹ In this study, the rats were fed Novelose 330 or raw control starch diet throughout the study. After 1 week of diet feeding, the rats were injected with DMH at 1 week intervals for 20 weeks. Young et al. observed increased epithelial proliferation, ACF density, and colorectal tumor formation in Sprague–Dawley rats fed diets containing raw potato starch (RS2), and an addition of raw wheat bran to this resistant starch diet suppressed tumorigenesis.¹¹ These rats were fed the diets for 31 weeks throughout the initiation and after the initiation (treatment with DMH injection) stages. Conversely, Thorup et al. found a lowered number of ACF in Wistar rats fed a raw potato starch diet compared with rats fed sucrose, raw cornstarch, or a basic semisynthetic diet.⁸ In this study, the carbohydrate in the basic diet contained 90% starch (45% cornstarch and 45% potato starch) and 10% sucrose/dextrin. All other diets used the respective test starches to replace the carbohydrate. Rats were injected with AOM once a week for 2 weeks. Then they were assigned to their respective diets.

SCFAs are thought to play an important role in the process of inhibiting colon carcinogenesis because SCFAs stimulate cell proliferation and induce apoptosis, which in turn affects carcinogenesis. SCFAs, including acetic, propionic, and butyric acids, can be major end products of microbial fermentation of some dietary polysaccharides including resistant starch after they escape absorption in the small intestine and are then digested in the large intestine. It was reported that resistant starch feeding increased cecal and colonic SCFA concentrations. Kleessen et al. reported a stimulation of bifidobacteria and lactobacilli and a higher SCFA concentration in male Wistar rats by a retrograded potato starch.³² Sakamoto et al. showed an

increased butyrate concentration in colonic content and in feces after the Sprague–Dawley rats were fed 3% resistant starch.¹⁰ In the water-boiled starch diet study, we did not observe a difference in fecal or colonic SCFA concentration between groups, but a significantly increased cecal SCFA amount in rats fed w-SAC and w-HA diet was observed. It was suggested that total SCFA excretion might also be relevant to the ability of resistant starch to reduce colon cancer risk, considering higher cecal content weight and thus SCFA amounts available for colonocytes.¹⁷ Differences in total SCFA amount between w-SAC and w-HA might be related to their efficacy in decreasing ACF occurrence (Table 3). Other end points altered by the gut bacteria fermentation are an increased cecal weight and decreased cecal pH.^{16,33} The results of an elevated cecal weight and decreased pH in cecal content by SAC and a change in SCFA content in our study indicated a microflora fermentation function stimulated by the processed resistant starch, which in turn shows a potential to suppress colon carcinogenesis.

Butyrate is an important SCFA because it is reported to reduce proliferation of colorectal cancer cells and stimulate proliferation in normal colorectal epithelial cells as observed in *in vitro* studies.^{34,35} Meanwhile, butyrate can induce apoptosis of colorectal epithelial cells and increase the differentiation and cell migration in colon cancer cells.^{36,37} In human colonic mucosa, butyrate was found to cause an increased transcriptional regulation of several pathways such as the citric acid cycle (TCA cycle), fatty acid metabolism, electron transport, TNF- α signaling, and oxidative stress pathways.³⁸ In some *in vitro* studies, butyrate was applied to the SW620 colon carcinoma cell line or HT29 colonic adenocarcinoma cell line, and microarray analysis demonstrated that some genes that regulate apoptosis, DNA synthesis, repair,

and recombination were up-regulated, whereas numerous oncogenes, cell cycle regulators, and transcription factors were down-regulated.^{38–40}

Increased fecal butyrate and propionate concentrations by w-HA and w-SAC feeding were observed in our study, suggesting that distal SCFA concentration might be associated with decreased ACF occurrence by w-SAC or w-HA. Colon cancer risk is higher in the distal colon compared with the proximal colon, and studies showed that AOM induced 63% more tumor in the distal colon than in the proximal colon.⁴¹ Considering the physiological significance of SCFAs, especially propionate and butyrate, in maintaining colon health and inducing hyperproliferative cell apoptosis, increased SCFAs in the distal colon may have important implications in the prevention of colon cancer.¹⁵ Studies have shown that the apoptotic index measured 6 h after an acute dose of AOM (10 mg/kg) in distal crypts was significantly correlated with fecal SCFA concentration but not with cecal SCFAs in Sprague–Dawley rats fed Hi-Maize starch or non-starch polysaccharides (wheat bran and cellulose) for 4 weeks.⁴²

The reduction of colorectal pH has been studied as a marker for the inhibition of carcinogenicity. Cecal pH may be related to cecal fermentation and a high production of SCFAs. Decreased fecal pH has been associated epidemiologically with lower colon cancer risk by studying different ethnic populations in urban and rural South Africa.⁴³ An acidic cecal environment inhibits growth of pathogenic bacteria⁴⁴ and may also decrease secondary bile acid levels, which were suggested as promoters for colon carcinogenesis.⁴⁵ In our study, the cecum pH was significantly decreased by SAC or HA, but SCFA concentration ($\mu\text{mol/g}$ wet wt) was not appreciably affected. It is possible that other organic acids might contribute to the decreased cecal pH observed. Kishida et al. reported that although the cecal pH was decreased from 7.1 in gelatinized normal cornstarch to 5.4 by 21 days of high-amylose cornstarch feeding in Wistar rats, no difference was observed in total cecal SCFA concentration between the two groups.⁴⁶ On the other hand, succinic acid was significantly correlated with decreased cecal pH ($r = -0.978$, $p < 0.05$) in their study with a >4-fold increase in rats fed high-amylose starch. However, some studies have suggested that a very low pH might increase the risk of carcinogenesis because a very low pH may stimulate epithelial cell proliferation, which in turn could enhance the chemically initiated carcinogenesis.^{40,47}

Elevated cecal tissue and content weights were found in rats fed diets containing w-SAC and w-HA compared with those fed w-CS, and the same trend was observed in total cecal weight rats fed r-SAC and r-HA compared with those fed r-CS. The increase in the cecal weight is likely related to the increase in fermentation in the cecum and the production of SCFA by gut microflora in animals fed resistant starches. The cecum is the main fermentation site in rodents, where the increased digesta by resistant starch and the increase in microbes would produce SCFA. It has been reported that SCFA reduced smooth muscle contractility and fluid output, which would help support the accumulation of gut contents in the cecum.⁴⁸

In the raw starch experiment, 5% cellulose was included in all diets. In the water-boiled experiment, 5% cellulose was removed from the ingredients and its proportion was made up by the starch under study. The removal was made on the basis of our desire to optimize the amount of experimental starch and reports suggesting the suppression of colonic carcinogenesis by cellulose. For example, cellulose diets and nonfiber diets were fed to AOM-treated (5 mg/kg body weight) Sprague–Dawley rats for

50 weeks, and cellulose diets were reported to significantly lower the induction rate of tumor in rats compared nonfiber diets.⁴⁹

In conclusion, our results showed that a water-boiled cooking method on SAC significantly enhanced its ability to inhibit MDF, a preneoplastic lesion that is used as biomarkers for colon tumor in comparison with w-CS or w-HA. Incorporating w-SAC in the diet inhibited preneoplastic lesions at the promotion stage in male Fisher 344 rats, compared with the rats fed w-CS and w-HA cooked by the same method. The F344 rats fed w-SAC developed significantly fewer MDF in the water-boiled starch diet study and had a trend toward decreasing ACF number. It is notable that a similar SAC used in a human study caused a lower circulating glucose and insulin response than the same load of wheat starch following a single meal in humans.¹³ To our knowledge, this is the first time the inhibition of preneoplastic colonic lesions was demonstrated in rats fed cooked starch. Boiling the starches in water is a common process in cooking, so human diets contain cooked starch. It will be important to assess other promising cooking methods and the impact of cooking on other starches in future research.

■ AUTHOR INFORMATION

Corresponding Author

*Postal address: Department of Food Science and Human Nutrition, 220 MacKay Hall, Iowa State University, Ames, IA 50011-1123. Phone: (515) 294-9873. Fax: (515) 294-6193. E-mail: dbirt@iastate.edu.

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Notes

Safety. Azoxymethane is a potent carcinogen able to induce colon cancer in rats and mice. It might cause harm to humans if swallowed, inhaled, or absorbed through the skin.

■ ABBREVIATIONS USED

ACF, aberrant crypt foci; AOM, azoxymethane; CRC, colorectal cancer; CS, control cornstarch diet; HA, high-amylose cornstarch diet; MDF, mucin-depleted foci; r-CS, raw control cornstarch diet; r-HA, raw high amylose cornstarch diet; RS, resistant starch; r-SAC, raw stearic acid complexed high-amylose cornstarch diet; SAC, stearic acid complexed high-amylose cornstarch diet; SCFAs, short-chain fatty acids; w-CS, water-boiled control cornstarch diet; w-HA, water-boiled high-amylose cornstarch diet; w-SAC, water-boiled stearic acid complexed high-amylose cornstarch diet.

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