Assessing the inhibition of azoxymethane-induced preneoplastic lesions in the rat colon by cooked resistant corn starches and identifying potential genetic targets

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Assessing the inhibition of azoxymethane-induced preneoplastic lesions in the rat colon by cooked resistant corn starches and identifying potential genetic targets

by

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ABSTRACT

Resistant starch (RS) is a complex carbohydrate that reaches the large intestine and is fermented by the colonic microflora. Two types of novel starches were processed: (1) stearic-acid complexed high-amylose cornstarch (SAC) which contains high resistance and (2) Guat from an inbred corn line with a high resistant starch content and ARXGuat from a hybrid of two corn lines AR (digestible starch) and Guat (resistant starch). The inhibition by SAC on colorectal carcinogenesis was compared with high amylose starch (HA) and normal corn starch (CS), and the study for the same purpose on Guat and ARXGuat starches was compared with AR starch. In all of the studies, starch diets were fed to Azoxymethane (AOM) - induced Fisher 344 rats for 8 weeks with 50-55% of the diet replaced with RS. The cooking method of water-boiling was applied to both studies. A bread-baked method was also applied to the SAC study for the comparison of the cooking methods. In the SAC study, the amount and concentration of Short Chain Fatty Acids (SCFAs) were assessed. Global gene expression was also studied in the colon mucosa of rats fed different diets or injected with AOM/saline by Affymatrix Microarray. Target genes differentially expressed after AOM treatment, or by the feeding of SAC were selected and identified by semi-quantitative real-time polymerase chain reaction analysis (semi-qRT-PCR). In our studies, SAC cooked by either the water-boiling or bread-baking method markedly reduced the Aberrant Crypt Foci (ACF) and Mucin Depleted Foci (MDF) numbers, and an enhanced ACF multiplicity was observed compared with cooked HA or CS within their cooking groups. Aberrant crypt foci numbers were dramatically decreased in the rats fed starches cooked by the bread-baking method compared with that in rats fed starch prepared by the water-boiling method.
Increased cecal weights and decreased cecal content pH, as well as increased total SCFAs or individual SCFA (butyrate, acetate) amounts were obtained by the same comparison. Microarray screening identified 3368 induced and 3060 repressed genes by AOM treatment and 544 induced and 859 repressed genes by SAC treatment, among which seven genes were confirmed by semi-qRT-PCR. In the Guat and ARXGuat study, Guat was found to cause a slight but significant increase in the ACF multiplicity, but no significant differences were found in total number of ACF, MDF or AC in rats fed either Guat or ARXGuat, relative to those fed AR. Collectively, the studies suggested the inhibitory effect of novel RS SAC on colonic preneoplastic lesions after the starches were cooked. It also revealed that the rats fed starch diets prepared by bread-baked cooking method had decreased preneoplastic lesion numbers compared with those fed water-boiled starch diets.
CHAPTER 1: GENERAL INTRODUCTION

Introduction

Resistant starch is a starch escaping digestion in the small intestine and fermenting in large intestine (Englyst et al., 1992). It serves as a potential source of fermentable substrate to produce short chain fatty acids by gut microflora (Scheppach et al., 1995). It also decreases cecal and fecal pH (Phillips et al., 1995) and increased their body weights (Gibson et al., 1996). Resistant starch has been reported to provide health benefits to humans and protect against diseases such as obesity, diabetes, chronic inflammatory bowel diseases, and cancers in cell culture systems and animal models (Higgins et al., 2011; Brennan, 2005; Jacobasch et al., 1999; Le Leu et al., 2009). On average, intake of high fiber diet is poor in Western societies. Resistant starch, however, can be a good alternative for dietary fiber since it is well tolerated and can be added up to 6 times the average daily intake in the normal Western diet (Van Munster et al., 1994).

In recent years, more and more studies have accumulated suggesting the benefit of RS on chronic inflammatory bowel diseases and colon cancer. Intestinal microflora, comprising several hundred species and forming a large ecosystem in its host, was revealed to play an essential role in the resistance to these diseases. Carbohydrates are the main substrate for the microflora. Resistant starch, an indigestible form of carbohydrate, is fermented by gut microflora and forms SCFA, hydrogen, methane and other products (Cummings et al., 1997). Butyrate is considered the most effective inhibitor of colorectal cancer (CRC) among the 3 main components of SCFA (butyrate, acetate, and propionate) and plays an important role in inhibiting proliferation and activating apoptosis of colon epithelial cells based on studies in colon cancer cell lines (Velazquez et al., 1996). Butyrate stimulates the expression of tumor suppressor proteins APC and E-cadherin, and
inhibits the accumulation of beta-catenin in the nucleus and thus deactivates oncogenes (Velazquez et al., 1997).

Resistant starch has been found in a wide range of natural plants. Four types of RS have been described including physically inaccessible starch, naturally highly resistant starch to \( \alpha \)-amylase digestion, retrograded resistant starch, or chemically modified starch, respectively. Chemical modification is an effective way to increase the resistance of starch granules to enzyme hydrolysis. One of the most popular modification methods is to conduct esterification of starch using anhydride, propylene oxide, or octenyl succinic anhydride (Chung et al., 2008). In our study, SAC is a production of linear molecules of amylose combined with stearic acid forming a single helical complex. This complex has an increased resistance to amylase hydrolysis presumably because the structure of the complex prevents it from fitting the enzyme binding site and being hydrolyzed (Hasjim, 2009).

Another kind of manmade RS came from natural plant breeding programs, in which a new RS line was bred from parent lines and inherits high resistant traits from them. Some of these RS have been put into the market, such as BarleyMax, a hybrid variety of barley, and Hi-maize resistant starch, highly resistant maize starch isolated from a special hybrid of high amylose starch. In our study, one maize line that was originally from Guatemala and one from Argentina were inbred as Guat and AR, respectively, and they were then crossed to make the ARXGuat hybrid. The resistance of the starches and the potential benefits of these starches in the rat large bowel were tested in our study.

It was the first time that the starches were cooked and evaluated for their effect on colorectal carcinogenesis. Cooking is a gelatinization process for starches. A fully gelatinized process requires sufficient amounts of water and appropriate temperature. In our study, neither the water-boiled method nor the bread-baked method provided full
gelatinization to the starches. The less gelatinized the RS is, the higher resistant starch content it gains (Donavan et al., 1979). Thus the resistant starch contents of SAC were different after the starch was cooked by the two different cooking methods (32.7 ± 0.8% in bread-baked SAC diet and 27.2 ± 0.1% in water-boiled SAC diet, respectively).

The studies in this thesis examined the inhibitory effect of cooked RS from 2 starch modification methods, SAC or cornline breeding (Guat and ARXGuat), in the colon of azoxymethane-induced Fisher 344 rats by observing the occurrence of aberrant crypt foci and mucin depleted foci. Both lesions are widely considered as carcinogen-inducedpreneoplastic lesions and are highly correlated to the risk of colon cancer incidence in humans (Corpet and Tache, 2002). Also, mucin-depleted foci were reported as a better pre-cancerous predictor than aberrant crypt foci (Femia et al., 2004; Pretlow et al., 1990).

A global gene screening was also conducted in the studies of water-boiled SAC compared to CS in our rat model. Microarrays provided powerful analysis of a large number of different mRNAs and identified genes and gene families whose expression levels were changed by diet in previous studies (Kato et al., 2004; Park et al., 2008). Microarrays were also applied to CRC patients as an assessment of gene alteration (Galamb et al., 2005; Croner et al., 2005). These previous studies provided references for our studies as that AOM treatment in rats were mimicking CRC incidence in human, and we also hoped to find target genes associated with diet treatment using similar microarray techniques.

Lowered cecal pH and increased cecal weights were observed in rats fed cooked SAC and HA compared with rats fed CS. The same trend was also observed in rats fed cooked Guat compared with those fed AR. These cecal changes are related to the accumulation of SCFA that comes from the fermentation of RS. In this process, gut microflora might play an important role as they use RS as a substrate to produce SCFA,
which impacts the incidence of CRC by modulating the proliferation, apoptosis and
differentiation of epithelial cells (Siavoshian et al., 2000; Gibson et al., 1992; Diez-
Gonzalez et al., 1999). Resistant starch, in turn, can modify the gut microbial population
after it is fed to animals, which may affect the ability of gut microflora on colonic
fermentation (Silvi et al., 1999).

The purpose of the studies included in this dissertation is to assess the inhibitory
effect of some RS on colonic preneoplastic lesions. These RS include the complex of
high-amylose starch and stearic acid SAC, and RS from traditional breeding Guat and
ARXGuat. The hypothesis of the studies is that our RS will reduce the risk of colon
preneoplastic lesions through changes in gene expression levels and changes in gut
microbial metabolism.

**Dissertation Organization**

This dissertation examines the inhibitory effect of cooked RS (SAC, Guat, and
ARXGuat) against colorectal preneoplastic lesions in an azoxymethane-induced rat model
using ACF and MDF as biomarkers for CRC. The studies suggested that cooked SAC
was efficient as an inhibitor of preneoplastic lesions. The enhanced fermentation in the
large bowel and increased SCFA amount were also revealed and presumably related to
this inhibitory effect of colon lesion incidence by the function of gut microflora. Global
gene expression was screened for the effect of AOM treatment and diet treatment
respectively. Genes or signaling pathways that related to carcinogenesis were mostly up-
regulated in AOM-induced colon samples, and the common genes which showed
differential expressions in both AOM treatment and diet treatment were mostly down-
regulated in SAC treated colon samples compared with the controls, indicating SAC
efficiently activated the cancer suppression process and inactivated functions of
oncogenes genetically. In the study of cooked Guat and ARXGuat starches in the same animal model, no inhibitive effect was seen compared to digestible control starch (AR).

In the first paper, “Inhibition of Azoxymethane-induced Preneoplastic Lesions in the Rat Colon by a Cooked Stearic Acid Complexed High-amylose Cornstarch”, the hypothesis that water-boiled SAC would be effective in inhibiting colon preneoplastic lesions in AOM-induced F344 rats and that high moisture cooking would be important on the suppression of colon carcinogenesis by this SAC was tested. The study confirmed that water-boiled SAC markedly reduced MDF numbers compared with the water-boiled HA or CS. Increased cecum weight, decreased cecal pH, and increased amount of cecal total SCFA, or fecal butyrate and propionate were also observed in rats fed water-boiled SAC. This indicated that an enhanced fermentation and SCFA accumulation by gut microflora may play an important role in this process of preneoplastic suppression. It was also observed that uncooked SAC did not show reduction in ACF or MDF occurrence compared with CS. In this study, Dr. Jay-Lin Jane and Dr. Jovin Hasjim in the Department of Food Science and Human Nutrition in Iowa State University modified the high-amylose VII resistant starch to SAC and analyzed the resistant starch content of the starches or diets. Dr. Suzanne Hendrich and Dr. Li Li from the same department analyzed the SCFA amount and concentration in cecum and feces of Fisher 344 rats after they were treated and sacrificed. I set up the animal model and analyzed the efficiency of SCA on colonic preneoplastic lesion suppression in both cooked and uncooked method. I wrote the manuscript for this collective work, and the paper was published in Journal of Agricultural and Food Chemistry.

The second paper was titled “Inhibition of Azoxymethane-induced Preneoplastic Lesions in the Rat Colon by a Stearic Acid Complexed High-amylose Cornstarch using Different Cooking Methods and Assessing the Potential Gene Targets”. This study tested
the hypothesis that SAC could prevent the incidence of preneoplastic lesions in the colon of AOM-treated Fisher 344 rats when this SAC was cooked in either of the two methods, water-boiled or bread-baked and the intake of SCA would trigger some expression changes at the genetic level. This study revealed that SAC cooked by both methods profoundly reduced the incidence of total ACF, large ACF and MDF compared with HA or CS in each respective cooking method. Moreover, rats fed bread-baked SAC showed dramatically fewer preneoplastic lesions than rats fed water-boiled SAC. Increased cecal contents and decreased cecal pH, as well as increased total SCFA and butyrate, acetate and propionate were observed in the cooked SAC group by either cooking method. Overexpression of Genes such as HSPD1, HSPH1, MCM6, RAC1, CD55, and TFRC were tested in rats after AOM induction, and the same genes were down-regulated after water-boiled SAC diet was fed. In this study, Dr. Suzanne Hendrich and Dr. Li Li collected data for the SCFA analysis. I modified the HA into SAC, tested its effect on carcinogenesis in the AOM-induced Fisher 344 rats, collected endpoints and did the analysis. Dr. Jiqing Peng from ISU GeneChip Facility conducted the Microarray. Dr. Man-Yu Yum and Tieming Ji of the Statistics Department of ISU helped me with the analysis of microarray data. I also wrote the manuscript.

The third manuscript, “A Study on the Effect of water-boiled Guat209 and ARXGuat a hybrid of Guat209 and AR16035 corn starches on Azoxy methane-induced Preneoplastic Lesions in the Rat Colon”, assumed that Guat, a resistant starch, would inhibit colon preneoplastic lesions compared with a digestible starch AR in AOM-treated F344 rats after the starches were water-boiled. And the hybrid of these two corn lines, ARXGuat, would inherit the high resistant feature of Guat and also show an inhibitory effect on pre-cancerous lesions after water-boiling. However, this study showed no lesion suppression from either water-boiled Guat or ARXGuat, although enhanced cecal weights and
reduced cecal pH were observed. For this study, Dr. Linda Pollak selected the corn lines. Dr. Marvin Paul Scott and Susan Duvick conducted the breeding work of the corn lines. I was responsible for the data collection from the rat model, data analysis and manuscript writing.

The research papers follow the general introduction and literature review. A general conclusion is provided at the end of the dissertation.

**Literature Review**

**Colon Cancer (CRC)**

CRC is one of the leading causes of cancer death worldwide. CRC was estimated to be the third most common cancer in both men and women, and is more common in developed countries. In the USA alone, an estimated 102,900 cases of colon and 39,670 cases of rectal cancer were predicted to occur in 2010, among which 51370 deaths were expected, accounting for 9% of all cancer deaths (American Cancer Society, 2010). In Europe, 413,000 people were diagnosed with colorectal cancer and among them 207,000 died in 2006. It has been the highest incidence cancer and the second-most deadly after lung cancer (Europacolon, 2011). The highest incidence rates of CRC occur in North America, Western Europe and Japan, while the lowest rates occur in Africa, Asia and Central America. The rates have been increasing largely in economically developing countries (Jemal, 2011). Some research showed that colorectal cancer incidence rates for both males and females primarily increased in the economically transitioning countries including Eastern European countries, most parts of Asia, and select countries of South America based on the data from 1983-87 to 1998-2002 (Center et al., 2009).

Colorectal cancer, also known as bowel cancer, is the cancer caused by uncontrolled cell growth in the colon or rectum of the gastrointestinal tract (American Cancer Society,
Colorectal cancers start in the lining of the bowel, most are benign as colorectal polyps or adenoma, but some develop into cancer over time (Falterman et al., 1974). Colorectal cancers can be adenocarcinomas, lymphomas or squamous cell carcinomas. The latter two are not as common and not as well characterized (Beart, 1990; Juturi et al., 1999). In our studies, we focused on pre-neoplastic lesions that originate in the colorectal epithelium. Epidemiologic studies have revealed a number of risk factors for colorectal cancer including age, family history of CRC or inflammatory bowel disease, smoking, alcohol consumption, obesity, and diet (Giovannucci, 2002; Wu et al., 1987).

Colorectal cancer can occur from familial (hereditary) genetics or as a sporadic genetic insult following an environment impact. Most colorectal cancers occur as sporadic cases. Most hereditary colorectal cancers are Familial Adenomatous Polyposis (FAP) and Hereditary Nonpolyposis Colorectal Cancer (HNPCC). About 20% of colorectal cancers occurred from familial, 5% of the incidence came from genetic syndromes such as (FAP and HNPCC, and around 75% occurred sporadically (Fearon and Vogelstein, 1990; Hendon et al., 2005). Familial Adenomatous Polyposis is an autosomal dominant syndrome characterized by multiple adenomatous polyps. Familial Adenomatous Polyposis may occur in the gastric, duodenal, jejunoileal, and colorectal areas. Patients with FAP usually have CRC within 8–10 years. Some genes on chromosome 5q21 are associated with FAP and are likely to contribute to colorectal tumorigenesis, including adenomatous polyposis coli tumor-suppressor gene (APC) and mitotic checkpoint proteins complex gene (MCC). The MCC gene was considered to cause FAP by somatic mutation in sporadic colorectal carcinomas. The APC gene is involved in cellular processes for the development of CRC (Kinder et al., 1991; Joslyn et al., 1991). Hereditary Nonpolyposis Colorectal Cancer is an autosomal dominant condition with an increased risk of developing colorectal cancer at an earlier age than
sporadic colorectal cancer. Individuals with HNPCC have about an 80% lifetime risk for CRC. Two-thirds of these cancers occur in the proximal colon (Rodney et al., 2001).

Carcinogenesis in the colon epithelium is a multistep process including different epithelial cell growth events in each step and commonly considered as an “adenoma-carcinoma” model, followed by malignant transformation (Kinzler and Vogelstein, 1996). In this model, accumulation of multiple genes, rather than their sequence, determines tumorigenesis. Two kinds of genetic alterations happen in the process: for the inherited syndromes such as APC and HNPCC, germline mutations are inherited, and for sporadic cancers, somatic mutations occur in a stepwise manner (Fearon and Vogelstein, 1990). Mutations in the APC gene, a tumor suppressor gene that occurs in both inherited and sporadic tumors, usually appear in the early stage of tumorigenesis. The mutation of APC leads to the loss of APC function as part of a protein complex targeting β-catenin for its degradation and thus activates β-catenin/Tcf-4 acts for proliferation in intestinal crypt epithelial cells (Bright-Thomas and Hargest, 2003). Among the genes that change in the process of tumorigenesis, K-ras and p53 are also key genes. The mutation of k-ras gene follows APC mutation, found in 50% of sporadic colorectal cancers. Ras oncogene mutation switches on the transmission of extracellular growth signals to the nucleus (Smith et al., 2002). The p53 suppressor gene is the most commonly mutated gene in human cancer occurring in the late development of CRC since the mutation of p53 gene could be identified in up to 75% CRCs but rarely found in adenomas and aberrant crypt foci (Manne et al., 1997).

Diet and Life Style

Environmental factors play a major role in the development of colorectal cancer. Diet and lifestyle are two of the main environmental factors that affect the occurrence of CRC.
One study reported data from a population-based study conducted in northern California, Utah, and Minnesota in 1991-1995. In this study, the lifestyle patterns for both men and women were divided by dietary variables, body size, medication, supplementation, alcohol, and physical activity. Previous studies suggested that consuming a Western style diet (high in meat, refined grains and sugar, and low in vegetables and fiber) and physical inactivity were most related to the high incidence rate of CRC. High levels of physical activity, consuming high levels of calcium and folate, keeping energy balance and maintaining an appropriate body weight were also reported to reduce the incidence of CRC (Slattery, 2000). Migrant studies of populations moving from areas with different colorectal cancer incidence also help the research on the environment impact on CRC. It was reported that CRC was sharply increased in Korean-Americans compared with Koreans in both genders (Lee et al., 2007).

Dietary fiber was widely accepted as a protective factor for CRC. But the concept became controversial as research accumulated. In a pooled study of 13 prospective cohort studies for 6 to 20 years of follow-up across studies, dietary fiber intake was inversely associated with the risk of colorectal cancer in age-adjusted analyses. However, high dietary fiber intake was not associated with a reduced risk of colorectal cancer after accounting for other dietary risk factors (Park et al., 2005). A 16-year-follow-up study in women aged from 34 to 59 years old with no history of cancer, inflammatory bowel disease, or familial polyposis revealed no association between the intake of dietary fiber and the risk of colorectal cancer (Fuchs et al., 1999). As studies were conducted, it was realized that the fiber type might be important for its association with colorectal cancer inhibition. A prospective study in men from 1986 to 1994 indicated a modest reduced risk of distal colon adenoma with increasing intake of fiber from fruit but not cereals or vegetables. Soluble fiber appeared to be inversely associated with distal colon adenoma,
but insoluble fiber did not have this association. No consistent relation was found
between fiber and rectal adenomas or hyperplastic polyps (Platz et al., 1997). Notably,
one of the reasons that no association was seen for fiber with CRC might be that human
fiber intake is generally low. For example, the adequate intake for 45,491 women 50
years and older was estimated at 21 g/day/woman (Food and Nutrition Board, Institute of
Medicine, 2002). A study of older women with a mean follow-up time of 8.5 years
revealed no association between fiber intake and CRC, but the 10th percentile of dietary
fiber intake was 5.4 g, and the 90th percentile was only 18.2 g, indicating an inadequate
intake of fiber for a large group of women in that age range (Mai et al., 2003).

Another common nutritional recommendation over the last 20 years for avoiding
colorectal cancer was to reduce the intake of dietary fat. A cohort study in men suggested
that intakes of total fat, saturated fat, and animal fat were not related to risk of CRC.
However, red meat intake was associated with an elevated risk of CRC. Men who ate
beef, pork, or lamb as a main dish five or more times per week had a relative higher risk
compared to men eating these foods less than once per month (Giovannucci, 1994). A
prospective study in women found that animal fat was positively associated with the risk
of CRC. In this study, results showed the relative risk of CRC in women who ate beef,
pork, or lamb as a main dish every day was sharply increased compared with those who
ate these foods less than once a month. The ratio of the intake of red meat to the intake of
chicken and fish was also an increased factor for CRC. Processed meats and liver were
also significantly associated with increased risk. Fish and chicken without skin were
related to decreased risk. No association for vegetable fat was observed (Willett, 1990).

Observations also revealed a connection between the increased intake of Folic acid
supplementation (Benito et al., 1991; Bird, 1995; Giovannucci et al., 1998), calcium and
Vitamin D (De Stefani et al., 1997; Pritchard et al., 1996; Kampman et al., 1994; Grau et
al., 2003), and high consumption of fruits and vegetables (Michels et al., 2000; van Duijnho¬ven et al., 2009) and the decreased occurrence of CRC, yet some of the findings were still controversial.

**Starch**

Starch is a carbohydrate produced by all green plants. It is common in the human diet, e.g. in staple foods such as potatoes, wheat, maize (corn), rice, and cassava. Starch contains two types of glucans: amylopectin and amylose. Amylopectin is highly branched molecules with α-(1->4) glycosidic-linked short linear chains connected by α-(1->6) glycosidic linkages. Amylopectin is packed into a semi-crystalline structure by the branch chains. Amylose is comprised of primarily linear molecules with α-(1->4) glycosidic linkages; some consist of a few branches. Amylose presents in an amorphous form. Amylose content is about 15-30% in normal starch, and 50% or more in high amylose starch (Nakamura, 2002). The crystallinity of high-amylose starch is less than that of the normal starch due to the greater amount of amylose in high-amylose starch.

Gelatinization of starch causes an irreversible change of the starch from semi-crystalline structure to amorphous structure when starch granules are heated to an appropriate temperature with a sufficient amount of water. During the gelatinization process, the starch granules swell, and the double helices in the granules dissociates. Gelatinization temperature and enthalpy change of gelatinization are two main properties of gelatinization, which are impacted by three main factors: structure of the amylopectin, amylose content, and phosphate-monoester derivatives of the starches. Amylose presents in starch granules in an amorphous form and reduces the crystallinity of the starch granules, which causes a reduced enthalpy change of starch gelatinization. Then amylopectin presents more in a crytallinity structure. High-amylose cornstarch has a high
gelatinization temperature (more than 100°C) due to its long branch-chains of amylopectin since long branch-chains spread more through the crystalline formation and stabilizes the crystalline structure compared with short branch chain amylopectin. Phosphate-monoester derivatives have negative charges thus providing repulsion forces between each other. Consequently, starches with a high proportion of the phosphate-monoester derivatives on its amylopectin have a low gelatinization temperature (Jane et al., 1999).

Retrogradation happens during the storage of gelatinized starch where gelatinized, amorphous starch molecules recrystallize in a double helical structure. Retrogradation of the starch is impacted by the amylose content, amylopectin structure, lipid content, storage temperature, and moisture content of starch paste. Amylose retrogrades faster than amylopectin. The presence of lipids and phospholipids make starch harder to retrograde since the lipids restrict the swelling of starch granules and the dispersion of starch molecules during cooking, and keeps the starch molecules close enough to resist retrogradation. Lipids can also form a complex with amylose, which also helps to resist retrogradation (Slade and Levine, 1987; Jane et al., 1999). Phosphate-monoester derivatives provide repulsive forces and reduce the retrogradation of starch.

Enzyme hydrolysis of starch molecules produces glucose as an energy source for plant metabolism, as well as for animal and human energy generation. Enzyme hydrolysis is slower in starch granules than in gelatinized amorphous starch molecules. Enzyme hydrolysis is also slower in large starch granules than in smaller granules. The structure of amylopectin, amylose content, lipid content, granular size, and polymorphism are all factors to impact susceptibility of the starch granules to enzyme digestion (Tester et al., 2004).
Resistant starch is not absorbed in the small intestine and is thus passed on to the larger intestine. Resistant starch is fermented in the large intestine by the gut microflora producing short chain fatty acids and other organic acids. Resistant starch can be classified into five types according to their structures and their enzyme resistance: RS type 1 (RS1) is physically inaccessible starch protected by a protein matrix or cell wall material, such as whole grains, legumes, and pasta. RS1 in legume seeds is protected by the cell wall, which prevents the starch granules from getting enough water when cooking. Thus the starch granules cannot fully swell and be gelatinized and are less digestible. RS1 in whole grains or in pasta is surrounded by a protein matrix, which forms a barrier for amylase hydrolysis and swelling during cooking. As a result, the center of the starch granules is not fully gelatinized and is less susceptible to amylase hydrolysis (O’Dea et al., 1980; Granfeldt et al., 1991). Resistant starch type 2 (RS2) is uncooked native semi-crystalline granular starch which displays the B- and some C-type polymorphs. Green banana starch, high-amylose cornstarch, and uncooked potato starch are all included in this category. RS2 turns from a semi-crystalline structure to a highly digestible amorphous structure during heat processing and consequently loses its resistance to enzymes during cooking. RS type 3 (RS3) is retrograded starch from the cooked starch. Gelatinized starch molecules retrograde from amorphous to a double helices structure during storage. This crystalline structure of RS3 is enzyme resistant. RS3 has a melting temperature above 120°C, so most RS3 remains crystalline after heat processing. Starches containing a large portion of amylose or long branch-chains of amylopectin are easily produced into RS3. On the contrary, starches with short branch chains of amylopectin are usually not able to form RS3 because the double helices and short branch chains formed are not long enough to produce stable crystals (Sievert and Pomeranz, 1990). Resistant starch type 4 (RS4) is chemically modified or cross-linked
starch, with decreased water solubility and accessibility of enzymes to hydrolyze the starch molecules. The hydrophobic moieties of the starch granules also have fewer enzyme binding sites for hydrolysis. Thus, they are less accessible for enzyme hydrolysis. A newly developed type 5 RS (RS5) consisting of amylose-lipid complex has been introduced recently (Hasjim et al., 2010). One hypothesis is that the linear molecules of amylose entangle with lipids and form a single helix complex, and the hydrocarbon chains of the lipid stay in the center of the helix through hydrophobic interaction with the amylose molecules. The amylose-lipid complex exists as amorphous or crystalline complex. The amorphous complex has lower dissociation temperature than the crystalline complex. Incubating the amorphous complex at temperatures above their dissociation temperatures and below the dissociation temperature of the crystalline complex will convert amorphous complex into crystalline complex (Biliaderis and Seneviratne, 1990). The crystalline complex is more resistant to amylase hydrolysis than the amorphous complex (Seneviratne and Biliaderis, 1991).

The non-digestible feature of resistant starch results in a low glycemic index since RS dilutes the digestible carbohydrate in food (Douglass, 1975). Consequently, RS improved the insulin sensitivity in humans and possibly prevent or reduce disease processes related to metabolic syndrome such as non-insulin-dependent diabetes, hypertension, and lipid abnormalities (DeFronzo and Ferrannini, 1991). Resistant starch also decreased the incidence of obesity by reducing the total energy intake. Since foods rich in RS stay in the digestive system longer, they may prevent the hypoglycemia-induced hunger and promote satiety, thus reduce food intake. On the other hand, since RS is not a good source of energy intake, the body has to use fat in adipose tissue to provide energy (Raben et al., 1994; Ranhotra et al., 1996). Resistant starch was also reported to decrease serum triglyceride and cholesterol concentrations, and reduce the risk of
cardiovascular disease (Ranhotra et al., 1996). Hasjim reported that RS5 reduced postprandial plasma glucose and insulin levels in humans. Twenty male human-subjects were fed bread made from 60% (dry basis) RS5 or white bread. Subjects fed RS5 had their postprandial plasma glucose and insulin levels reduced to 55% and 43%, respectively, compared with those fed control white bread (100%). The results suggested that the RS5 can be used for the intervention of diseases such as diabetes and obesity (Hasjim et al., 2010). Other studies showed evidence that resistant starches could lower serum plasma cholesterol after they were processed by heat and moisture treatment. One study gave 4 diets to six-month-old ovariectomized female Wistar rats 7 days after ovariectomization for 21 days: heat-moisture treated high-amylose corn starch; gelatinized high-amylose corn starch; and gelatinized normal corn starch. The dietary fiber contents were 64.5, 19.3, 2.4, and 0.1% measured by AOAC 991.43. The study showed that total plasma cholesterol concentrations decreased with increasing dietary fiber content, and hypocholesterolemic effect of HACS in ovariectomized rats appeared to be more effective with starch treated with heat-moisture (Liu et al., 2007).

Resistant starch has been observed to prevent colon preneoplastic lesions or CRC in recent years. In a short term animal study, male Wistar weanling rats were given 2 AOM injections, and RS was fed to rats 3 weeks after the first AOM injection by low-RS composition (7.6 g/kg body weight), middle-RS composition (15.2 g/kg body weight) or high-RS composition (22.8 g/kg body weight). Normal cornstarch was used as control. Rats were killed 13 weeks after initial injection of AOM. Incidence of ACF, number of Aberrant Crypts (AC), and the crypt multiplicity showed significant decreases in RS treated animals compared with controls, and there was a reverse accordance of ACF incidence, AC, and crypt multiplicity with the percentage of RS in the diet (Liu et al., 2008). A long term animal study also proved that feeding dietary RS (high amylose
cornstarch, HA) protected against AOM-induced colon carcinogenesis. Male Sprague-Dawley rats were provided 10% HA, 20% HA or control cornstarch diets. After eating their experimental diets for 4 weeks, the rats were injected AOM twice and then maintained on their dietary regimen until 25 weeks after second AOM injection. Both doses of HA significantly decreased the incidence and multiplicity of adenocarcinomas in the colon compared with the control group (Le Leu et al., 2007). In a human study, patients with recently removed colonic adenoma(s) were fed native amylomaize starch (RS) or maltodextrin (control) for 4 weeks. Bile acid concentration and secondary bile aid decreased sharply in humans fed RS relative to control. Secondary bile acids are promoters of colon carcinogenesis in laboratory animals (Valhouny et al., 1984). Epidemiologic studies also showed a positive correlation between fecal bile acid concentration and CRC risk (Hill, 1991). The results from this experiment thus might correlate RS with a reduced risk of CRC (Grubben et al., 2001).

Although no study, to our knowledge, has showed the impact of starch properties and structures on different cooking methods, previous studies have shown that the heat-moisture treatment (HMT) of starches can impact the molecular structures and the properties of starches, which suggests an impact of starch properties and their effects on colon carcinogenesis. Heat-moisture treatment is defined as a physical modification that involves treatment of starch granules. Three conditions are required for the treatment: 1) moisture < 35% moisture w/w, 2) temperature at 84 – 120 °C, 3) treatment time at 15 min – 16 h. Some studies showed a decreased granular swelling and amylose leaching by HMT in legume (Hoover and Vasanthan, 1994) and tuber (Tattiyakul et al., 2006) starches. Hoover et al. (Hoover and Manuel, 1996) also reported that HMT decreased the swelling factor (amylomaize V > waxy maize > dull waxy maize > normal maize), amylose leaching (amylomaize V > normal maize), pasting viscosities (amylomaize V >
normal maize), acid hydrolysis (amylomaize V > normal maize > waxy maize > dull waxy maize), and enzyme hydrolysis (amylomaize V > normal maize > dull waxy maize > waxy maize). Shin et al. (Shin et al., 2005) tested structural characteristics of potato and sweet potato RS and suggested that partial acid hydrolysis, autoclaving-cooling, and HMT are good methods for increasing the RS content of these starches. These studies also suggested that the reduction in granular swelling and amylose leaching on HMT were due to an association of less branched starch chains during HMT. The association of the starch chains occurred within the amorphous and crystalline regions of the granules, involving the interaction between amylose-amylose and/or amylopectin-amylopectin chains. Heat-moisture treatment was also reported to decrease the enzyme hydrolysis of some resistant starches under certain treatment conditions. Kweon et al. (Kweon et al., 2000) reported a decrease in the digestibility of normal and Hylon V (57% amylose) maize starches after an HMT treatment (110 °C/16 h in the moisture range 15 - 21%). At 18% moisture, digestibility decreased by about 20, 32, 7, and 14%, respectively in normal, waxy, Hylon V, and Hylon VII (71% amylose) starches. Notably, starch digestibility increased when HMT condition changed. Starch digestibility increased on HMT (110 °C/16 h) at moisture contents in the range 24 - 27% in normal and Hylon V starches, and at 27% moisture in waxy and Hylon VII starches.

Studies also showed that HMT can increase resistant starch content. One study compared the effect of RS formation in normal and high amylose (ae VII) maize starches on HMT (30% moisture at 100- 140 °C, 80 min.) and ae-VII maize starch increases the amount of boiling-stable RS of 13, 25, and 34% at 100, 120, and 140 °C respectively by the AOAC method (Brumovsky et al., 2001). Another study reported that Hylon V maize starch (~50% amylose) increased resistant starch by 19% and decreased slow digestible starch and rapidly digestible starch levels by 12% and 6%, respectively, compared to raw
Hylon V maize starch on HMT (45% moisture, 110 °C, 4 h) and phosphorylation (sodium trimetaphosphate/sodium tripolyphosphate) (Sang and Seib, 2006).

In our studies described in this dissertation, starches used for comparison came from two different sources. In the first two studies, a high-amylose starch and stearic acid complex (SAC) was processed as a RS5. HA and a normal corn starch were used as positive and negative controls to compare the resistant and preneoplastic lesion inhibitive features with SAC. In the third study, traditional breeding of corn lines was conducted. AR 16035 (AR) and Guat 209 (Guat) were inbreds from the Latin American exotic cornlines. AR 16035 (AR) and Guat 209 (Guat) were then crossed to get the new corn line ARXGuat, with the hope that ARXGuat would inherit the high-yielding property of AR and high RS property of Guat. Starches in both experiments were cooked, which was a gelatinization process. Resistant starch contents were increased in the RS diets since they were not fully gelatinized mainly due to the higher gelatinization temperatures than the temperatures provided by the cooking methods. The starches were fed to rats as fresh as possible, and they were not stored in refrigerator to avoid retrogradation and its impact on the resistant starch contents of the RS.

Animal Model

Animal models are essential tools to study the mechanisms underlying the initiation and progression of CRC. Naturally mutant or genetically modified animals, such as APC

\[ \text{min}^{+/+} \]

mice, are used to mimic and study hereditary CRC, whereas chemically induced rodent CRC models are broadly developed to mimic non-hereditary tumorigenesis.

Azoxymethane (AOM), an organotrophic colon carcinogen, does not interact with DNA directly but is activated by cytochrome P450 and then follows a hydroxylation step. Further metabolism by colonic microflora produces methyldiazonium (Sohn et al., 2001;
Fiala et al., 1977). It initiates cancer by inducing mutagenic lesions in target cells by the alkylation of DNA at the O6 position of guanine. Azoxymethane is a downstream metabolite of dimethylhydrazine (DMH), and showed a higher potency and enhanced stability than DMH, thus AOM is now more frequently used than DMH, but both are used for many studies (Moriya et al., 1982; Rosenberg and Liu, 1995).

Azoxymethane is predominantly used as an agent for tumor initiation, but some reports its usage for tumor promotion (Bissahoyo et al., 2005). Azoxymethane treatment is mostly performed in rats, but studies have been performed with AOM in mice. Azoxymethane rodent models for CRC are widely used to study sporadic (nonfamilial) forms of colorectal cancer, similar to those in sporadic forms of the human CRC (Moser et al., 1990; Papanikolaou et al., 1998). Azoxymethane-induced tumors also resemble human CRC at the molecular level. Azoxymethane-induced tumors display aberrant expression of APC (Maltzman et al., 1997), which causes elevated β-catenin signaling and transcription of candidate target genes such as cyclin D1 and c-myc (Takahashi et al., 2000). Azoxymethane rodent models also showed a mutation of Ras, but p53 allelic loss was rarely seen (Okamoto et al., 1993). On the other hand, the tumor initiation induced by DMH does not appear to involve APC or p53 mutations (Moen et al., 1996). Repetitive administration of AOM produces tumors in the distal colon of rodents, and AOM-induced carcinogenesis is organ-specific (Shamsuddin and Trump, 1981; Druckery, 1972; Papanikolaou et al., 1998, Delker et al., 1999).

Azoxymethane is one of the most successful carcinogens used in the induction of CRC in rodent models. Meta-analysis revealed that the use of AOM-induced rodent models of carcinogenesis predicted chemopreventive efficacy in humans better than other models (Corpet and Pierre, 2005). It also has the advantage of low cost, simple application, and high potency and reproducibility. Markedly, although AOM has many
advantages, the tumors induced by AOM rarely show the feature of mucosal invasiveness and metastasis, which are common in human CRC (Boivin et al., 2003; Nambiar et al., 2003; Shamsuddin, 1984).

Repeated intraperitoneal injections of AOM to rodents are highly efficient in the induction of colon tumors in rodents. The susceptibility of different rodent strains to AOM, intestinal microflora, and the AOM preparation method also impact the induction of CRC by AOM. In rat studies, a standard protocol for F344 rats is treating animal with no more than two injections of 15 mg AOM per kg body weight (Kawamori et al., 1999).

Dextran sodium sulfate (DSS), a pro-inflammatory reagent, induces rodent models for colitis-associated tumor development, especially that by chronic colitis in ulcerative colitis or Crohn’s disease (Okayasu et al., 1990). DSS is usually applied together with AOM. A two-stage colon tumor model may use a single injection of AOM and a single cycle of DSS for 20 weeks to mimic colitis-driven tumor development (Tanaka et al., 2003; Suzuki et al., 2006). One of the disadvantages of DSS is that the model, either with or without carcinogen, needs a long period or repeated administration of DSS to induce colitis and colitis-related CRC, and the occurrence and multiplicity of the tumors are relatively low (Okayasu et al., 2002).

Another rodent model to develop tumors uses 2-Amino-1-methyl-6-phenylimidazo [4, 5-b] pyridine (PhIP). Aberrant crypt foci (Takahashi et al., 1991), putative preneoplastic lesions of the colon (Bird, 1987), and CRCs (Ito et al., 1991; Hasegawa et al., 1993) have been reported to be induced by PhIP in F344 male rats. Mutations and suppressed expression of Apc gene was frequently observed in PhIP-induced colon tumors (Kakiuchi et al., 1999; Ubagai et al., 2002), resembling the process in human CRC case (Kinzler and Vogelstein, 1996; Polakis, 2000).
Some animals widely used in the testing of CRC occurrence by environment factors are Fisher 344, Sprague-Dawley or Wistar rats. Usually male rodents of 5-6 week old, 180-200 g in weight are ideal. Two injections of AOM at 15mg/kg body weight are popularly conducted.

**Preneoplastic lesions**

*Aberrant Crypt Foci (ACF)*

Aberrant Crypt Foci have been identified as putative precancerous lesions of the colon. Aberrant crypt foci were first described by Bird in methylene blue–stained colonic mucosa from rodents treated with AOM in 1987 (Bird, 1987). ACF are featured as: (1) having altered luminal openings; (2) exhibiting thickened epithelia; and (3) being larger than adjacent normal crypts (Bird et al., 1989). ACF can be divided into 3 categories histologically: nondysplasia, dysplasia, and a mixed type of both hyperplasia and dysplasia. Nondysplasia can be further divided into 2 subtypes: ACF with normal mucosa and ACF with hyperplasia. Aberrant crypt foci with normal mucosa lack significant modifications of the epithelium lining the crypts; they have enlarged crypts (at least 1.5 times larger than normal) with only slightly enlarged and elongated nuclei, but no mucin depletion. Crypt cells with positive staining of PCNA and Ki-67 remain at the lower part of the crypts. Hyperplasia has larger or longer crypts than normal crypts, sometimes showing apical branching. The luminal opening is serrated and slightly elevated from the surrounding normal mucosa, with partial mucin depletion, but without dysplasia. Nuclei are enlarged or sometimes crowded without stratification. Cells with positive staining of PCNA and Ki-67 remain at the lower and middle parts of the crypts. Dysplasia is also called microadenoma. Crypts of dysplasia have enlarged, elongated and sometimes stratified and depolarized nuclei. The number of goblet cells is decreased.
obviously and mucin is depleted. The major site of PCNA and Ki-67 positive staining is extended to the upper part of the crypts. Dysplastic ACF occurs commonly in FAP patients, but not in sporadic patients. Sporadic ACF patients have less frequent APC mutations than FAP patients, and more frequent methylation. The third type is a mixed type of hyperplasia and dysplasia.

Aberrant crypt foci were first reported in both experimental animals and human as a potential biomarker of human colorectal cancer (Roncucci et al., 1991; Pretlow et al., 1991; Tudek et al., 1989; McLellan, 1988). Aberrant crypt foci were considered to play an important role in the adenoma-carcinoma sequence in epidemiologic studies supporting ACF as a biomarker for colorectal carcinoma. Genetic abnormalities were also described in ACF (Gupta et al., 2007). Another review summarized publications of ACF incidence as well as ACF numbers or size, with subject characteristics, such as age or family history of CRC. The review concluded that ACF can be used as an intermediate endpoint due to the relationship of ACF to the known risk factors for CRC (Stevens et al., 2007).

Using ACF in a rodent model as a biomarker is possible in a short term of experimental period of 8 weeks. Aberrant crypt foci are specific to colon carcinogenesis, and are induced by colon specific carcinogens in a dose dependent manner, and size and crypt multiplicity increase with time as the disease develops (Bird, 1995). Several factors play a critical role in the incidence of ACF and tumors, including: carcinogen dosage, rodent strain, administration method, and the intervention time at the carcinogenic state, etc. For example, the frequency of carcinogen injection affects the number and growth features of ACF. Testing the ACF number and other features of rats injected with AOM once a week for 1, 2 or 4 weeks showed that the rats injected 4 times or twice had a similar number of ACF at week 6 after the last AOM injection. At week 18, rats with 4
AOM injections induced a significantly higher number of ACF with advanced growth features than those had 2 AOM injections.

The ACF incidence in rodents is strongly associated with tumor occurrence. In one study, male Sprague-Dawley rats were fed diets with cholic acid (CHA), a colonic tumor promoter (Magnuson et al., 1993). In this study, crypt multiplicity, rather than the number of ACF at early time points was considered a good predictor of tumor incidence. The increased crypt multiplicity in rats fed CHA suggested that the growth of a small number of ACF caused an increased number of tumors compared to a control AIN76 diet. In another study, the incidence of ACF at an early time period was compared with the incidence of tumors later in F344 rats treated with AOM and CRC chemopreventive agent, sodium phytate. The results showed that the development of larger ACF (with four or more crypts) was predictive of the tumor incidence (Pretlow et al., 1992).

Aberrant crypt foci appear within two weeks after carcinogen injection, and each ACF evolve from one altered crypt. As time progresses, a single crypt expands by crypt branching or multiplicity and develops into more than one crypt (Bird, 1998). Aberrant crypt foci in colons are in a dynamic state. Some may remodel or regress or even get eliminated; others may progress forward to the next stage. Thus the number of ACF may vary with time (Cheng and Lai, 2003). One study explained how ACF grow in different stages of carcinogenesis. Sprague-Dawley rats were injected with a single dosage of 1,2-dimethylhydrazine-HCL at varying time points ranging from 2 to 57 weeks. Two weeks after the second injection, a large number of primal ACF (1-3 crypts/foci) were observed in the rat colons. From post-carcinogen injection at week 6, an increasing number of intermediate (4-6 crypts/foci) or advanced (>6 crypts/foci) ACF and a decreased incidence of primal lesions were observed. Beyond 24 weeks post-carcinogen injection, which can be included in the progression stage, the incidence of advanced ACF reached a
peak and occurrence of microadenomas, adenomas, and adenocarcinomas were observed (McLellan et al., 1991).

The time of carcinogen administration is vital in determining the yield of the preneoplastic lesions. An agent can be a tumor inhibitor or promoter depending on the time point it is fed to the animals relative to the carcinogen administration. When a compound is added to the diet at the first AOM injection, the experiment is directed at the effect to prevent the incidence and growth of ACF at the initiation and post-initiation stage by the compound. If the compound is fed at a later time point, e.g. 4-8 weeks after first AOM injection, the emphasis is the impact on ACF at the post-initiation and promotion stages (Bird and Good, 2000). Cholic acid (CHA), a primary bile acid, was generally expected as a colon carcinogenesis promoter, but one study showed how different feeding time points before or after carcinogen treatment affected the result of the colon carcinogenesis (Baijal et al., 1998). In this study, Male Sprague-Dawley rats were injected twice, 1 week apart, with AOM. Following the first AOM injection, animals were randomly assigned to two groups and fed control AIN-93G diet (CON) or control diet containing 0.2% CHA (CHA). Three weeks after the first injection, the animals were randomly assigned to CON or CHA diets within each present diet group, thus four treatments were formed: CON-CON, CON-CHA, CHA-CHA, and CHA-CON. After 3, 12, and 20 weeks following the first carcinogen injection, the rats were killed and the number and crypt multiplicity of ACF were counted, and tumors were evaluated at week 20. Numbers of medium (4-6 crypts/focus) and large (> or = 7 crypts/focus) ACF and average crypt multiplicity were significantly higher in CHA-CHA and CHA-CON compared with CON-CON and CON-CHA. CHA-CHA had the highest tumor incidence followed by CHA-CON, and tumor multiplicity and number of tumors per rat in CHA-CON were similar to CHA-CHA. CON-CHA produced a tumor outcome similar to CON-
CON, it did not enhance colonic tumor incidence comparing to the other two groups. These results suggested that transient exposure to CHA (CHA-CON) was sufficient to induce an increased ACF and tumor yield, yet a delayed intervention with CHA (CON-CHA) did not enhance colonic tumor incidence. It could be concluded that CHA was effective in enhancing colon carcinogenesis during early phases and ineffective in post-initiation phases. In our studies, rats were injected with AOM twice with one week between injections. Resistant starch diets were fed 3 days after the second AOM injection, which fell into the promotion stage of tumorigenesis.

Distribution of ACF could be in the proximal, mid and distal section of the colon in AOM-injected Fisher 344 rats. In a previous study, F344 rats were treated with 2 doses of azoxymethane (15 mg/kg body weight, s.c.) one week apart, and sacrificed 6, 12, 20, 30 and 36 weeks after the first carcinogen injection. Control groups of untreated rats were sacrificed at the same time points. The number of ACF per area, their multiplicity (number of crypts per focus), ACF frequency and multiplicity according to each colonic site, histology of ACF and macroscopic lesions were recorded and compared between AOM-injected and control groups. In the AOM-injected group, higher ACF incidence happened in the mid colon than in the distal colon and rectum, and they were rare in the proximal colon and cecum. On the other hand, adenomas and well-differentiated adenocarcinomas were found in the distal colon. All proximal neoplasms were signet ring cell carcinomas, suggesting signet ring cell carcinomas had a different pathway of growth than ACF. It also indicated that ACF distribution was not correlated to adenoma and adenocarcinoma distribution (Ghirardi et al., 1999).

All of our studies utilized AOM induced Fisher 344 rats as our animal model. Azoxymethane was administrated before the feeding of RS to the rats, so that the prevention of preneoplastic lesions in the promotion stage could be assessed. The rats
were fed RS or relative controls for 8 weeks, and then euthanized for the endpoint test. Preneoplastic lesions were collected and counted in the distal region of the rat colon.

During the formation of ACF, or when cells turn from ACF to adenomas, a lot of genes are changed in their expressions. Some of the key genes are listed below.

β-catenin, a transcriptional activator, has an important role in epithelial tumorigenesis. β-catenin belongs to the β-catenin-Tcf oncogenic pathway. Overexpression of β-catenin protein was reported in both humans and rats (Takahashi et al., 1998; Morin et al., 1997). In ACF with hyperplasia, β-catenin was found at the cell membrane, same as in normal colon epithelium. In ACF with dysplasia, as well as in adenoma and carcinoma, β-catenin was localized less in the membrane and more in the nuclei and cytoplasm (Hao et al., 2001).

There was no difference between hyperplastic ACF and normal mucosa when comparing microsatellite instabilities and mutations of APC, H-ras, k-ras, p53, DCC, and DNA repair genes hMLH1, hMSH2 (Sedivy et al., 2000). But hyperplastic ACF had frequent K-ras mutations (Otori et al., 1995), chromosome 1p loss (Chan et al., 2002), and CpG island Methylation (Chan et al., 2002). Of these gene alterations, K-ras mutation was one of the major events in ACF formation (Stopera et al., 1992). The mutation rate of K-ras in ACF was similar to that of small adenomas, and was higher in occurrence than in CRC (Stopera et al., 2000).

COX and c-myc, two oncogenes, were also found to have an increased mRNA or protein expression in carcinogen induced rats (Furukawa et al., 2002). The expression of the oncogenes cfos, ras, bcl-2 and p53 were abnormal in ACF (Otori et al., 1999). The APC gene is considered a “gatekeeper gene”, maintaining the stability of the colon epithelium. A decreased mRNA expression of APC was observed in ACF both in human and in carcinogen treated rats (Furukawa et al., 2002), but the mutation rates of APC in
ACF was lower or undetectable compared to those in adenomas and carcinomas, suggesting that an APC mutation was not present during the initiation stage of ACF (Yuan et al., 2001). If a ras gene mutation occurred first, ACF would be nondysplasia; if an APC mutation occurred first, ACF would be dysplasia, and a K-ras mutation and other gene mutations would follow (Takayama et al., 2001). Accordingly, in sporadic colorectal carcinogenesis, K-ras mutations mainly occurred during the formation of ACF. APC mutation occurred when ACF turned into adenomas. In FAP, somatic mutations of APC occurred first during ACF formation, followed by K-ras mutations ((Jen et al., 1994).

The mismatch repair (MMR) system is a DNA repair process. Inactivation of MMR can result in instability of the whole genome and an increased rate of spontaneous mutations of other vital genes to carcinogenesis. MMR was also found to be involved in cell-cycle regulation, and p53-dependent apoptotic response to a variety of DNA damages (Zhang et al., 1999). Germline mutations of MMR, especially hMSH2 and hMLH1, increase the incidence of HNPCC (Peltomaki et al., 2001).

CpG island methylation, an epigenetic alteration, and microsatellite instability (MSI), a genetic phenotype alteration, also play important roles in the development of ACF. Silencing of genes mediated by CpG island methylation may inactive the gene, and further cause the incidence of ACF or CRC. For example, hMLH1 gene promoter was aberrantly methylated in sporadic CRC (Veigl et al., 1998). MSI could occur either in dysplastic or hyperplastic ACF from HNPCC patients. MSI was also seen in some sporadic ACF. In HNPCC patients, carcinomas with high levels of MSI were a hallmark (Augenlicht et al., 1996).

*Mucin Depleted Foci (MDF)*

MDF, another preneoplastic biomarker in rat colon carcinogenesis, feature absent or scarce mucous production. It is a focal lesion formed by crypts with a lumen, which is
often distorted when compared with normal surrounding crypts (Caderni et al., 2003). Elevation of the lesion above the surface of the colon, and multiplicity of the foci are also frequent features of MDF. MDF can be identified using Alcian blue, which stains acid mucin in blue. If stained by alcian blue-neutral red, the epithelial layer of the mucosa is shown as a reddish background stained by neutral red, and the acid mucin was stained by alcian blue as blue spots. MDF are viewed as a reddish background surrounded by normal shaped and sized blue spots. MDF can also be stained by high-iron diamine Alcian blue. MDF occur at a lower incidence than ACF in rats when the same dosage of carcinogen was administered, but MDF are more dysplastic than common ACF. In a study comparing the relationship among ACF, MDF and BCAC in male F344 rats given DMH, MDF were suggested to have more potential in predicting CRC than ACF, and MDF with more than 4 crypts were shown to overexpress $\beta$-catenin (Yoshimi et al., 2004).

MDF were suggested as a subgroup of ACF and may predict tumor outcome better than ACF. Male F344 rats were first fed synbiotics, an intestinal cancer reducer, or control diet for 10 days. Rats were then injected AOM twice, 1 week apart. Aberrant crypt foci or MDF were collected 15 weeks after the first AOM injection and tumors were detected 31 weeks after first AOM injection. MDF appeared 7 weeks after AOM treatment. The number and multiplicity of MDF at 15 weeks after first AOM injection were correlated with carcinogenesis. On the contrary, ACF number was not correlated with tumor outcome (Caderni et al., 2003).

**Beta-catenin-accumulated crypts (BCAC)**

BCAC are pre-malignant lesions of CRC, featured as dysplastic crypts with overexpress of $\beta$-catenin. BCAC are frequently accompanied by Paneth cells. The number of the crypts/lesion and histological abnormality of BCAC were found to significantly
increase with time, with higher cell proliferative activity than in ACF. The most common β-catenin gene mutation in BCAC is the same as that detected in colon tumors, suggesting that BCAC are the pre-malignant lesion and that these mutations play gatekeeper roles in the development of colon tumors. BCAC, as well as colon tumors, have β-catenin gene mutations more frequently than typical ACF in both cytoplasm and nuclei, indicating the potential of BCAC to progress into malignant lesions. β-catenin mutations were detected in both well differentiated and poorly differentiated adenocarcinomas. The above results indicated that activation of β-catenin signaling pathway is not only important in the initiating stage, but also in the promotion stage of colorectal carcinogenesis (Yamada et al., 2003). Among the early lesions, ACF showed more frequent K-ras mutations than BCAC, indicating that K-ras mutations are closely associated with the formation of typical ACF.

Chemopreventive agents or tumor promoters have been applied to alter the development of BCAC. Peucedanum japonicum, an herb in the Ryukyu Islands, was reported to inhibit BCAC in AOM-injected male F344 rats (Morioka et al., 2004). Azoxyymethane-treated F344 rats were fed CHA (0.5%) diet during the post-initiation phase significantly reduced the total number, multiplicity and size of ACF, as well as the number of large ACF (>4 aberrant crypts/focus). CHA significantly enhanced the multiplicity, size and the number of large BCAC (> 6 crypts/lesion), but not the incidence of BCAC when compared with the control diet group (Hirose et al., 2003).

In summary, ACF and its subset MDF are among the most widely used precancerous biomarkers that predict the incidence of sporadic CRC. BCAC, putativepreneoplastic lesions independent from ACF, have recently been suggested as intermediate biomarkers for colon carcinogenesis. All these preneoplastic lesions can be induced by AOM treatment. ACF and MDF can be easily observed using an optical microscope after dye
staining, while BCAC have to be detected by immunohistochemistry. Some studies also showed that ACF were more sensitive than BCAC in assessing the impact of continuous feeding of certain diets (Hirose et al., 2003). ACF/MDF detection thus might be a simpler, more sensitive way to predict colon carcinogenesis, but a combination of the two methods could be considered a more reliable prediction and thus both of these lesions were assessed in our studies.

**Short Chain Fatty Acids (SCFA)**

Among SCFAs, acetate is the most abundant in fecal and cecal samples, followed by propionate and butyrate. Other organic acids, such as lactate and succinate, are also found in SCFA, but in much smaller amounts (Hoverstad and Bjorneklett, 1984; Takahashi et al., 1993).

Consequences of fermentation in the large bowel include the increase of cecal or fecal weight, reduced transit time, and increased cecal pH, which have possible implications for protection against large bowel cancer. First, an increased fecal weight was observed mainly due to the increase in bacterial cell mass (Stephen and Cummings, 1980). Resistant starch and other fermentable fibers are only partially fermented, so their residual polysaccharides absorb water and add to the fecal weight (McBurney et al., 1985). Consequently, a reduced transit time is observed, and the contents of the large bowel lumen are diluted, which would reduce the time putative carcinogens are in contact with the large bowel mucosa (Burkitt, 1969). Short Chain fatty acids are relatively weak acids and raising SCFA concentrations through fermentation decreases digesta pH. When high-amylose starch, wheat bran, or oat bran was fed, pH values decreased significantly since these are fermentative substrates (Noakes et al., 1996; Lampe et al., 1993; Kashtan et al., 1992). Short Chain fatty acids concentration may be the most important impact
factor for the decrease of pH. One report revealed that there was a negative relationship between fecal SCFA and pH (Segal et al., 1995). The lowered pH values may prevent the pathogenic bacteria from rapid growth. In a swine study, the increased content of SCFA was reported to be related to the lowered numbers of potential pathogens such as *Salmonella* (Prohaszka et al., 1990).

Studies were reported that the consumption of RS can increase SCFA in rat cecum and colon. Rats fed 350 g raw potato starch/kg have a higher concentration of SCFA in the cecum, compared with rats fed digestible wheat starch (Younes et al., 1996). Feeding rats with raw pea also resulted in an increased butyrate proportion while the proportion of acetate, isobutyrate, isovalerate and valerate decreased compared with rats fed maize starch (Goodlad and Mathers, 1990).

Resistant starch or dietary fibers were reported to protect against colorectal cancers. A significantly increased concentration of acetate and propionate in intestinal contents was observed in rats treated AOM and fed high amylose maize starch. A decrease in the number of ACF was also observed in the same group of rats. When clostridium butyricum, an anaerobic prokaryote that utilizes HA and produces butyrate and acetate, was fed to rats together with HA, a more significant increase of acetate and propionate concentration, as well as a decrease in ACF was found (Nakanishi et al., 2003). Rats with tumors induced by dimethylhydrazine were fed different fibers and assessed the number and size of tumors. Significantly fewer tumors were observed in the rats fed wheat bran compared with those fed guar or oat bran. The concentration of fecal butyrate strongly and negatively correlated with tumors (Weaver et al., 1988).

Short Chain fatty acids were found to have paradoxical effects on the proliferation of colon epithelial cells. Short Chain fatty acids stimulate proliferation in normal colonic epithelium, while butyrate and propionate reduce proliferation of cancer cells. An *in vitro*
study used biopsy specimens obtained from normal individuals at routine colonoscopy. Tissues were incubated with sodium salts of SCFAs and cell proliferation was found to rise significantly in all incubations (Scheppach et al., 1992). The effect of SCFA on cell proliferation was also reported to be dose-respond. Luminal and serosal n-butyric acid (0, 0.1, 1, or 10 mmol/L, adjusted to neutral pH) were assessed on the epithelial cell proliferation of pig colonic mucosa in organ culture. Crypt cell production rate was used as the measurement of proliferative activity. With 0 or 0.1 mmol/L serosal n-butyric acid, luminal n-butyric acid increased crypt cell production rate at 1 mmol/L, and decreased crypt cell production rate at 10 mmol/L when compared to the controls with 0 mmol/L luminal n-butyric acid. With 1 or 10 mmol/L serosal n-butyric acid, luminal n-butyric acid depressed organ culture taking crypt cell production rate dose-dependently. This experiment indicated that n-butyric acid stimulated colonic epithelial cell proliferation at low concentration and inhibited it at high concentration (Inagaki and Sakata, 2005).

Of all the SCFAs, butyrate is considered to mostly relate to CRC inhibition. Butyrate is well recognized as an anti-proliferative agent, differentiation-inducing agent, and apoptosis-inducing agent. Butyrate works as an anti-proliferative agent through inhibiting DNA synthesis and cell growth via inhibition of histone deacetylase, while other SCFA are much less active in this respect (Kruh, 1982). The effect is reversible when butyrate is removed from culture media. Butyrate enhanced repair and survival of human adenocarcinoma cell lines which were suffered from ultra violet radiation damage. The mechanism was revealed as the histone acetylation which allows the enzymatic accessibility of chromatin in permeabilised cells (Smith, 1986). As a differentiation-inducing agent, SCFA reduced cloning efficiency and induces the activity of membrane glycoproteins such as alkaline phosphatase, a commonly used marker of differentiation, and altered the subcellular distribution pattern of the disaccharidases in some human
colorectal cancer cell lines (Chung et al. 1985). Other SCFAs did not show such an effect compared with butyrate (Whitehead et al. 1986). Butyrate did not show this effect in all cell lines, though. In one line derived from familial adenomatous polyposis, butyrate was reported to increase malignancy (Berry and Paraskeva, 1988). Apoptosis is considered a final stage of colonic epithelial cell differentiation pathways. Studies have accumulated in epithelial cell culture models proving the active effect of butyrate in cell apoptosis. For example, butyrate was detected and associated with apoptosis in cell lines derived from colonic adenomas and carcinomas (Hague et al., 1993). Butyrate also induced typical apoptotic morphology of condensed chromatin and an associated DNA fragmentation pattern when it was added in culture medium of unattached cells (Wylie et al., 1980). Propionate and acetate were also found to induce apoptosis in colon tumor cell lines, such as AA/C1 and RG/C2, but both propionate and acetate induced apoptosis in a less level compared with butyrate (Hague et al., 1995)

**Gut microflora, SCFA and CRC**

There is a large population of bacteria in rodent and human cecum and colon. Many species of bacteria live in the mammalian large intestine, with up to $10^{11} - 10^{12}$ cells/g of luminal contents (Simon and Gorbach, 1984). About 60% of fecal solids are also bacteria, with anaerobic bacterial far outnumbering aerobic bacteria (Salminen et al., 1998). In human feces, more than 50 genera and 400 species of bacteria have been identified, most of which are anaerobes, such as bacteroides, bifidobacteria, eubacteria, streptococci, and lactobacilli (Gibson and Roberfroid, 1995; hill, 1995). A number of bacteria can secrete hydrolases which the human host cannot secrete to hydrolyze carbohydrate. Of all the bacteria living in the colonic lumen, some can infer important health benefits to the host, and others can be pathogens in certain circumstances. For example, some genera of
microflora are related to the incidence of CRC in different directions. Bacteria of the
bacteroides and clostridium genera were reported to increase the incidence of CRC in
animals, whereas other genera such as lactobacillus and bifidobacteria prevent colonic
tumorigenesis (Horie et al., 1999; Singh et al., 1997; Pool-Zobel et al., 1996). One major
metabolic function of gut microflora is to ferment non-digestible dietary residues by
various enzymes and biochemical pathways provided by these gut microflora, and
produce energy and nutrients for both themselves and the host. Non-digestible
carbohydrates, such as resistant starch, are used as fermentable substrate to generate
SCFA as metabolic endpoint, and the fermentation differs in the different part of large
intestine. In the cecum and right colon, fermentation is very intense, resulting in high
SCFA amount, acidic pH, and rapid growth of bacterial. In the left colon, SCFA are at a
lower concentration, the pH is close to neutral and bacterial populations are close to
static. On the other hand, a higher incidence of polyps was observed in the distal colon,
which might relate to the lower SCFA concentration in this region (Govers et al., 1999).
In particular, butyrate is the major energy source for the colonic mucosa and has been
implicated in the protection of CRC. As evidence, butyrate inhibits cell proliferation and
stimulates cell differentiation in human neoplastic epithelial cell lines (Siavoshian et al.,
2000), and reversely express phenotypic markers of differentiation in neoplastic cells or
normal epithelial cells (Gibson et al., 1992). In addition, apoptoses may be increased in
neoplastic cells and inhibited in normal epithelial cells by adding butyrate (Diez-
Gonzalez et al., 1999). Butyrate was also observed to modify gene expression by being
involved in chromatin hyperacetylation and DNA methylation in the colon (Csordas,
1996; Pool-Zobel et al., 1996), but how gene expression alterations by butyrate are related
to CRC is not known yet. Studies have been accumulated to explore the link between
SCFA, protection impact against CRC and RS. It has been presumed that RS may affect
the colonic fermentation and further inhibit CRC by modifying the gut microbial population. A study on human flora-associated rats showed increased colon butyrate and increased numbers of gut microflora that produce SCFA, such as lactobacilli and bifidobacteria in rats fed a retrograded high amylose starch compared to sucrose-fed rats (Silvi et al., 1999).

In our studies assessing the inhibitive effect of cooked SAC on carcinogenesis, cecal and fecal SCFA amount and concentrations were tested from fresh cecum content and feces of rats after 8-week feeding of SAC or the controls. The cecal weight change and pH alteration were also assessed as a reflection of RS fermentation in the large bowel. These results could be linked with the incidence of ACF/MDF in the rat colon as an indirect proof of gut microflora to utilize RS and impact carcinogenesis.

In our studies, we assumed that a reduced preneoplasia, either ACF or MDF, occurred when the cooked resistant starch diets were fed to the rats. The cooked resistant starch diets were fermentated by gut microbes and produced SCFA. The inhibitive effect of the cooked resistant starch diets might come from the impact of both the metabolism of SCFA and the changed gut microbial population.

**Genetics of colorectal cancer**

CRC is a genetic disease. The initial genetic alteration of a cell starts aberrant proliferation, followed by the accumulation of more mutations. Thus some subclones developing enhanced growth properties transform to malignant and invasive cancers. This process is mainly a result of mutational activation of oncogenes and mutational inactivation of tumor suppressor genes. Alteration of stability genes, such as Microsatellite instability (MSI) and mismatch repair (MMR) are also important events responsible for tumorigenesis.
Genetic alterations in oncogenes

One important oncogene alteration is ras mutation. Ras gene mutation was usually a point mutation and was found in approximately 50% of colorectal carcinomas and adenomas which are greater than 1 cm in size, but they were found in less than 10% of the adenomas with size smaller than 1 cm (Vogelstein et al., 1988), indicating that ras gene mutations may be responsible in the later adenoma progression, rather than the initiating event in most tumors (Pretlow et al., 1993). Mutated ras protein is active in pathways such as RAF/MAPK, JUN and PI3K (Downward et al., 2003).

Oncogene alteration was also seen as gene amplification and rearrangement, including the amplification of meu, c-myc, and c-myb, as well as the rearrangement of trk gene. c-myc is highly expressed in most colorectal carcinomas, and it was frequently revealed in the distal colon (Steward et al., 1986).

Tumor suppressor genes

The mutation of tumor suppressor genes mainly occurs as an event of loss of specific chromosomal regions. The mutation of p53 gene occurs with the allelic loss of chromosome 17p in colorectal tumors. P53 protein regulates DNA repair during replication and induces apoptosis. Wild-type p53 gene, identified as a tumor suppressor, inhibits colorectal tumor growth. p53 mutations occur mostly from the transition from adenoma to cancer, and have been identified in 40-50% sporadic colorectal cancers (Baker et al., 1989).

Another common allelic loss in colorectal tumor occurs in chromosome 18q, leading to the gene mutation of DCC and SMAD4/2. DCC gene functions in cell adhesion. Reduction of its expression in colorectal epithelial cells may alter adhesion of normal cell-cell and cell-extracellular matrix interaction (Hoffman and Edelman, 1983). Inactivation of SMAD4 is associated with cancer progression through TGF-β/SMAD
signaling pathway. SMAD4 gene expression was found in 16-25% colorectal cancer cases, and SMAD2 gene mutation was found in 6% of the cases (Thiagalingam et al., 1996; Eppert et al., 1996).

APC gene mutation is critical in the early development of colorectal cancer. Loss of function mutations in APC or activating mutations of β-catenin result in the stabilization and accumulation of β-catenin in the nucleus, activating the transcription factor T-cell factor (Tcf-4), and further activating the β-catenin/Tcf-4 pathway which switch the cells from differentiation to proliferation in the intestinal crypt epithelial cells (Van de Wetering et al., 2002).

**MSI and MMR**

MSI are characterized by expansions or contractions in the number of tandem repeats of simple DNA sequences. MSI are euploid tumors without allelic losses (Ionov et al., 1993). MMR genes are responsible for correcting the ubiquitous nucleotide base mispairs and small insertions or deletions that occur during DNA replication (Papadopoulos et al., 1995). Frequent mutations of MSI includes the TGF β-RII, BAX, TCF4, Caspase 5, HIF1 α, BRAF and PI3KCA (Popat et al., 2005). In human CRC, MMR enzymes, including hMSH2, hMLH1, hPMS1, hPMS2, and hMSH6, have since been shown to be responsible for MSI (Bronner et al., 1994; Nicolaides et al., 1994). MMR related genes include hMSH2 (human mutS homolog 2), hMLH1 (human mutL homolog 1), hPMS1 and hPMS2 (human postmeiotic segregation 1 and 2), hMSH6 (human mutS homolog 6), and hMLH3, a mismatch repair gene that interacts with MLH1 (Papadopoulos et al., 1995).

A well-known colon model for tumorigenesis was proposed as an adenoma-carcinoma sequence. In this model, transition from normal epithelium to adenoma and carcinoma is associated with acquired molecular events, including the mutation and
alteration of the genetic events described above. The accumulation of germline or somatic mutations, rather than their sequence with respect to one another, determines the biological behavior of the tumor. At least four to five gene mutations occur in the malignant tumor, while fewer are required for benign tumors. In some tumor suppressor genes, the mutation of one allele may be sufficient for an altered cellular phenotype, so these tumor suppressor genes might not be recessive at the cellular level (Fearon and Vogelstein, 1990).

**Overall Summary**

In our studies, the RS were obtained by different methods that were assumed to improve their resistance in the gastrointestinal tract. Our goal was to determine the health benefits of these RS to humans after they were cooked. Specifically, we would like to know the ability of these cooked RS on the reduction or prevention of colon cancer. To reach this goal, we first evaluated the inhibitory effects of the RS with known resistance on colorectal preneoplastic lesions in an AOM-induced F344 rat model for a short term of 8 weeks. We also measured the SCFA amount and concentrations, as well as cecal fermentation, as indirect evidence of altered gut microbial metabolism by the fermentation of the RS. Global gene screening was applied to colon samples from AOM-treated rats fed RS diet or CS diet, or the CS diet-fed rats treated with AOM or saline, respectively, by microarray assessment to discover differentially expressed genes after RS diet treatment or AOM treatment, respectively. Several potential target genes that were differentially expressed during the treatment of SAC on AOM-induced rats were assessed by RT-qPCR as preliminary studies to explore the molecular genetic mechanism of the carcinogenesis inhibition by cooked RS. These differentially expressed genes were
selected for RT-qPCR because they were previously shown to correlate with colon cancer occurrence, thus having potential to explain how the modulation of the genes by cooked RS may have impacted the incidence of CRC. Further, some of these genes were of particular interest since they showed totally opposite directions of expression by AOM treatment and cooked RS diet treatment respectively, indicating their potential to reverse the progress of AOM induced CRC after cooked RS diets were fed.

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CHAPTER 2: INHIBITION OF AZOXYMETHANE-INDUCED PRENEOPLASTIC LESIONS IN THE RAT COLON BY A COOKED STEARIC ACID COMPLEXED HIGH-AMYLOSE CORNSTARCH

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\textbf{KEYWORDS:} stearic acid complexed high-amylose cornstarch; resistant starch; cooking; aberrant crypt foci; mucin depleted foci; short chain fatty acids

\textbf{Abstract}

We evaluated a novel stearic-acid-complexed-high-amylose-cornstarch (SAC) for the prevention of preneoplastic lesions in the colon of azoxymethane (AOM)-treated Fisher344 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 MDF numbers compared with w-HA or w-CS. r-HA significantly decreased ACF numbers compared with r-CS or r-SAC. Increased cecum weight and decreased cecum pH were observed in SAC or HA groups. The highest amounts of total or individual SCFAs in cecum, and butyrate or propionate in feces were observed in the AOM-treated w-SAC group. This study revealed the effectiveness of a novel resistant starch on inhibiting colonic preneoplastic lesions and the importance of high moisture cooking on the suppression of colon carcinogenesis by this resistant starch.

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, which is not digested and absorbed in the small intestine of healthy individuals. Resistant starch 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 the 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. A newly developed type 5 RS consisting of amylose-lipid complex has been introduced recently (13), and the effects of this starch on colon cancer prevention were not 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 prevent 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 $10^{10}$ to $10^{11}$ 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 intestines 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 amylose-lipid complex (RS5) 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, i.e., 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), but it can offer better potency and stability than DMH (18, 19). Because starchy food in 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 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., Minneapolis, MN), 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 based on the standard diet recommended by the American Society for Nutritional Sciences for mature rats (AIN-93M) (20) (Table 1). In the two experiments, the 3 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 the 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 minutes to room temperature. The water-boiled starch pastes were then mixed with the powder containing the rest of AIN-93M diet ingredients and made into diets. Diets were prepared every two 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 were measured by the Megazyme/AOAC method 2002.02 (21), and the resistant content of the diets in the cooked diet experiment were assessed by AOAC method 991.43 (22). Water content of these samples was determined by drying the diet in an oven at 105°C for 3 hours. 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 forty-five 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 relative humidity at 60%±5%, and fluorescent lights were on from 6 AM to 6 PM. 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.
Carcinogen Treatment. Azoxymethane was purchased from Midwest Research Institute (Kansas City, MO) and F344 rats were injected at seven 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 most lesions and did not show toxicity in the rats.

Cecal Weight and pH. Five-week-old F344 rats were fed with control cornstarch diet (r-CS or w-CS for the respective experiments) for 2 weeks. Then 2 AOM or saline injections were administered to the rats one week apart. During the injection period and three days after the second injection, the rats were fed with r-CS or w-CS for the raw and water-boiled starch experiments, respectively. Then the rats were divided randomly into 3 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 3 starch diets were fed to the diet groups respectively for 8 weeks. Body weight was measured weekly. Fresh food was provided every two days in a pre-weighed 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 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 75mm were trimmed and fixed in 10% formalin for 24 hours before they were made into specimens. Colons were stained with 1% alcian blue in acetic acid (pH 2.5), then counterstained with 1% neutral red to view ACF and MDF. Aberrant crypt foci were defined as crypts that: (i) have altered luminal openings; (ii) exhibit thickened epithelia; (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.** Short Chain fatty acids were measured in the water-boiled starch experiment only. Fresh fecal samples were collected two days before rats were sacrificed. Cecal and colonic contents were collected during the autopsy at the end of the study. All the samples were kept at -50°C until analysis. Short Chain fatty acids, 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 10min; 1mL supernatant was mixed with 100 µmol 2-ethyl-butyric acid (Sigma-Aldrich, St. Louis, MO) as internal standard. Hydrochloric acid was added to protonize SCFAs, followed by diethyl ether (2mL) extraction and derivatization with N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide (Sigma-Aldrich, St. Louis, MO) at 80°C for 20 minutes. Short Chain fatty acids silyl derivatives (1ul) were injected in a split mode into a Gas Chromatograph (Model HP 6890N) (Hewlett-Packard, Roseville, CA) equipped with a flame ionization
detector and a SPB5 capillary column (30 m×0.25mm i×d, 1µm film; Supelco, Inc). Helium was used as carrier gas. The initial oven temperature was held at 70°C for 4 min, and was increased at 7°C/min to 160°C and retained at this temperature for 5 min. The injector temp was 200°C, and the detector temperature was 220°C. A standard SCFAs mixture containing acetate, propionate and butyrate (Sigma-Aldrich, St. Louis, MO) 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 SCFAs pools were calculated as the product of concentration (µmol/g) of individual acid, or their sum (total SCFAs) and gram of the cecal content weight.

**Statistical Analysis.** Body weight, food disappearance, cecal content pH, cecal weight, ACF and MDF number, total amount and concentration of SCFAs were analyzed using 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 mean ± standard deviation. All statistical analyses were performed using SAS software (SAS Institute) and P-value < 0.05 was considered significant.

**Results**

**Resistant Content of the Experimental Diets.** Resistant content 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 were substantially greater compared with that of w-CS diet. For the raw starch diet experiment, the resistant content 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 resistant content of 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 the 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 eight 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 comparing the end and the beginning body weights and 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 raw starch diet was 18.9±1.4 g and 15.4±1.8 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 g and 11.3±2.1 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 weight 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 (Figure 1A; Figure 2A and 2B).
Cecal content pH was significantly decreased in rats fed SAC and HA (P-value < 0.05) (Figure 1B; Figure 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 the rats fed r-CS or r-SAC (P-value < 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 and 2E).

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 r-CS, r-HA and r-SAC diets, respectively. A significant decrease was seen in the large ACF number in rats fed r-HA diet compared with the rats fed r-CS or r-SAC diet (P-value < 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 w-CS, w-HA and w-SAC diets, respectively. No significant difference was observed between these groups.

All the above data were not compared between the raw starch experiment and water-boiled starch experiment since the two experiments were not conducted at the same time.

**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 SCFAs detected in cecum, colon and feces. Propionate was the second most abundant SCFAs in cecum contents and feces, and least abundant was butyrate. Short Chain fatty acids concentration was highest in cecum, followed by colon, and then feces. In general, no difference was observed in the individual or total SCFAs 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

Type 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 feeding rats 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 since 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 the starches, an inhibitory effect of w-SAC on colon carcinogenicity was shown compared with w-HA or w-CS diet; yet compared with the r-HA or r-CS diets, the r-SAC did not inhibit the colon pre-neoplasia. Comparing the resistant content of the diets in the raw starch diet experiment and the water-boiled starch diet experiment, we observed that the water-boiling process resulted in a dramatic difference in the resistant content of w-CS, w-HA and w-SAC diet. Yet in the raw starch diet experiment, the resistant content of r-HA and r-SAC were not different. Considering the fact that 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 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. But for SAC, we hypothesize that the lipid and amylose formed a complex which prevented the amylose from leaching and starch granules from swelling. Thus 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 the 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 (1987) reported ACF as a biomarker for colon cancer in 1987 (28). Femia et al. (2004) demonstrated that the number of MDF, its 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. (1990) 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. (2006) showed that hydrothermally treated Novelose 330, a commercially developed RS3 prevented colon tumors in 1,2-dimethylhydrazine-treated Sprague-Dawley rats when incorporated into diet (31). In this study, the rats were fed with Novelose 330 or raw control starch diet throughout the study. After 1 week of diet feeding, the rats were injected DMH at 1-week intervals for 20 weeks. Young et al. (1996) 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 1,2-DMH injection) stages. Conversely, Thorup et al. (1995) found a lowered number of ACF in Wistar rats fed raw potato starch diet compared with rats fed sucrose, raw cornstarch or a basic semi-synthetic 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 since SCFAs stimulates cell proliferation and induces apoptosis, which in turn impacts carcinogenesis. Short Chain fatty acids, 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 SCFAs concentrations. Kleessen et al. (1997) reported a stimulation of bifidobacteria, lactobacilli, and a higher SCFAs concentration in male Wistar rats by a retrograded potato starch (32). Sakamoto et al. (1996) 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 difference in fecal or colonic SCFAs concentration between groups, but a significantly increased cecal SCFAs amount in rats fed w-SAC and w-HA diet was observed. It was suggested that total SCFAs excretion might also be relevant to the ability of resistant starch to reduce colon cancer risk, considering higher cecal content weight and thus SCFAs amounts available for colonocytes (17). Differences in total SCFAs 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 stimulates 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 citric acid cycle (TCA-cycle), fatty acid metabolism, electron transport, TNF-alpha 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, while numerous oncogenes, cell cycle regulators and transcription factors were down regulated (38-40).

Increased fecal butyrate and propionate concentration by w-HA and w-SAC feeding was observed in our study, suggesting that distal SCFAs 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 proximal colon (41). Considering the physiological significance of SCFAs, especially propionate and butyrate, in maintaining colon health and inducing hyper-proliferative cell apoptosis, increased SCFAs in distal colon may have important implications in the prevention of colon cancer (15). Studies have shown that apoptotic index measured 6h after an acute dose of AOM (10mg/kg) in distal crypts was significantly correlated with fecal SCFAs 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 bacteria (44) and may also decrease secondary bile acid levels, which were suggested as promoters for colon carcinogenesis (45). In our study, cecum pH was significantly decreased by SAC or HA, but SCFAs concentration (μmol/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 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 SCFAs concentration between the two groups (46). On the other hand, succinic acid was significantly correlated with decreased cecal pH ($r = -0.978$, P-value < 0.05) in their study with more than 4-fold increase in rats fed high amylose starch. But some studies have suggested that a very low pH might increase the risk of carcinogenesis because very low pH may stimulate epithelial cell proliferation, which in turn could enhance the chemically initiated carcinogenesis (40, 47).

An elevated cecal tissue and content weight was 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 weigh 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 contractibility 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 based on our desire to optimize the amount of experimental starch and on reports suggesting the suppression of colonic carcinogenesis by cellulose. For example, cellulose diets and non-fiber diets were fed to AOM-treated (5mg/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 with non-fiber 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 and 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.
Abbreviations

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.

Safety

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

Acknowledgements

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Figure Captions

Figure 1. Endpoints 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.

The ACF were measured in the distal 75mm of the colon.

ACF were only seen in AOM-treated rats. Values are means ±SD. a,b,c bars with different letters in each panel are significantly different P-value < 0.05.

Figure 2. Endpoints 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.

The ACF and MDF were measured in the distal 75mm of the colon.

ACF were only seen in AOM-treated rats. Values are means ±SD. a,b,c bars with different letters in each panel are significantly different P-value < 0.05.
Figures

Figure 1

Panel A: Total cecal weight (g) across treatments.

Panel B: Cecal content pH across treatments.

Panel C: Average ACF across treatments.

Key:
- AOM, r-CS
- AOM, r-HA
- AOM, r-SAC
- Saline, r-CS
- Saline, r-HA
- Saline, r-SAC
Figure 2

A. Cecal tissue weight (g)

B. Cecal content weight (g)

C. Cecal content pH

D. Average ACF

E. Average MDF

- □ AOM, w-CS
- □ AOM, w-HA
- □ AOM, w-SAC
- ■ Saline, w-CS
- □ Saline, w-HA
- □ Saline, w-SAC
### Table 1: Diet Ingredients for the Raw Starch Diet Experiment and the Water-boiled Starch Diet Experiment¹.

<table>
<thead>
<tr>
<th>Diet Ingredient</th>
<th>Raw Starch Diet (%)</th>
<th>Water-Boiled Starch Diet (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>starch</td>
<td>50.0</td>
<td>55.0</td>
</tr>
<tr>
<td>casein</td>
<td>20.0</td>
<td>20.0</td>
</tr>
<tr>
<td>Dextrose</td>
<td>15.0</td>
<td>15.0</td>
</tr>
<tr>
<td>cellulose (insoluble fiber)</td>
<td>5.0</td>
<td>0.0</td>
</tr>
<tr>
<td>mineral mix (AIN-93)</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>choline</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>methionine</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>vitamin mix (AIN-93)</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>corn oil</td>
<td>5.0</td>
<td>5.0</td>
</tr>
</tbody>
</table>

¹Starches 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).
### Table 2: Resistant Starch Contents of the Three Diets Prepared by Two Methods (% dry feed basis)

<table>
<thead>
<tr>
<th>Starch group</th>
<th>Resistant Starch Content of Diets in Raw Starch Experiment (%)</th>
<th>Resistant Starch Content of Diets in Water-boiled Starch Experiment (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control normal cornstarch diet</td>
<td>0.4±0.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.9±1.5&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>High amylose cornstarch diet</td>
<td>13.2±0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.3±3.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Stearic acid complexed high-amylose cornstarch diet</td>
<td>12.2±0.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.8±5.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are expressed as 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. 

<sup>a,b,c</sup> indicates values in each column with different letters are significantly different at P-value < 0.05.
### Table 3: Effect of Experimental Starch Diets on Cecal, Colonic and Fecal SCFA Concentrations in Fisher344 Rats Fed the Diets in the Raw Starch Diet Experiment.

<table>
<thead>
<tr>
<th>group</th>
<th>Saline, w-CS</th>
<th>Saline, w-HA</th>
<th>Saline, w-SAC</th>
<th>AOM, w-CS</th>
<th>AOM, w-HA</th>
<th>AOM, w-SAC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cecum (µmol)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total SCFA</td>
<td>131.6 ± 28.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>653.2 ± 78.8&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>643.9 ± 158.1&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>177.2 ± 67.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>569.7 ± 217.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>890.5 ± 260.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Acetate</td>
<td>85.8 ± 23.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>417.5 ± 63.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>342.4 ± 116.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>118.3 ± 47.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>371.0 ± 160.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>544.9 ± 179.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Propionate</td>
<td>29.1 ± 5.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>165.9 ± 88.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>188.9 ± 47.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>37.0 ± 13.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>138.1 ± 52.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>217.4 ± 113.1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>n-Butyrate</td>
<td>16.6 ± 4.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>69.7 ± 37.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>112.6 ± 69.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21.9 ± 7.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>60.6 ± 21.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>128.2 ± 55.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Cecum (µmol/g wet wt)</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Total SCFA</td>
<td>41.1 ± 12.6</td>
<td>49.0 ± 6.5</td>
<td>34.2 ± 6.3</td>
<td>61.5 ± 23.3</td>
<td>43.1 ± 16.1</td>
<td>55.8 ± 14.0</td>
</tr>
<tr>
<td>Acetate</td>
<td>26.8 ± 9.3&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>31.5 ± 6.5&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>18.2 ± 5.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>41.1 ± 16.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>28.2 ± 12.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>34.5 ± 10.0&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Propionate</td>
<td>9.1 ± 2.7</td>
<td>11.8 ± 4.4</td>
<td>10.0 ± 1.8</td>
<td>12.9 ± 4.9</td>
<td>10.3 ± 3.2</td>
<td>13.6 ± 7.3</td>
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<tr>
<td>n-Butyrate</td>
<td>5.2 ± 1.8</td>
<td>5.8 ± 4.0</td>
<td>6.0 ± 3.5</td>
<td>7.5 ± 2.2</td>
<td>4.6 ± 1.5</td>
<td>7.8 ± 2.7</td>
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<tr>
<td><strong>Colon (µmol/g wet wt)</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Total SCFA</td>
<td>22.4 ± 6.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>30.2 ± 8.0&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>20.2 ± 5.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>29.3 ± 13.8&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>17.6 ± 6.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>30.9 ± 10.9&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Acetate</td>
<td>16.4 ± 4.0</td>
<td>21.0 ± 4.2</td>
<td>11.8 ± 3.7</td>
<td>20.8 ± 8.9</td>
<td>13.6 ± 5.1</td>
<td>21.1 ± 6.8</td>
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<tr>
<td>Propionate</td>
<td>3.0 ± 1.4</td>
<td>4.9 ± 3.5</td>
<td>4.8 ± 1.4</td>
<td>4.2 ± 2.4</td>
<td>2.7 ± 1.3</td>
<td>5.6 ± 4.2</td>
</tr>
<tr>
<td>n-Butyrate</td>
<td>3.0 ± 1.4</td>
<td>4.3 ± 3.5</td>
<td>3.5 ± 2.9</td>
<td>4.4 ± 2.7</td>
<td>1.3 ± 0.9</td>
<td>4.2 ± 3.1</td>
</tr>
<tr>
<td><strong>Feces (µmol/g wet wt)</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total SCFA</td>
<td>16.8 ± 6.6</td>
<td>16.2 ± 1.0</td>
<td>13.4 ± 3.5</td>
<td>10.7 ± 3.3</td>
<td>16.1 ± 6.4</td>
<td>16.6 ± 6.0</td>
</tr>
<tr>
<td>Acetate</td>
<td>14.9 ± 5.5</td>
<td>10.3 ± 5.6</td>
<td>7.7 ± 1.6</td>
<td>9.6 ± 2.9</td>
<td>12.7 ± 5.1</td>
<td>11.3 ± 3.9</td>
</tr>
<tr>
<td>Propionate</td>
<td>1.5 ± 1.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.6 ± 3.1&lt;sup&gt;*&lt;/sup&gt;</td>
<td>4.4 ± 1.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.9 ± 0.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.4 ± 1.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.5 ± 1.8&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>n-Butyrate</td>
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<td>2.2 ± 2.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.4 ± 1.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.2 ± 0.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.0 ± 0.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.8 ± 1.3&lt;sup&gt;c&lt;/sup&gt;</td>
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Data are expressed as mean±SD.

<sup>a,b,c</sup> indicates values in each row with letters and without a common letter differ, P-value < 0.05.
CHAPTER 3: INHIBITION OF AZOXYMETHANE-INDUCED PRENEOPLASTIC LESIONS IN THE RAT COLON BY A STEARIC ACID COMPLEXED HIGH-AMYLOSE CORNSTARCH USING DIFFERENT COOKING METHODS AND ASSESSING POTENTIAL GENE TARGETS

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Abstract

We evaluated a novel stearic-acid-complexed-high-amylose-cornstarch (SAC) for the prevention of preneoplastic lesions in the colon of azoxymethane (AOM)-treated Fisher344 rats fed cooked resistant starches at 55% of the diet for 8 weeks when two cooking methods were applied respectively: water-boiling and bread-baking methods.
Water-boiled SAC (w-SAC) diet was compared with water-boiled CS (w-CS) or water-boiled HA (w-HA), and bread-baked CS (b-CS) was compared with bread-baked HA (b-HA) and bread-baked SAC (b-SAC), respectively. Global gene expression profiles of colon mucosa from rats fed different diets in the water-boiled group were determined. Either w-SAC or b-SAC markedly reduced ACF and MDF numbers compared with HA or CS within their cooking groups. Increased cecal contents and decreased cecal pH were observed in SAC or HA groups. The total SCFAs or each individual SCFA (butyrate, acetate, and propionate) were observed to increase in certain SAC groups. Differentially expressed genes were found for AOM or diet treatment in rats fed w-CS or w-SAC, and 7 genes (CACYBP, RhoA, MAPK1, TOPBP1, HSPH1, MCM6, HSPD1) were confirmed with semi-quantitative real time-PCR. This study revealed that SAC cooked by both methods inhibited colonic preneoplastic lesions and identified potential gene targets.

1. Introduction

Colorectal cancer is one of the leading causes of cancer deaths worldwide. In the USA alone, an estimated 102,900 cases of colon and 39,670 cases of rectal cancer were predicted to occur in 2010, among which 51,370 deaths were expected, accounting for 9% of all cancer deaths (American Cancer Society, 2010). Colorectal cancer was estimated to be the third most common cancer in both men and women. Diet has been reported as an important modifiable factor for control risk of colon cancer (Rogers et al., 1993).

Resistant starch (RS) is the sum of starch and products of starch degradation that is not digested and absorbed in the small intestine of healthy individuals. Traditionally, RS was classified into four categories: RS1, physically inaccessible starch (e.g. coarsely
ground whole grains and legumes); RS2, crystalline (uncooked) starch granules with the B- or some C-type crystalline structure (e.g. raw potato, banana, and high-amylose maize starch); RS3, retrograded amylose, which can be found in cooked and chilled potatoes; and RS4, chemically modified starch. A newly developed type 5 RS consisting of amylose-lipid complex has been introduced recently (Hasjim et al., 2010).

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 (Sengupta et al., 2006; Scheppach et al., 1995). These physiological properties produced by RS have been proposed to prevent colorectal cancer development (Scheppach et al., 1995). Of the three main SCFAs (butyrate, acetate, and propionate), butyrate has been extensively studied and has been considered to be the most potent for protection against colon carcinogenesis (Hijova and Chmelarova, 2007; Topping and Clifton, 2001).

Hasjim (Hasjim et al., 2010) reported that SAC, a RS5, reduced postprandial plasma-1 glucose and insulin levels in humans. Twenty male human subjects were fed bread made from 60% (dry basis) SAC or white bread. Subjects fed SAC had their postprandial plasma glucose and insulin levels reduced to 55% and 43%, respectively, compared with those fed control white bread (100%). The results suggested that the SAC can be used for the intervention of diseases such as diabetes and obesity.

In our previous study, we discovered that water-boiled SAC had a better inhibitory impact on colon carcinogenesis in Fisher 344 rats than uncooked forms. This
discovery is notable because humans generally consume cooked cornstarch. In this study, we explored the impact of different cooking methods on the colorectal carcinogenesis further. To our knowledge, this is the first time the influence of different cooking methods on the ability of RS to prevent preneoplastic lesions have been reported. We also conducted global gene expression analysis by microarray and identified several potential genetic targets using qRT-PCR that may be important in colon cancer prevention by water-boiled SAC.

2. Materials and Methods

2.1. Diets

Three starches were incorporated into diets and fed to the rats, CS (Cargill Gel™ 03420; Cargill Inc., Minneapolis, MN), HA (AmyloGel 03003; Cargill Inc., Minneapolis, MN) and SAC (processed from Department of Food Science and Human Nutrition, Iowa State University) (Hasjim et al., 2010) as the negative control, positive control, and experimental diet, respectively. The diets were formulated based on the standard diet recommended by the American Society for Nutritional Sciences report for mature rats (AIN-93M), except that 5% cellulose was not included, and starch content was set at 55% (Reeves, 1997). All three starches were cooked by two different methods: water-boiled method and bread-baked method (Table 1). For the water-boiled diets, the starches were mixed with water gradually and cooked on the range top of a stove. The starch-water mixture was slowly stirred throughout the cooking process until a paste was formed. The paste was then cooled to room temperature and added into the rest of AIN-93M diet ingredients. For the bread-baked diets, each starch was mixed with bread flour in the proportion of 5:2. First 2.5 g yeast were mixed with 100 g warm water and held for at
least 10 min, and then 300 g of starch and bread flour mixture were mixed together. Next, 2.0 g salt was dissolved with 65 g hot water and 12.5 g shortening, and heated on a boiling water bath until melted. The melted shortening, salt solution and yeast were then added into the starch-bread flour mixture and mixed slowly for 3.5 min to form dough. The dough was put in a greased pan, covered with a moist towel and let rise for 1 hour. The dough was then put in a loaf pan and let rise for another hour. The raised dough was baked in an oven for 25 minutes at 400°F, and then cooled for 1 hour at room temperature. The bread was then dried, ground and mixed with other ingredients of the modified AIN-93M diet. Diets were prepared and fed to rats every two days.

Notably, a key strategy was to keep the CS, HA and CSA at 55% of the diet and thus in the bread-baking diets, the composition of vitamins and minerals were less than the standard AIN-93M rat diet (Table 1B). In the bread-baking diet ingredients, the vitamin mix was included at 56% of the recommended amount in the AIN-93M diet, and mineral mix was contained at 61% of the amount in the AIN-93M diet. L-lysine, DL-tryphophan, L-histidine, and DL-threonine were added to the diet since they were needed by rats and not sufficiently obtained from the wheat protein and other ingredients of the bread-baked diet (Table 1C). Eighteen amino acids from each diet were listed and compared, among which 7 are essential amino acids (Table 1C). All of the amino acids in the water-boiled diet exceeded the recommended amount for the AIN-93M diet. All of the essential amino acids were present in the bread-baked diet, but isoleucine and leucine were somewhat low compared with the AIN-93M ingredient (86% and 79% of the recommended amount in AIN-93M diet, respectively). There were another 6 amino acids from the bread-baking diet that were 55-97% of what was recommended for the AIN-93M diet. Comparing the bread-baked diet with water-boiled diet, two amino acids
(Glutamic acid and glycine) from the bread-baking diet had higher amounts than that contained in the water-boiled diet, and all the other amino acids in the bread-baking diet were 38% - 96% of the respective amino acid content in the water-boiled diet. The total amount of amino acids were higher in the bread-baked diet than in the AIN-93M ingredient (14.83% of bread-baked diet and 14.05% of the AIN-93M diet), but less than that of the water-boiled diet (20.07%). Although most of the amino acids were sufficient compared with their recommended amount in the AIN-93M ingredient, they were mostly less than the respective amount in the water-boiled diet. The reason for the lower amount of vitamins, minerals and selected amino acids was that bread flour, wheat gluten, shortening, yeast and salt had to be added in addition to the resistant starch or control starch to shape and make the bread in the bread-baking process. Thus, with 37.97% the above components of the total, the remaining ingredients had to be reduced to keep the diets at a composition of 55% corn starch.

2.2. Carcinogen treatment

AOM was purchased from Midwest Research Institute (Kansas City, MO) and diluted by phosphate buffered saline (PBS) to 2mg/ml. AOM solution was injected by intraperitoneal injection to 60 rats at seven weeks of age at the dosage of 20 mg AOM/Kg rat body weight. The remaining 30 rats were injected with physiological saline (VWR International, Inc., Batavia, IL) by the same method, and they served as controls.

2.3. Animals and housing

Five-week-old male Fischer 344 (F344) rats were obtained from Charles River laboratory (Wilmington, MA). Ninety animals were housed individually in a plastic cage with stainless-steel, wire-mesh top. A stainless-steel wire floor was placed on the bottom
of each cage to prevent the rats from eating their own feces. The rats were housed in an animal room at the temperature of 22±1°C and relative humidity at 60%±5% with a 12-hour light cycle. Diet and water were provided ad libitum.

F344 rats were first injected with AOM or saline at 6 weeks of age. They were injected with AOM for a second time one week later. From their arrival until 3 days after the second injection, the rats were fed with w-CS or b-CS. Then the rats were divided randomly into 6 diet groups (w-CS, w-HA, w-SAC, b-CS, b-HA and b-SAC) with 5 saline-injected rats and 10 AOM-injected rats in each diet group. The rats were fed the 6 diets respectively for 8 weeks. Body weight was measured weekly and food disappearance was calculated every 2 days when freshly prepared diet was provided. The rats were then killed by carbon dioxide euthanasia and opened the thoracic cavity and abdominal cavity.

2.4. Resistant starch content (RC) and water content (WC)

Diet samples were collected right after they were made. Water content of these samples was determined by Sartorius moisture analyzer MA30 (Bohemia, NY). Resistant starch contents were then assessed by AOAC method 991.43 on a dry-weight basis (Horwitz, 2003).

2.5. Cecal weight and pH, liver weight

Cecum was collected from rats and cut open. The cecal content weigh and pH were measured. Cecal tissue was rinsed in PBS, quickly blotted with a paper towel and weighed. The liver was also removed and weighed.
2.6. ACF and MDF

The rinsed colons were cut open longitudinally, laid flat, and flushed with PBS. The distal 75mm of the colon measured from the anus was fixed in 10% formalin for 24 hours before processing into specimens. Colons were first stained with 1% alcian blue in acetic acid (pH 2.5), and then counterstained with 1% neutral red to view total ACF number, ACF multiplicity and MDF number. The proximal and distal ACF number, ACF multiplicity and MDF number were also counted. ACF was also grouped in to small (1-3 crypts) or large (>=4 crypts) by size and large ACF numbers were also enumerated.

2.7. SCFA

Fresh fecal samples were collected two days before rats were sacrificed. Cecal and colon contents were collected during the necropsy at the end of the study. All the samples were kept at -80°C until analysis.

After thawing, cecum, colon or fecal samples were diluted with distilled water at a ratio of 1:1, 1:2.5 or 1:3, respectively. Extraction of SCFA and Gas Chromatography analysis were performed by following the methods described (Sayar, 2007) with slight modification. Briefly, 500 μL supernatant collected after centrifuging the homogenous slurry at 3,000 g for 10 min, was added to 250 μL concentrated hydrochloric acid containing 50 μL 2-ethyl-butyric acid (0.1 M) (Sigma-Aldrich, St. Louis, MO) as internal standard. After 2 × 1 mL diethyl ether extraction, 200 μL upper ether phase was silylated with 20 μL N-(tert-butyldimethylsilyl)-N-methyltrifluoro-acetamide (Sigma-Aldrich, St. Louis, MO) at 80 °C for 20 min. SCFA derivatives were stored at room temperature for 24 h in the dark before Gas Chromatography analysis.
SCFA silylated derivatives were analyzed by a Gas Chromatography equipped (HP 6890N) with a flame ionization detector using a SPB5 30 m × 0.25 mm i.d. × 1 μm capillary column (Supelco, Inc). A split mode was used and the Gas Chromatography conditions were as follows: injection volume (1 μL), initial temperature at 70 °C for 5 min raised to 160 °C at 7 °C/min and final temperature at 160 °C for 4 min, injector temperature (200 °C), and detector temperature (220 °C). Quantifications of SCFA were based on the standard curves established using internal standard 2-ethyl-butyric acid. All standards and samples were analyzed in replicates. SCFA data was presented as mean values ± standard deviation. The concentration of SCFA was expressed as µmol/g wet weight. Cecal SCFA amounts were calculated as the product of the concentration (µmol/g) of individual acids, or the pooled SCFA amount.

2.8 RNA Processing and Microarray

Colon samples were collected for Microarray studies from 3 rat experiments (experiment 1, 2 and 3). All of these rats were treated exactly the same way as in the preneoplastic lesion study. Colon mucosa was scraped from the surface of colon tissue and collected immediately in liquid nitrogen after rats were killed and used for RNA extraction later. Total RNA was isolated from rat colon mucosa using Trizol (Invitrogen, Carlsbad, CA). In one microarray analysis study, colon mucosa samples came from 2 rat experiments (experiment 1 and 2). Twenty total RNA samples were isolated from distal rat colon mucosa (the colon section from anus to 75mm above anus) in either AOM w-SAC rat group or AOM w-CS rat group to compare the effects on gene expression levels caused by the two diets fed to AOM treated rats with 10 samples in each group (6 from the distal section of rat colons in one experiment, 4 as replicates from the other experiment). In a second comparison of the gene expression difference impacted by AOM
treatment, nineteen RNA samples were isolated from either the distal (anus to 75mm above anus) or the adjacent rat colon mucosa (the next 75mm from the distal section) in the rats fed w-CS and treated either AOM or saline from 2 experiments (experiment 1 and 3). In this comparison, ten samples were collected from the rats fed w-CS and injected AOM (6 samples from distal colons of one experiment and 4 samples from the adjacent colons of the other experiment), and 9 samples were collected from the rats fed the same diet and injected with saline (6 samples from distal colons of one experiment and 3 samples from the adjacent colons of the other experiment). A further purification was conducted with RNeasy Protect Mini Kit (QIAGEN, Valencia, CA), and RNase-Free DNase (QIAGEN) was performed to remove contaminating DNA. Integrity, quality, and quantity of isolated RNAs were confirmed with RNA 6000 Nano LabChip kit with Agilent 2100 Bioanalyzer (Agilent Biotechnologies, Palo Alto, CA).

These RNA samples were then applied to an Affymetrix oligonucleotide microarray using GeneChip Rat Genome 230 2.0 (Affymetrix, Santa Clara, CA) for gene expression analyses, and each replicate was analyzed on an individual GeneChip. The labeling of RNA was conducted with the GeneChip 3’ IVT Express Kit and control reagent package according to manufacturer’s instructions (Affymetrix, Santa Clara, CA), and the process was performed by a GeneChip fluidics station 450 and a GeneChip Scanner 3000 7G (Affymetrix, Santa Clara, CA). All techniques for the GeneChip array were carried out according to the manufacturer’s instructions by the Microarray Facility at Iowa State University. Samples from AOM w-CS group and saline w-CS group were compared for the effects AOM conducted on the gene expressions. Samples from AOM w-SAC and AOM w-CS rats were analyzed for the effects of SAC diet on the gene expression level.
2.9 Semi-quantitative real time-PCR

DNA-free RNA was reverse-transcribed using the iScript cDNA synthesis kit (BioRad, Hercules, CA). Four genes were chosen to verify the AOM effects on gene expressions, and 5 genes were selected for the diet effects. These genes were chosen for quantitative real-time PCR analysis (qRT-PCR) based on their significant differential expression in treatments False Discovery Rate (FDR) less than 0.05 and Fold Change (FC) more than 1.2 either up-regulated or down-regulated, and their relevance to biological pathways of interest. GAPDH was used as control. The primers used to confirm differentially expressed genes were designed using the software PrimerQuest (Integrated DNA Technologies, Inc., Coralville, IA) and synthesized by Integrated DNA Technologies, Inc. A MyiQ thermal cycler using the SYBR green supermix kit was used for the qRT-PCR procedure according to the manufacturer’s instructions (BioRad, Hercules, CA). Conditions for the qRT-PCR were: 95 °C for 3 min, and 40 cycles of 95 °C for 15 s, annealing temperature of the primer for 30 s, and 72 °C for 30 s, followed by 95 °C for 1 min, and 55 °C for 1 min. The following genes were selected for qRT-PCR to confirm microarray results: MAPK1, CACYBP, RhoA, and APC were selected for the expression levels in AOM injected rats in comparison with saline injected rats in the w-CS diet groups. TOPBP1 was tested for its gene expression in AOM-injected rats fed w-SAC in comparison with AOM-injected rats fed w-CS. The expression of HSPH1, MCM6, and HSPD1 were assessed both in AOM-injected rats fed w-SAC compared with those fed w-CS, and in AOM-injected rats compared with saline injected rats which were fed w-CS. The primer sequences and annealing temperatures were presented in Table 2.
2.10 Statistical Analysis

Two-way AVONA was performed through GLM Procedure in SAS for cecal weight, pH, preneoplastic lesions, and SCFA (SAS Institute, 2010). One-sided t-test was then applied to test the directions of the differences between groups. All values were reported as mean ± standard deviation, and P-value < 0.05 was considered significant.

The microarray experiment was designed as a balanced incomplete block design with replications as fixed blocks and injection levels (AOM, saline) and diet levels (CS, SAC) as the factors and analyzed for the differences of gene expression. An unbalanced incomplete block design was carried out to the samples testing the gene expression changes caused by AOM treatment. The normalized log signal intensities were fitted to the linear model. The moderated t-test (Smyth et al., 2004) and q-value procedure (Storey and Tibshirani, 2003) were employed to control FDR at 0.05 level for AOM treatment analysis and Diet treatment analysis. FC was also taken into consideration in prioritizing genes for further study. Within genes of FDR < 0.05 for the analyses of the either treatment, an up-regulated or down-regulated 1.2-fold change or more were considered biologically significant (Strath et al., 2009; Jickling et al., 2010).

DAVID 6.7 was used to identify pathways with significant differentially expressed genes. Gene ontology functional classifications based on the differentially expressed genes were also obtained. The rat Affymetrix background was applied when conducting the above procedures (Huang et al., 2009a, 2009b). Cluster and TreeView programs (Eisen et al., 1998) were used to develop heat map with genes that had FDR < 0.01 and FC > 1.5 both in the direction of up-regulation and down-regulation.
3. Results

3.1. Resistant starch content (RC) and water content (WC) of the experimental diets

Resistant starch contents of the water-boiled and bread-baked diets are shown in Table 3. Resistant starch content decreased in the order of SAC, HA and CS within each cooking method (bread-baked or water-boiled). Water content of all 6 diets was 49-60%. Only w-CS had significantly higher water content compared with all the other diets (Table 3).

3.2. Body weight, food disappearance and liver weight

No significant difference was seen in the body weight gain or food disappearance over the period of eleven weeks. Rats fed the water-boiled starches and bread-baked starches gained 187 ± 14 and 187 ± 17 g body weight respectively over the 11 weeks feeding time. Average daily food disappearances of rats in water-boiled and bread-baked groups were 16.6 ± 1.8 and 15.8 ± 2.1 g in dry weight, respectively. Liver weight collected from rats fed the water-boiled and bread-baked starches were 8.9 ± 0.5 and 8.9 ± 0.8 g, respectively. No effects of diet or AOM were observed on liver weight of the rats at final sacrifice.

3.3. Cecum Weight and pH

In the rats fed both the water-boiled starches and the bread-baked starches, cecal content weight and cecal tissue weight were significantly higher in rats fed SAC and HA compared with that in rats fed CS. Cecal contents pH was significantly lower in rats fed SAC and HA (P-value < 0.05) than in the CS groups (Fig. 1).
3.4. ACF and MDF

Total ACF number, ACF multiplicity, and Total MDF number were assessed in the distal 75mm of colon tissue in both water-boiled and bread-baked diet groups. Both lesions were seen in AOM-treated rats only (Fig. 2).

In the water-boiled groups, ACF number decreased significantly in rats fed w-SAC compared with the rats fed w-CS or w-HA (P-value < 0.05). In the bread-baked group, ACF number assessed in rats fed b-SAC was also dramatically lower than that of rats fed b-CS or b-HA diet. Comparing the two cooking methods, ACF number was significantly lower in the rats fed the bread-baked starches (29 ± 12, 29 ± 7, and 17 ± 7 ACF for b-CS, b-HA, and b-SAC, respectively) then in the rats fed the water-boiled starches (225 ± 52, 256 ± 86, and 121 ± 46 ACF for w-CS, w-HA, and w-SAC, respectively) (Fig. 2, panel A).

In both rats fed the water-boiled and the bread-baked starches, a small but significant elevation of ACF multiplicity was seen in the rats fed SAC compared with rats fed CS or HA diets. In the water-boiled treatment groups ACF multiplicity significantly increased from the CS < HA < SAC groups (Fig. 2, panel B).

In rats fed water-boiled starches, a significant reduction of MDF was seen in the rats fed w-SAC compared with rats fed w-CS or w-HA diets. In the groups fed the bread-baked starches, rats fed b-SAC diet had fewer MDF than rats fed b-CS or b-HA diets. There was a trend of higher MDF number in the rats fed the water-boiled starches (10.7 ± 4.1, 8.7 ± 3.5, and 2.1 ± 1.5 MDF for w-CS, w-HA, and w-SAC, respectively) than in the
bread-baked starches (6.1 ± 2.6, 4.9 ± 3.0, and 1.9 ± 1.4 MDF for w-CS, w-HA, and w-SAC, respectively) (Fig. 2, panel C).

Total ACF number, ACF multiplicity, and total MDF number were assessed throughout two segments of the collected colon tissue: the proximal colon and distal colon. A very similar result was observed in the significance or the trends between dietary groups compared with what we observed in the entire distal 75 mm colon tissue (Fig. 3 and 4).

The numbers of large ACF (crypts/foci >= 4) were also counted and compared in the whole collected colon tissue or in the proximal and distal segments of the colon tissue. A similar trend was observed as was seen in the total ACF numbers. Large ACF numbers were significantly lower in the rats fed w-SAC compared with w-CS or w-HA either in the whole collected colon tissue or in the distal or proximal part of the collected colon in the rats fed the water-boiled starch. In the rats fed the bread-baked starch, Large ACF numbers were significantly reduced in the rats fed b-SAC compared with b-CS in distal, proximal or total assessed colon. Comparing the two starch cooking method groups, large ACF numbers were higher in the rats fed the water-boiled starches than those fed the bread-baked starches (Fig. 5).

3.5. SCFA

SCFA amount in the cecum (µmol/cecum), calculated as cecum weight times cecal SCFA concentration, were similar in animals fed the same starch diets regardless of the preparation method (bread-baked or water-boiled) and carcinogen treatment (AOM or saline) (Table 4). Total amounts of acetate and the sum of SCFA in the cecum were
significantly greater in animals fed b-HA and b-SAC compared with b-CS, or w-HA and w-SAC compared with w-CS. Total amount of butyrate or propionate in cecum were higher in rats fed HA or SAC diets compared with CS diet groups. No differences were observed in the cecal SCFA amount between 8 groups fed HA or SAC. For µmol per g cecal contents, acetate and total SCFA tended to be lower in rats fed water-boiled starch diets compared with those fed bread-baked starch diets.

No differences were observed in colon SCFA µmol/g concentrations between the diets when comparing amongst rats fed either bread-baked diets or water-boiled diets.

In fecal samples, no significant difference was seen in rats fed water-boiled diets in total SCFA or acetate. In the bread-baked diet group, a significant increase of total SCFA or acetate was observed in rats treated with AOM and fed b-HA compared with rats fed b-CS. Fecal butyrate did not differ among treatments.

Interestingly, minor SCFA, including isobutyrate, isovalerate and valerate, were only detected in animals fed control starch diets. There were no significant differences in isovalerate and valerate among groups in cecal, colonic and fecal samples (data not shown). Isobutyrate concentrations in the cecum were significantly greater in the rats fed w-CS and treated with saline (0.88 ± 0.26 µmol/g wet weight) or AOM (0.94 ± 0.30,) than in rats fed b-CS (0.35 ± 0.15 in saline group and 0.39 ± 0.28 in AOM group). There was no such difference in colonic and fecal samples.
3.6 Global Gene Differential Expression

In the microarray analysis which compared colon mucosa samples collected from rats fed w-CS and injected AOM or saline, mRNA expression showed that a total of 6404 genes were expressed differentially with fold changes 1.2 times or more and FDR less than 0.05. Of these 6404 genes, 3368 genes were up-regulated and 3060 genes were down-regulated, with 7% of the selected genes showing more than 2 fold change. Other selected genes had 1.2-2 fold change (Table 5).

In the microarray analysis which compared gene expression changes of the AOM-treated rats after they were fed w-SAC compared with those fed w-CS, 1403 genes were identified to be differentially expressed with FDR < 0.05 and fold change > 1.2, among which 544 genes were up-regulated and 859 genes were down-regulated (Table 5). The majority of the selected genes (96%) showed moderate alteration in expression of fold change between 1.2–1.9. Twenty genes showed a fold change between 2 - 4.9 with up-regulated expression and 33 genes in this fold change range had down-regulated expression.

Of the differentially expressed genes, 152 genes were differentially expressed in both the Microarray Analysis for AOM treatment and diet treatment with FDR < 0.05 and FC > 1.2 (Figure 6, 7). Among these common genes, 83% had a down-regulated expression in the diet study (AOM w-SAC compared against AOM w-CS, p-value < 0.05) and an up-regulated expression in the AOM study (AOM w-CS compared with saline w-CS, p-value < 0.1). These genes were picked out as potential target genes related to the inhibitory effect of SAC on AOM induced preneoplastic lesions. Some of the target
genes that have reported connection to colorectal cancer were HSPD1, HSPH1, MCM6, Rac1, Cd55 and TFRC (Table 6A).

3.7 Confirmation of Differential Gene Expression

Semi-quantitative real-time PCR (qRT-PCR) analysis was conducted of expression levels of genes differentially expressed in the microarray experiment in rat colon with AOM or saline injections; and with w-CS or w-SAC diet feeding (Table 7). The expression levels of 3 genes (CACYBP, RhoA, and MAPK1) were confirmed from the microarray results that the expression levels of these genes were significantly increased in the AOM injected rats compared with saline injected rats when both groups were fed w-CS diet (P-value < 0.05). TOPBP1 was confirmed to have a decreased expression in the AOM treated w-SAC fed rats compared with rats fed w-CS (P-value < 0.05). HSPH1, MCM6, and HSPD1 were confirmed to have both a decreased expression in the diet study (AOM w-SAC compared against AOM w-CS, P-value < 0.05) and an increased expression in the AOM study (AOM w-CS compared with saline w-CS, P-value < 0.1). APC did not show significant expression changes.

4. Discussion

SAC is an amylose-lipid complex which is a newly developed type of resistant starch – RS5 (Hasjim et al., 2010). It is hypothesized that the lipid in the complex forms a single helical complex with linear molecules of amylose and keeps the starch granules in a more intact form and more resistant to enzyme hydrolysis. With each cooking method, SAC diets suppressed both the total ACF number and the total MDF number compared with HA or CS diet. The same conclusion was revealed by either proximal or distal total
ACF number or MDF number data. The results of total, proximal and distal large ACF numbers (crypts \( \geq 4 \)), suggested to have a greater potential to the cancer progress than small ACF, were consistent with the total ACF or MDF numbers. ACF multiplicity increased slightly but significantly in SAC compared with CS in either the whole 75 mm of colon observed, or either the proximal or distal portion of this colon segment. Interestingly, when we compared the two cooking methods, rats fed starches prepared by the bread-baking method had fewer total ACF, total MDF, or large ACF in all parts of the colon compared with rats fed starches prepared by the water-boiling method.

In one of our previous studies, we compared water-boiled starch diets with uncooked starch diets. The resistant starch contents of the uncooked diets did not show any difference between the SAC and HA diets (12.2 ± 0.7% in uncooked SAC compared with 13.2 ± 0.8% in uncooked HA), and the water-boiling method obviously increased the differences between these two diets (25.8 ± 5.0% in w-SAC against 14.3 ± 3.0% in w-HA) (Zhao et al., 2011). The resistant starch content values from this study also suggested that cooking is an important procedure for increasing the resistant starch content (32.7 ± 0.8% in b-SAC against 24.5 ± 0.2% in b-HA), and this increasing trend might correlate with the enhanced inhibitory effect of SAC on colorectal carcinogenesis in F344 rats.

The influence of the two cooking methods on the resistant starch contents might come from the properties of starch gelatinization procedure. Cooking starch is a starch gelatinization process requiring sufficient amounts of water and appropriate temperature when heating starch granules, which causes an irreversible change of a starch from semi-crystalline structure to amorphous structure (Tester and Karkalas, 1996; Tufvesson et al., 2003). In our water-boiled cooking process, water was sufficiently added to the starch
(water: starch >= 2.5:1, w/w), and the starch paste gained a water-boiled temperature of 95°C. With the bread-baking cooking method, bread contained an insufficient percentage of water (34.2%) in the total weight. On the other hand, the bread baking temperature was 204°C on the surface of the bread, while the internal temperature of the bread was around 82 - 93°C. It is likely that a less complete starch gelatinization was gained in the bread than in the water-boiled paste. Starch gelatinization temperatures of SAC and HA were measured by Hasjim. SAC displayed a thermal transition from 78.9°C ($T_O$) to 125.3°C ($T_c$), while HA showed a thermal transition from 70.4°C ($T_O$) to 125.3°C ($T_c$) (Hasjim et al., 2009). For both SAC and HA, although the crust temperature of bread exceeded the starch gelatinization temperature, both the internal bread temperature and the water-boiled paste temperature were lower than $T_c$, and the internal temperature of bread was not as high as the temperature of the water-boiled paste. Importantly, when the starch is heated with a limited amount of water, as was the case with the bread-baking procedure, the gelatinization temperature would be expected to increase. Research suggested that the less water content the starch retains, the higher temperature of the endotherm was required when purified potato starch granules were heated, and this shift to higher temperature was believed to be due to the decreased melting point of starch crystallites by solvent water (Donovan, 1979). Based on the fact that the bread-baking process contained an insufficient amount of water and increased gelatinization temperature, we believe the bread-baking method led to less adequate starch gelatinization so that less amylose-SA complex was dissociated than with water-boiled starches. Consequently, resistant starch content was higher in starch cooked by the bread-baking method than in the water-boiled starch for SAC or HA. In our study, rats fed bread-baked SAC or HA showed a dramatic reduction of either MDF number, or total ACF number and large ACF number compared with the rats fed water-boiled RS. Connecting the higher resistant starch content in bread-
baked SAC and HA with their elevated inhibitory ability on colonic preneoplastic lesions compared with the water-boiled SAC and HA, respectively, it may imply that bread-baked RS can gain a better carcinogenesis inhibitory effect than the water-boiled RS through obtaining a higher starch resistance by this cooking method. There was no significant difference in the resistant starch content of CS because the gelatinization temperature for CS is between 45°C and 75°C (Buckow et al., 2007). CS was expected to be completely gelatinized in either of the cooking methods.

Other than the differences in the resistant starch content, differences also existed between water-boiled and bread-baked diet when comparing other ingredients. Water-boiled diet reflected a modified AIN-93M maintenance purified diet, and the primary change was replacing cellulose with corn starch as was discussed in our previous study (Zhao et al., 2011). In the bread-baked diet, vitamins and minerals were included at lower levels (56% and 61% of what was present in the water boiled diets). It has not been reported if the reduced amount of vitamins and minerals will impact the colon preneoplastic lesions in a short time study. The present reports were focused on the effect of vitamin and mineral deficiency to the colon cancer occurrence in a long term observation. Of all the vitamins, Vitamin D deficiency has been widely associated with the incidence of colon cancer. In an animal study, the growth of MC-26 colon cancer xenografts in Balb/c mice was enhanced after these mice were fed Vitamin D deficient diet for 3 months (Tangpricha et al., 2005). Vitamin B-12-deficient diet was also observed to create aberrations in both base substitution and methylation of colonic DNA in weanling male Sprague-Dawley rats, which might increase susceptibility to carcinogenesis (Choi et al., 2004). Notably, none of the reports presented the effects on the low levels of multiple nutrition components or multiple deficiencies to the rat colon
carcinogenesis. Notably, in the studies mentioned above, the deficiency of the respective vitamin means none supplement of that vitamin in the diet at all. E.g. in the study by Tangpricha et al., a vitamin D–sufficient diet contained cholecalciferol of 500,000 IU/kg food, while a vitamin D–deficient diet was identical except that it contained no cholecalciferol. In the study by Choi et al., vitamin B-12 (as cyanocobalamin) sufficient diet maintained 50 ug vitamin B-12/kg diet, while the deficient diet had the exact same diet without cyanocobalamin. In our study, no obvious signs of weight change, deficiency, sickness or abnormal activity of rats fed bread-baked diets were observed during the process of the study. Furthermore, bread-baked diet seemed to have a more significant inhibition of ACF and MDF than the water-boiled diet. This might be because the vitamin and mineral insufficiency was not severe, or the negative effect of the lower intakes did not appear because it was in a short-term study of 10 weeks.

In our study, an increased apparent cecal fermentation (lower cecum pH and higher cecal content weight) of SAC comparing with HA or CS was observed within each cooking method. An increased SCFAs or individual major SCFA (butyrate, acetate, and propionate) were found in some SAC groups compared with CS groups. Although no reports have been found discussing the function of SCFAs in the cooked resistant starches, some previous studies showed the change in cecum fermentation and SCFAs in rats after they were fed heat-moisture treatment (HMT)-treated resistant starch. HMT is defined as a physical modification that involves treatment of starch granules. Three conditions are required for the treatment: 1) moisture < 35% moisture w/w, 2) temperature at 84 – 120 C, 3) treatment time at 15 min – 16h. Novelose 330 is a retrograded RS3 with RS content of 40.4%. A hydrothermal treatment was conducted with Novelose 330 to increase the RS content up to 75%. The fermentation of
hydrothermal-treated Novelose 330 produced higher concentrations of short chain fatty acids and butyrate than untreated Novelose 330. Furthermore, the fermentation of hydrothermal-treated Novelose 330 was shown to occur beyond the caecum and throughout the colon, yet the fermentation of untreated Novelose 330 occurred in the caecum and proximal colon. Total bile acid concentration increase was strongly inhibited in hydrothermal-treated Novelose 330, which in turn was related to the low pH and high concentrations of butyrate. Since bile acid, especially some secondary bile acids like deoxycholic acid (DCA) and lithocholic acid (LCA), has been reported to be implicated in the promotion of colon tumorigenesis in animals and man, the low pH and high butyrate concentration may indicate a protection of the mucosa of the distal colon from toxic and inflammatory damage (Jacobasch et al., 2006).

Putative targets of w-SAC that may be involved in the reduction of preneoplasia were identified as genes that were up-regulated by AOM induction (compared with saline) and down-regulated by w-SAC intake (compared with w-CS), such as HSPH1, MCM6, Rac1, CD55, TFRC and HSPD1. Of these genes, Rac1 is a key gene in several pathways, such as Rac1/PAK1 and ILK/IQGAP1/Rac1 signaling transduction pathway, and enhances colon cancer cell migration and invasion (Zhu et al., 2011; Tang et al., 2011; Wang et al., 2010). CD55 is a decay accelerating factor and considered a colon cancer marker (Bjørge and Jensen, 1996; Koretz et al., 1992). Tfrc is a target gene of Wnt pathway, which regulates development, cell growth and proliferation (Röhrs et al., 2009). MCM6 has been reported as a biomarker of cell proliferation and its expression has been observed to relate to cutaneous melanoma, Merkel cell carcinoma, and lung cancer (Nioi et al., 2008; Gammbichler et al., 2009; Kadara et al., 2009). MCM6 also played a critical role in regulating the initiation of DNA replication and cell cycle in the butyrate induced
cell cycle arrest in bovine cells (Li and Li, 2008). HSPD1 is an apoptosis-related gene and has been reported to be overexpressed in human colon cancer tissues, human gastric carcinoma cell line, and human ovarian cancer cell line (Jung et al., 2011; Nagahama et al., 2008; Li et al., 2010). HSPH1 was also reported to be overexpressed in squamous cell carcinoma (Muchemwa et al., 2006). Meanwhile, a few other genes, i.e. CD48, were down-regulated by AOM induction and up-regulated by w-SAC intake. CD48 is an immune response gene and a member of NF-kappa B gene family (Ramos et al., 2011). CD48 can increase the immunogenicity on some poorly immunogenic tumors in mice when combined to B7-1 (Li et al., 1996).

Our study indicated for the first time that the preneoplastic lesions (ACF and MDF) were inhibited by SAC diet prepared by either of the two cooking methods, water-boiling and bread-baking, at the promotion stage in male Fisher 344 rats. This is the first report in the literature where different cooking methods were compared for their inhibitory effects on the preneoplastic lesions. A genome-wide microarray expression analysis was also conducted, and promising genes for inhibiting colon carcinogenesis by SAC intake were identified. Further studies are needed to explain the impact of different starch cooking methods on colon tumorigenesis and the application of the promising genes in the prevention of colon cancer, so that humans can benefit from improved dietary strategies for colon cancer prevention.

**Abbreviations**

ACF, Aberrant Crypt Foci

AOM, Azoxymethane
Safety

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

Acknowledgements

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**Figure Captions**

**Fig. 1. Cecal weight and pH measured when rats were killed**
(9 weeks after the first AOM injection; n = 5 for saline-injected rats in each diet group, n = 10 for AOM-injected rats in each diet group):
A. Cecal tissue weight (g); B. cecal content weight (g); C. cecal content pH.
Values are means ±SD. a,b,c,d,e,f bars with different letters in each panel are significantly different at P-value < 0.05. One sample was collected from each rat. Five samples were collected from saline-injected rats and 10 from AOM-injected rats in each diet group.

**Fig. 2. Colon lesion numbers and multiplicities in rats fed water-boiled or bread-baked starch diets killed 9 weeks after the first AOM injection** (n = 5 for saline-injected rats in each diet group, n = 10 for AOM-injected rats in each diet group):
A. total ACF number; B. ACF multiplicity; C. total MDF number. The ACF and MDF were measured in the distal 75mm of the colon from anus.
ACF were only seen in AOM-treated rats. Values are means ±SD. a,b,c,d bars with different letters in each panel are significantly different at P-value < 0.05. One sample was collected from each rat. Five samples were collected from saline-injected rats and 10 from AOM-injected rats in each diet group.

**Fig. 3 Proximal colon lesion numbers and multiplicities in rats fed water-boiled or bread-baked starch diets killed 9 weeks after the first AOM injection** (n = 5 for saline-injected rats in each diet group, n = 10 for AOM-injected rats in each diet group):
A. proximal total ACF number; B. proximal ACF multiplicity; C. proximal total MDF number.
ACF were only seen in AOM-treated rats. Values are means ±SD. a,b,c,d bars with different letters in each panel are significantly different at P-value < 0.05. One sample
was collected from each rat. Five samples were collected from saline-injected rats and 10 from AOM-injected rats in each diet group.

**Fig. 4. Distal colon lesion numbers and multiplicities in rats fed water-boiled or bread-baked starch diets killed 9 weeks after the first AOM injection**

(n = 5 for saline-injected rats in each diet group, n = 10 for AOM-injected rats in each diet group):

A. distal total ACF number; B. distal ACF multiplicity; C. distal total MDF number.

ACF were only seen in AOM-treated rats. Values are means ±SD. a,b,c,d. bars with different letters in each panel are significantly different at P-value < 0.05. One sample was collected from each rat. Five samples were collected from saline-injected rats and 10 from AOM-injected rats in each diet group.

**Fig. 5. Large ACF numbers and multiplicities in rats fed water-boiled or bread-baked starch diets killed 9 weeks after the first AOM injection** (n = 5 for saline-injected rats in each diet group, n = 10 for AOM-injected rats in each diet group):

A. proximal large ACF number; B. distal large ACF number; C. total large ACF number.

The ACF was measured in the distal 75mm of the colon from anus.

ACF were only seen in AOM-treated rats. Values are means ±SD. a,b,c. bars with different letters in each panel are significantly different at P-value < 0.05. One sample was collected from each rat. Five samples were collected from saline-injected rats and 10 from AOM-injected rats in each diet group.
Fig. 6. Numbers of common genes that were differentially expressed under each respective treatment group when compared to respective control. N = 10 in either AOM w-SAC rat group or AOM w-CS rat group for the comparison of gene expression level impacted by the diet treatment (n = 6 from one experiment; n = 4 from another experiment; Colon tissues were collected from distal session of the colons from each experiment). For the analysis of gene expression change caused by AOM induction, n = 10 in AOM w-CS rat group (n = 6 from distal colon tissue; n = 4 from adjacent colon tissue), and n = 9 in saline w-CS rat group (n = 6 from distal colon tissue; n = 3 from adjacent colon tissue).

Fig. 7. Heat map showing the differential expression of the genes impacted by the AOM treatment (AOM vs. saline) and the diet treatment (w-SAC vs. w-CS) with FDR < 0.01 and FC > 1.5 for each of the treatment groups.
Figures

Fig 1.

(A) Cecal Tissue Weight (g)

(B) Cecal Content Weight (g)

(C) Cecal Content pH
Fig. 2

(A) Total ACF Number

(B) ACF Multiplicity (crypts/focus)

(C) Total MDF Number

- AOM, b–CS
- AOM, b–HA
- AOM, b–SAC
- AOM, w–CS
- AOM, w–HA
- AOM, w–SAC
Fig. 3

(A) Proximal Total ACF Number

(B) Proximal ACF Multiplicity (crypts/focus)

(C) Proximal Total MDF Number

- AOM, b-CS
- AOM, b-HA
- AOM, b-SAC
- AOM, w-CS
- AOM, w-HA
- AOM, w-SAC
Fig 4.

(A) Distal Total ACF Number

(B) Distal ACF Multiplicity (crypts/focus)

(C) Distal Total MDF Number

- AOM, b–CS
- AOM, b–HA
- AOM, b–SAC
- AOM, w–CS
- AOM, w–HA
- AOM, w–SAC
Fig. 5

(A) Proximal large ACF numbers

(B) Distal large ACF numbers

(C) Total large ACF numbers

bread-baking method  water-boiling method
Fig. 6

- 1403 genes
- 6404 genes

- Differentially expressed genes caused by different diet treatments with FDR < 0.05 and FC > 1.2
- Differentially expressed genes by AOM or saline treatment with FDR < 0.05 and FC > 1.2

152 genes in common.
### Table 1A: Ingredients for the Water-boiled Starch Diet and Bread-baked Starch Diet

<table>
<thead>
<tr>
<th>Diet Ingredient</th>
<th>water-boiled starch diet</th>
<th>bread-baked starch diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>starch</td>
<td>55.00%</td>
<td>55.00%</td>
</tr>
<tr>
<td>Bread flour</td>
<td>-</td>
<td>22.06%</td>
</tr>
<tr>
<td>wheat Gluten</td>
<td>-</td>
<td>10.98%</td>
</tr>
<tr>
<td>Shortening</td>
<td>-</td>
<td>3.63%</td>
</tr>
<tr>
<td>Yeast</td>
<td>-</td>
<td>0.74%</td>
</tr>
<tr>
<td>Salt</td>
<td>-</td>
<td>0.56%</td>
</tr>
<tr>
<td>casein</td>
<td>20.00%</td>
<td>-</td>
</tr>
<tr>
<td>Dextrose</td>
<td>15.00%</td>
<td>-</td>
</tr>
<tr>
<td>mineral mix (AIN-93)</td>
<td>3.50%</td>
<td>2.00%</td>
</tr>
<tr>
<td>choline</td>
<td>0.20%</td>
<td>0.14%</td>
</tr>
<tr>
<td>methionine</td>
<td>0.30%</td>
<td>0.16%</td>
</tr>
<tr>
<td>vitamin mix (AIN-93)</td>
<td>1.00%</td>
<td>0.55%</td>
</tr>
<tr>
<td>L-lysine</td>
<td>-</td>
<td>0.43%</td>
</tr>
<tr>
<td>DL-tryptophan</td>
<td>-</td>
<td>0.05%</td>
</tr>
<tr>
<td>L-histidine</td>
<td>-</td>
<td>0.20%</td>
</tr>
<tr>
<td>DL-threonine</td>
<td>-</td>
<td>0.20%</td>
</tr>
<tr>
<td>corn oil</td>
<td>5.00%</td>
<td>3.30%</td>
</tr>
</tbody>
</table>

1Starches were purchased from Cargill Inc., Minneapolis, MN and 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).
Table 1B. Nutrient Compositions of the Water-boiled Diet or Bread-baked Diet Ingredient

<table>
<thead>
<tr>
<th>Nutrient Constituent</th>
<th>water-boiled diet (%)</th>
<th>bread-baked diet (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>20.3</td>
<td>12.51</td>
</tr>
<tr>
<td>starch</td>
<td>55</td>
<td>55.00</td>
</tr>
<tr>
<td>salt</td>
<td>-</td>
<td>0.56</td>
</tr>
<tr>
<td>mineral (AIN-93)</td>
<td>3.5</td>
<td>2.13</td>
</tr>
<tr>
<td>choline bitartrate</td>
<td>0.2</td>
<td>0.14</td>
</tr>
<tr>
<td>vitamin mix (AIN-93)</td>
<td>1</td>
<td>0.56</td>
</tr>
<tr>
<td>Total lipid (fat)</td>
<td>5</td>
<td>7.56</td>
</tr>
<tr>
<td>other carbohydrate</td>
<td>15</td>
<td>20.43</td>
</tr>
<tr>
<td>other Fiber, total dietary</td>
<td>-</td>
<td>0.79</td>
</tr>
<tr>
<td>Sugars, total</td>
<td>-</td>
<td>0.07</td>
</tr>
<tr>
<td>Ash</td>
<td>-</td>
<td>0.26</td>
</tr>
</tbody>
</table>

Table 1C. Amino Acid Composition of Water-boiled Diet and Bread-baked Diet Ingredient

<table>
<thead>
<tr>
<th>Amino Acid Constituent</th>
<th>water-boiled diet (%)</th>
<th>bread-baked diet (%)</th>
<th>AIN-93M Ingredient amino acid composition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>0.56</td>
<td>0.32</td>
<td>0.39</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.70</td>
<td>0.58</td>
<td>0.49</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>1.29</td>
<td>0.51</td>
<td>0.9</td>
</tr>
<tr>
<td>Cystine</td>
<td>0.33</td>
<td>0.25</td>
<td>0.23</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>4.09</td>
<td>4.54</td>
<td>2.86</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.39</td>
<td>0.46</td>
<td>0.27</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.51</td>
<td>0.49</td>
<td>0.36</td>
</tr>
<tr>
<td>*Isoleucine</td>
<td>0.96</td>
<td>0.57</td>
<td>0.67</td>
</tr>
<tr>
<td>*Leucine</td>
<td>1.73</td>
<td>0.96</td>
<td>1.21</td>
</tr>
<tr>
<td>*Lysine</td>
<td>1.46</td>
<td>1.12</td>
<td>1.02</td>
</tr>
<tr>
<td>*Methionine</td>
<td>0.51</td>
<td>0.40</td>
<td>0.36</td>
</tr>
<tr>
<td>*Phenylalanine</td>
<td>0.96</td>
<td>0.68</td>
<td>0.67</td>
</tr>
<tr>
<td>Proline</td>
<td>2.36</td>
<td>1.60</td>
<td>1.65</td>
</tr>
<tr>
<td>Serine</td>
<td>1.10</td>
<td>0.61</td>
<td>0.77</td>
</tr>
<tr>
<td>*Threonine</td>
<td>0.77</td>
<td>0.55</td>
<td>0.54</td>
</tr>
<tr>
<td>*Tryptophan</td>
<td>0.21</td>
<td>0.19</td>
<td>0.15</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>1.01</td>
<td>0.39</td>
<td>0.71</td>
</tr>
<tr>
<td>Valine</td>
<td>1.14</td>
<td>0.60</td>
<td>0.80</td>
</tr>
</tbody>
</table>

* Essential amino acids

1 The nutrient compositions of the bread-baked diet ingredient was calculated based on the USDA National Nutrient Database for Standard Reference.

2 The amino acid composition of wheat gluten was calculated based on the publication of Wookey N (Wookey, 1979).
Table 2. Primer Sequences for Differentially Expressed Genes Confirmed by Quantitative Real Time PCR

<table>
<thead>
<tr>
<th>gene</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC</td>
<td>5’- GTCCCCCGTGTACTTTTCA -3’ 5’- GAGCACTGTGGGGATATGGT -3’</td>
</tr>
<tr>
<td>CACYBP</td>
<td>5’- AGAAGAGGTCAAAGTGCTGCTGGA -3’ 5’- ACCCTGTTGTAAGAGGAGCAACCA -3’</td>
</tr>
<tr>
<td>Rhoa</td>
<td>5’- TGCCCATCATCCTAGTTGGGAACA -3’ 5’- AGCGCCAATCCTGTTTGCCATATC -3’</td>
</tr>
<tr>
<td>MAPK1</td>
<td>5’- GCGCTACACACACCTCTCGT -3’ 5’- CACGGTGCAAGACGTAGCTG -3’</td>
</tr>
<tr>
<td>MCM6</td>
<td>5’- AAGTAT TCCCCGGAGTTAGA GG-3’ 5’- GACACCGCCTTT TACTTCATCATT -3’</td>
</tr>
<tr>
<td>HSPD1</td>
<td>5’-TCACCGGAAGCCCTTGGTATAAT-3’ 5’- ACCCTGGAGCTTTGACTGCTACAA -3’</td>
</tr>
<tr>
<td>TOPBP1</td>
<td>5’-TCATGTGACACACCTCCATCGTGA-3’ 5’- TTCATTCTGGGAGGGCTCTGT -3’</td>
</tr>
<tr>
<td>HSPH1</td>
<td>5’-TTAGCGTGAGCAGATACACAGCA-3’ 5’- GTCATTCATGAGCCGAACGAGTT-3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5’-GGGTGGTGCCAAAAGGGGTC-3’ 5’- GGAGTTGCTGTGTTGAATGCACA-3’</td>
</tr>
</tbody>
</table>

The primers used to confirm differentially expressed genes were designed using the software PrimerQuest (Integrated DNA Technologies, Inc., Coralville, IA).
Table 3. Resistant Starch Contents and Water Content of the Three Diets Prepared by Two Methods.

<table>
<thead>
<tr>
<th>Starch Groups</th>
<th>Resistant Starch Content on Dry Diet Basis (% ± sd)</th>
<th>Moisture Content of the Diets (% ± sd)</th>
</tr>
</thead>
<tbody>
<tr>
<td>b-CS</td>
<td>2.6 ± 0.1&lt;sup&gt;e&lt;/sup&gt;</td>
<td>52% + 2%&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>b-HA</td>
<td>24.5 ± 0.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>50% + 0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>b-SAC</td>
<td>32.7 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>49% + 1%&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>w-CS</td>
<td>2.9 ± 0.2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>60% + 1%&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>w-HA</td>
<td>16.4 ± 1.2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>53% + 2%&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>w-SAC</td>
<td>27.2 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>49% + 1%&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD (n = 3). Resistant starch contents of diets were measured by AOAC method 991.43.

a, b, c, d, e Indicates values in each column with different letters are significantly different at P-value < 0.05.
Table 4. SCFA Contents in Cecum, Colon and Feces of Fisher 344 Rats Fed Water-boiled Starch Diet or Bread-baked Starch Diet.

<table>
<thead>
<tr>
<th>Group</th>
<th>Bread-baked starch diet</th>
<th>Water-boiled starch diet</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline</td>
<td>AOM</td>
<td>Saline</td>
<td>AOM</td>
<td>Saline</td>
<td>AOM</td>
<td>Saline</td>
<td>AOM</td>
<td>Saline</td>
</tr>
<tr>
<td>Cecum (µmol/cecum)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total SCFA</td>
<td>332.1 ± 89.7 a</td>
<td>1823.4 ± 414.7 bc</td>
<td>203 ± 60.1 a</td>
<td>1601.4 ± 501.4 bc</td>
<td>1094.8 ± 244.2 b</td>
<td>327.1 ± 1489.8 ± 359.2 bc</td>
<td>1196.3 ± 379.6 ± 71.6 a</td>
<td>1032.0 ± 295.8 ± 846.5 b</td>
<td></td>
</tr>
<tr>
<td>Acetate</td>
<td>208.9 ± 73.9 a</td>
<td>1695.8 ± 398.7 bc</td>
<td>294.5 ± 27.9 a</td>
<td>1334.8 ± 437.6 bc</td>
<td>925.8 ± 269.2 b</td>
<td>284.6 ± 1379.1 ± 470.4 bc</td>
<td>326.8 ± 1010.6 ± 340.1 bc</td>
<td>132.5 ± 98.5 ± 53.0 ± 31.0 bc</td>
<td></td>
</tr>
<tr>
<td>Propionate</td>
<td>17.3 ± 3.9 a</td>
<td>57.9 ± 17.8 ac</td>
<td>17.1 ± 5.4 a</td>
<td>58.0 ± 44.5 abc</td>
<td>51.7 ± 4.4 a</td>
<td>19.2 ± 167.6 ± 105.4 c</td>
<td>26.2 ± 21.8 ± 107.8 ± 63.8 c</td>
<td>24.4 ± 5.3 ± 44.5 ± 3.6 a</td>
<td></td>
</tr>
<tr>
<td>n-Butyrate</td>
<td>25.8 ± 12.7 ab</td>
<td>69.7 ± 74.4 ab</td>
<td>23.7 ± 60.1 b</td>
<td>105.1 ± 39.0 ab</td>
<td>64.1 ± 23.3 ± 9.3 ab</td>
<td>54.8 ± 101.4 ± 50.9 b</td>
<td>30.9 ± 77.9 ± 41.7 b</td>
<td>53.0 ± 31.0 ab</td>
<td></td>
</tr>
<tr>
<td>Colon (µmol/g wt)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total SCFA</td>
<td>152.6 ± 52.5 bc</td>
<td>172.4 ± 45.7 c</td>
<td>135.0 ± 42.2 bc</td>
<td>138.2 ± 49.2 bc</td>
<td>124.2 ± 40.4 bc</td>
<td>114.1 ± 111.8 ± 40.1 bc</td>
<td>135.9 ± 113.0 ± 72.7 ± 30.1 a</td>
<td>89.5 ± 28.6 ± 28.6 ab</td>
<td></td>
</tr>
<tr>
<td>Acetate</td>
<td>132.6 ± 43.7 bc</td>
<td>160.4 ± 33.8 c</td>
<td>118.4 ± 38.2</td>
<td>119.4 ± 41.1</td>
<td>110.6 ± 18.1</td>
<td>99.3 ± 97.1 ± 40.4</td>
<td>124.1 ± 97.9 ± 32.4 ± 29.1</td>
<td>74.1 ± 28.1</td>
<td></td>
</tr>
<tr>
<td>Propionate</td>
<td>7.9 ± 2.5 ab</td>
<td>5.2 ± 3.6 ab</td>
<td>4.7 ± 4.3 ab</td>
<td>5.8 ± 4.4</td>
<td>6.7 ± 4.4</td>
<td>6.7 ± 6.7 ± 4.0 b</td>
<td>2.5 ± 0.2 ± 3.3</td>
<td>10.5 ± 6.9</td>
<td></td>
</tr>
<tr>
<td>n-Butyrate</td>
<td>12.2 ± 6.8</td>
<td>6.8 ± 4.6 ab</td>
<td>10.1 ± 6.2</td>
<td>7.9 ± 6.0</td>
<td>8.0 ± 3.2</td>
<td>4.1 ± 4.8</td>
<td>9.3 ± 4.4</td>
<td>8.8 ± 3.0</td>
<td></td>
</tr>
</tbody>
</table>

Col (µmol/g wt)
### Table 1: SCFA Concentrations in Feces

<table>
<thead>
<tr>
<th>Component</th>
<th>Total SCFA</th>
<th>Acetate</th>
<th>Propionate</th>
<th>n-Butyrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>41.5 ± 30.0</td>
<td>36.2 ± 26.0</td>
<td>2.9 ± 2.5</td>
<td>2.4 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>67.1 ± 18.0</td>
<td>60.3 ± 14.9</td>
<td>3.4 ± 3.2</td>
<td>3.3 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>104.3 ± 45.3</td>
<td>11.8 ± 3.7</td>
<td>2.2 ± 2.7</td>
<td>4.5 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>43.7 ± 31.0</td>
<td>39.8 ± 26.7</td>
<td>2.3 ± 1.5</td>
<td>1.6 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>84.2 ± 47.3</td>
<td>75.2 ± 41.0</td>
<td>2.9 ± 3.4</td>
<td>4.5 ± 4.4</td>
</tr>
<tr>
<td></td>
<td>86.4 ± 25.0</td>
<td>77.8 ± 27.1</td>
<td>4.6 ± 3.9</td>
<td>4.0 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>67.1 ± 21.9</td>
<td>58.2 ± 19.0</td>
<td>4.5 ± 1.9</td>
<td>4.3 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>70.5 ± 40.1</td>
<td>58.4 ± 10.3</td>
<td>10.2 ± 7.6</td>
<td>1.9 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>61.5 ± 23.5</td>
<td>56.1 ± 21.5</td>
<td>1.4 ± 0.9</td>
<td>4.1 ± 3.0</td>
</tr>
<tr>
<td></td>
<td>59.6 ± 25.4</td>
<td>52.7 ± 24.1</td>
<td>3.5 ± 1.4</td>
<td>3.4 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>53.9 ± 20.4</td>
<td>43.8 ± 20.4</td>
<td>7.0 ± 3.3</td>
<td>3.1 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>61.8 ± 19.6</td>
<td>61.8 ± 19.6</td>
<td>6.3 ± 3.7</td>
<td>3.9 ± 4.3</td>
</tr>
</tbody>
</table>

**Feces (µmol/g wt)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Total SCFA</th>
<th>Acetate</th>
<th>Propionate</th>
<th>n-Butyrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>26.9 ± 15.1</td>
<td>24.0 ± 12.7</td>
<td>1.7 ± 1.2</td>
<td>1.2 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>69.7 ± 39.5</td>
<td>90.0 ± 72.5</td>
<td>4.1 ± 3.1</td>
<td>1.2 ± 1.2</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD (n = 5 for saline-injected rats in each diet group, n = 10 for AOM-injected rats in each diet group). Indicates values in each row with letters and without a common letter differ, P-value < 0.05.
Table 5. The Distribution for Fold Change of Gene Expression.

<table>
<thead>
<tr>
<th></th>
<th>Fold Change</th>
<th></th>
<th></th>
<th></th>
<th>total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.2-1.5</td>
<td>1.5-2</td>
<td>2-5</td>
<td>5-10</td>
<td></td>
</tr>
<tr>
<td>AOM w-CS vs. saline w-CS (FDR &lt; 0.05)</td>
<td>up-regulated genes</td>
<td>2036</td>
<td>949</td>
<td>379</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>down-regulated genes</td>
<td>2468</td>
<td>510</td>
<td>82</td>
<td>0</td>
</tr>
<tr>
<td>AOM w-SAC vs. AOM w-CS (FDR &lt; 0.05)</td>
<td>up-regulated genes</td>
<td>435</td>
<td>89</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>down-regulated genes</td>
<td>708</td>
<td>118</td>
<td>33</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 6A. Some Genes Differentially Expressed Caused by Both AOM Treatment and w-SAC Diet Feeding. Fold Changes (FC) are More Than 1.2 for Either Up- or Down-regulations for All the Genes.

<table>
<thead>
<tr>
<th>Gene Title</th>
<th>Gene Symbol</th>
<th>AOM treatment</th>
<th>diet treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>heat shock protein 1 (chaperonin)</td>
<td>Hspd1</td>
<td>1.91</td>
<td>0.83</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.009</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.045</td>
<td>0.000</td>
</tr>
<tr>
<td>heat shock 105kDa/110kDa protein 1</td>
<td>Hsph1</td>
<td>1.84</td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.003</td>
<td>0.000</td>
</tr>
<tr>
<td>minichromosome maintenance deficient 6 (MIS5 homolog, S. pombe) (S. cerevisiae)</td>
<td>Mcm6</td>
<td>1.85</td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.002</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.019</td>
<td>0.000</td>
</tr>
<tr>
<td>ras-related C3 botulinum toxin substrate 1</td>
<td>Rac1</td>
<td>1.25</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.012</td>
<td>0.172</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.028</td>
<td>0.236</td>
</tr>
<tr>
<td>CD55 antigen</td>
<td>Cd55</td>
<td>6.16</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.000</td>
<td>0.193</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.000</td>
<td>0.251</td>
</tr>
<tr>
<td>transferrin receptor</td>
<td>Tfrc</td>
<td>3.35</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.000</td>
<td>0.008</td>
</tr>
</tbody>
</table>
Table 6B. Some Genes Differentially Expressed Caused by AOM or Diet Treatment. Fold Changes are More Than 1.2 for Either Up- or Down-regulations for All the Genes.

<table>
<thead>
<tr>
<th>Gene Title</th>
<th>Gene Symbol</th>
<th>AOM treatment</th>
<th>Diet treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>FC</td>
<td>P.Value</td>
</tr>
<tr>
<td>calcyclin binding protein</td>
<td>Cacybp</td>
<td>3.10</td>
<td>0.000</td>
</tr>
<tr>
<td>ras homolog gene family, member A</td>
<td>Rhoa</td>
<td>2.13</td>
<td>0.000</td>
</tr>
<tr>
<td>mitogen activated protein kinase 1</td>
<td>Mapk1</td>
<td>2.15</td>
<td>0.000</td>
</tr>
<tr>
<td>adenomatosis polyposis coli</td>
<td>Apc</td>
<td>1.66</td>
<td>0.000</td>
</tr>
<tr>
<td>Janus kinase 1</td>
<td>Jak1</td>
<td>3.33</td>
<td>0.000</td>
</tr>
<tr>
<td>Janus kinase 2</td>
<td>Jak2</td>
<td>2.13</td>
<td>0.000</td>
</tr>
<tr>
<td>signal transducer and activator of transcription 1</td>
<td>Stat1</td>
<td>2.26</td>
<td>0.000</td>
</tr>
<tr>
<td>v-raf murine sarcoma 3611 viral oncogene homolog</td>
<td>Araf</td>
<td>1.37</td>
<td>0.019</td>
</tr>
<tr>
<td>eukaryotic translation initiation factor 4, gamma 2 (predicted)</td>
<td>Eif4g2_predicted</td>
<td>4.04</td>
<td>0.000</td>
</tr>
<tr>
<td>phosphatidylinositol 3-kinase, catalytic, beta polypeptide</td>
<td>Pik3cb</td>
<td>1.66</td>
<td>0.000</td>
</tr>
<tr>
<td>v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog</td>
<td>Kras</td>
<td>1.36</td>
<td>0.028</td>
</tr>
<tr>
<td>CD9 antigen</td>
<td>Cd9</td>
<td>2.37</td>
<td>0.003</td>
</tr>
<tr>
<td>CD38 antigen</td>
<td>Cd38</td>
<td>2.36</td>
<td>0.001</td>
</tr>
<tr>
<td>CD59 antigen</td>
<td>Cd59</td>
<td>2.92</td>
<td>0.000</td>
</tr>
<tr>
<td>CD55 antigen</td>
<td>Cd55</td>
<td>6.16</td>
<td>0.000</td>
</tr>
<tr>
<td>CD48 antigen</td>
<td>Cd48</td>
<td>0.81</td>
<td>0.415</td>
</tr>
<tr>
<td>caspase 8</td>
<td>Casp8</td>
<td>2.41</td>
<td>0.000</td>
</tr>
<tr>
<td>caspase 3, apoptosis related cysteine protease</td>
<td>Casp3</td>
<td>3.40</td>
<td>0.000</td>
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<tr>
<td>interleukin 1 alpha</td>
<td>Il1a</td>
<td>2.23</td>
<td>0.001</td>
</tr>
<tr>
<td>transferrin receptor</td>
<td>TFRC</td>
<td>3.35</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Topoisomerase (DNA) II beta binding protein    Topbp1  0.76  0.045  0.050

FC stands for Fold Change
Fold change of AOM treatment reflects the multiple on gene expression level of AOM w-CS compared with that of saline w-CS. Fold change of diet treatment reflects the multiple on gene expression level of AOM w-SAC compared with that of AOM w-CS.
Table 7. Quantitative Real-time PCR Analysis of the Expression Levels of Genes Affected by AOM or Diet to Confirm Microarray Results (Data represent means ± standard deviation)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Effect Compared</th>
<th>real-time PCR analysis</th>
<th>p-value</th>
<th>Gene Expression Level</th>
<th>Confirmed Microarray Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>CACYBP</td>
<td>AOM w-CS vs. saline w-CS</td>
<td>-3.45 + 2.27</td>
<td>0.0150</td>
<td>increase</td>
<td>Yes</td>
</tr>
<tr>
<td>RhoA</td>
<td>AOM w-CS vs. saline w-CS</td>
<td>-2.84 + 2.52</td>
<td>0.0411</td>
<td>increase</td>
<td>Yes</td>
</tr>
<tr>
<td>MAPK1</td>
<td>AOM w-CS vs. saline w-CS</td>
<td>-3.36 + 2.46</td>
<td>0.0243</td>
<td>increase</td>
<td>Yes</td>
</tr>
<tr>
<td>APC</td>
<td>AOM w-CS vs. saline w-CS</td>
<td>-2.26 + 2.96</td>
<td>0.4379</td>
<td>no change</td>
<td>No</td>
</tr>
<tr>
<td>TOPBP1</td>
<td>AOM w-SAC vs. AOM w-CS</td>
<td>1.50 + 1.34</td>
<td>0.0031</td>
<td>decrease</td>
<td>Yes</td>
</tr>
<tr>
<td>HSPH1</td>
<td>AOM w-SAC vs. AOM w-CS</td>
<td>1.56 + 1.29</td>
<td>0.0015</td>
<td>decrease</td>
<td>Yes</td>
</tr>
<tr>
<td>MCM6</td>
<td>AOM w-SAC vs. AOM w-CS</td>
<td>1.64 + 1.68</td>
<td>0.0347</td>
<td>decrease</td>
<td>Yes</td>
</tr>
<tr>
<td>HSPD1</td>
<td>AOM w-SAC vs. AOM w-CS</td>
<td>0.91 + 1.28</td>
<td>0.0503</td>
<td>decrease</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>AOM w-CS vs. saline w-CS</td>
<td>-0.81 + 1.27</td>
<td>0.0767</td>
<td>increase</td>
<td>Yes</td>
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</tbody>
</table>
## Supplement 1. Functional Classes of Genes that were Significantly Increased or Decreased.

<table>
<thead>
<tr>
<th>Term</th>
<th>Enrichment Score</th>
<th>% genes differentially expressed in pathway</th>
<th>P-Value</th>
<th>Benjamini</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>up-regulation genes by AOM treatment</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alzheimer's disease</td>
<td>2.276</td>
<td>3.7</td>
<td>0.0053</td>
<td>0.5</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>1.854</td>
<td>2.3</td>
<td>0.014</td>
<td>0.6</td>
</tr>
<tr>
<td>RIG-I-like receptor signaling pathway</td>
<td>1.538</td>
<td>1.7</td>
<td>0.029</td>
<td>0.72</td>
</tr>
<tr>
<td>Hematopoietic cell lineage</td>
<td>1.495</td>
<td>2.0</td>
<td>0.032</td>
<td>0.66</td>
</tr>
<tr>
<td>Jak-STAT signaling pathway</td>
<td>1.409</td>
<td>2.5</td>
<td>0.039</td>
<td>0.65</td>
</tr>
<tr>
<td>Viral myocarditis</td>
<td>1.260</td>
<td>1.7</td>
<td>0.055</td>
<td>0.71</td>
</tr>
<tr>
<td>Pathways in cancer</td>
<td>1.187</td>
<td>4.5</td>
<td>0.065</td>
<td>0.71</td>
</tr>
<tr>
<td>Natural killer cell mediated cytotoxicity</td>
<td>1.174</td>
<td>2.0</td>
<td>0.067</td>
<td>0.68</td>
</tr>
<tr>
<td>Focal adhesion</td>
<td>1.071</td>
<td>3.1</td>
<td>0.085</td>
<td>0.72</td>
</tr>
<tr>
<td><strong>down-regulation genes by AOM treatment</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
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<td>6.4</td>
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<tr>
<td>Systemic lupus erythematosus</td>
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<tr>
<td>B cell receptor signaling pathway</td>
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<td>5.1</td>
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</tr>
<tr>
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<td>Prion diseases</td>
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<td>3.8</td>
<td>0.022</td>
<td>0.17</td>
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<tr>
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<tr>
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<td>2.886</td>
<td>2.7</td>
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<td><strong>up-regulation genes by w-SAC treatment</strong></td>
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<tr>
<td>Steroid biosynthesis</td>
<td>3.208</td>
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<td>Pathway</td>
<td>Enrichment Score</td>
<td>FDR</td>
<td>P-value</td>
<td>q-value</td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>------------------</td>
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<td>1.620</td>
<td>1.7</td>
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<td>Oocyte meiosis</td>
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<td>1.523</td>
<td>2.3</td>
<td>0.03</td>
<td>0.36</td>
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<tr>
<td>Fructose and mannose metabolism</td>
<td>1.377</td>
<td>1.7</td>
<td>0.042</td>
<td>0.41</td>
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<tr>
<td>Butanoate metabolism</td>
<td>1.357</td>
<td>1.7</td>
<td>0.044</td>
<td>0.38</td>
</tr>
<tr>
<td>Graft-versus-host disease</td>
<td>1.022</td>
<td>1.7</td>
<td>0.095</td>
<td>0.61</td>
</tr>
</tbody>
</table>

Enrichment score is the negative log of the p-value. Enrichment score of 1.3 is equivalent to non-log scale 0.05. Thus, more attention should go to groups with scores ≥ 1.3. Benjamini-Hochberg is a statistics for multiple comparison corrections. It corrects p-values to be more conservative in order to lower family-wise false discovery rate.
Supplement

Pathways that were significantly enriched by either AOM/saline treatments or different diet treatments were picked out by DAVID 7. Up-regulated signaling pathways, such as Jak-STAT signaling pathway, Cancer related pathways, Apoptosis, Natural killer cell mediated cytotoxicity, Focal adhesion, RIG-I-like receptor signaling pathway, Alzheimer's disease pathway, may be important pathways for the induction of preneoplasia by AOM (Table supplement 1).

Pathways were also clustered by genes affected by feeding w-SAC against w-CS in AOM-induced rats. DNA replication, ABC transporters, cell cycle and RNA degradation pathways were discovered from the down-regulated genes all with a p-value less than 0.05. The up-regulated genes in the diet effect genes were clustered mainly as lipid or carbohydrate metabolism pathways. Some other pathways involved in cell cycle (oocyte meiosis pathway, p-value < 0.03) or immune activity (graft-versus-host disease pathway, p-value < 0.1) were also clustered (Table supplement 1).
CHAPTER 4: A STUDY ON THE EFFECT OF WATER-BOILED GUAT 209 AND A HYBRID OF GUAT209 AND AR16035 CORN STARCH ON AZOXYMETHANE-INDUCED PRENEOPLASTIC LESIONS IN THE RAT COLON

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KEYWORDS: ARXGuat; resistant starch; cooking; aberrant crypt foci; mucin depleted foci; crypt/foci
Abstract

Guat209 (Guat) and AR16035 (AR) are two corn lines both from Latin American exotic populations. These two corn lines were crossed to form a new line ARXGuat. Guat is a resistant starch (RS) with 34.5% of resistant starch content, while AR and ARXGuat hold low resistant starch content of 1.2% and 1.9%, respectively. The prevention abilities of both water-boiled Guat and ARXGuat on preneoplastic lesions in the colons of azoxymethane (AOM)-treated Fisher344 rats were compared with water-boiled AR. Each starch composed 55% of the diet and was fed to the AOM-induced rats for 8 weeks. Our study showed a notable increase in the weights of the cecal content and cecal tissue, and a decreased cecum pH in the Guat-fed rats compared with those in the AR- or ARXGuat-fed rats. A slight increase of crypts/foci was observed in the AOM-injected rats fed Guat compared with those fed the other two diets. No differences in aberrant crypt foci (ACF) and mucin depleted foci (MDF) numbers, as well as aberrant crypt (AC) numbers were seen among the groups. This study suggested no inhibitory effect of cooked starches from either the inbred corn line Guat or hybrid corn line ARXGuat on AOM induced colorectal preneoplastic lesions induced in Fisher 344 rats.

Introductions

Colorectal cancer (CRC) is one of the most prevalent cancers in western countries and is rapidly increasing in developing countries. CRC is the third most common cancer in both men and women in the United States (American Cancer Society, 2010). It has been commonly believed that the incidence of CRC is greatly affected by food intake. Diets with RS have been reported to reduce CRC. Several studies have observed suppression of colon cancer or precancerous biomarkers by dietary resistant starch or
other dietary fibers in rat models (Le Leu et al., 2007a; Le Leu et al., 2007b; Clarke et al., 2008; Jacobsen et al., 2006; Caderni et al., 1994; Thorup et al., 1995). In other studies, indirect evidence was shown for the protective role of RS on CRC incidence both in rat studies and in human trials. In AOM-injected Sprague–Dawley rats fed high amylose cornstarch, increased fecal and cecal fermentation and concentrations of short chain fatty acids (SCFA), as well as a decreased cell proliferation in the colonic epithelium, were observed, which might indicate a correlation between the inhibitory effects of RS on CRC and the cecal physiological changes after RS intake (Le Leu et al., 2003). Studies of bile acids and secondary bile acids, two CRC promoting agents, were also among some of the indirect evidence. The concentration of bile acids and secondary bile acids were significantly reduced in colonic adenoma(s) removed patients after the intake of 45 g native amylo maize starch (resistant starch content of the starch was 62%) for 4 weeks, compared with control starch intake (45 g of maltodextrin) (Grubben et al., 2001). 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 (Caderni et al., 1991; Sakamoto et al., 1996; Young et al., 1996).

Resistant starch is a dietary starch that is not digested and absorbed in the small intestine, but fermented in the large intestine instead. Resistant starch can be classified into four main types: RS1 to 4. The RS5 classification was newly introduced (Hasjim et al., 2010). Resistant starch1 is physically entrapped starch such as coarsely milled grain. RS2 comprises starches highly resistant to α-amylases digestion by their nature. High amylose corn starch is rich in RS2. RS3 includes starches retrograded by a cook-and-cool process. RS4 are chemically modified starches, and RS5 are a complex of amylose and lipid.
AR and Guat are derivatives of the Argentine and Guatemala corn landrace and were inbred respectively (work conducted in the Department of Food Science and Human Nutrition, Iowa State University and Agricultural Research Service, U.S. Department of Agriculture). Guat is an exotic population with the amylose content up to 70% and is considered RS, but this cornline is not high in yield. AR was derived from a high-yielding Latin American exotic population and is known as digestive starch. Crosses were made between AR and Guat corn lines, and a new corn line, ARXGuat, was formed. This hybrid, ARXGuat, was hoped to retain both the high-yield from AR and the high resistant starch content from Guat. One important gene that may hold the high RS trait of Guat is the mutant of branching enzyme 1 gene (Sbe1). Corn starch with sbe1a mutant was reported to alter molecular structures resulting in a better resistance to pancreatic α-amylase, β-amylase, and isoamylase than wt starch (Xia et al., 2011). This mutant of Sbe1 was expected to be inherited from Guat to ARXGuat so that the hybrid starch can retain the high RS property from Guat. In our test of resistant starch content, Guat contained 34.5% of resistant starch, yet AR and ARXGuat were low in resistant starch content (1.2% and 1.9% respectively). The research reported here tested the hypothesis that both the inbred corn line Guat and the hybrid ARXGuat could inhibit colon preneoplastic lesions, ACF and MDF, in the colon of carcinogen-induced rats. All three starches were cooked by a water-boiling procedure in this study based on our previous observation that the cooking procedure could improve the impact of resistant starch against colon carcinogenesis, and starchy food are commonly served to humans cooked (Zhao et al., 2011).
Materials and Methods

Diets. AR, Guat, and ARXGuat were cooked by a water-boiling method and then mixed with other components into diets. The diet formulation was based on the standard diet recommended by the American Society for Nutritional Sciences for mature rats (AIN-93M) (Reeves, 1997), but the 5% cellulose in the AIN-93M was not included and the starch content was increased by this amount. Thus the proportion of the starch reached 55% (Table 1). The boiling procedure was described in a previous study (Zhao et al., 2011). Diets were made and fed fresh to the rats every two days. The resistant starch contents of the starches and diets were measured fresh by AOAC method 991.43 (Horwitz, 2003). The water content of these samples was determined by a Sartorius moisture analyzer MA30 (Bohemia, NY). The resistant starch contents and water contents were listed in Table 2.

Animals and Housing. Forty-five five-week-old male Fischer 344 (F344) rats were obtained from Charles River laboratory (Wilmington, MA). Each rat was housed individually in a plastic cage with stainless-steel, wire-mesh top, and a stainless-steel wire floor was placed on the bottom of each cage to prevent the rats from eating their own feces. The temperature of the animal room was 22±1°C and relative humidity at 60±5%, and fluorescent lights were on from 6 AM to 6 PM. 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 of Iowa State University.

Carcinogen Treatment. AOM was purchased from Midwest Research Institute (Kansas City, MO) and injected at the dosage of 20 mg AOM/Kg rat body weight (n=30 rats). The
rats were injected intraperitoneally one week after they arrived. A second injection was followed 1 week later. The remaining rats were injected with physiological saline as negative controls (n = 15).

**Diet Treatment.** AR was fed to all the rats from the day they arrived until 3 days after the second AOM injection. Then the rats were divided randomly into 3 diet groups with 15 rats in each diet group (10 AOM-injected rats and 5 saline-injected rats) and fed the respective diets for 8 weeks. Body weight was measured weekly. Food disappearance was measured by calculating the difference between the amount that was fed to the rats and the amount that remained at the end of each feeding time. The rats were then euthanized by CO$_2$.

**Cecal Weight and pH.** Right after the rat died, its cecum was collected and weighed as cecal weight with contents. The cecum was then cut open and cecal contents were scraped off from the cecal tissue to measure the cecal pH. Then the cecum tissue was rinsed in phosphate buffered saline, quickly dried with a paper towel and weighed as the cecal tissue weight. The difference of the cecal weight with content and cecal tissue weight was calculated as cecal content weight. The liver was also 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 50 mm were trimmed and fixed in 10% formalin for 24 hours before they were made into specimens. Colons were stained with 1% alcian blue in acetic acid (pH 2.5), then counterstained with 1% neutral red to view ACF and MDF using a light microscope.
Statistical Analysis. Body weight, food disappearance, liver weight, cecal endpoints, ACF and MDF number, as well as average crypts/foci were analyzed using Two-way AVONA performed through GLM Procedure in SAS (SAS Institute, 2010). One-sided t-tests were then applied to test the significant differences between groups. All values were reported as mean ± standard deviation and P-value < 0.05 was considered significant.

Results

Resistant Starch Content of the Experimental Diets. Resistant starch content of the AR, Guat, and ARXGuat were 5.4 ± 0.5%, 18.6 ± 0.9%, and 8.6 ± 0.6% on the dry diet weight of the diets, respectively. The water contents of the 3 diets were 20.0 ± 0.9%, 14.1 ± 0.9% and 13.0 ± 1.0%, respectively. For the starches, the resistant starch contents are 1.2 ± 0.1%, 34.5 ± 2.0% and 1.9 ± 0.1% for AR, Guat, and ARXGuat, respectively. Three samples were randomly selected and measured for each diet group (Table 2).

Body Weight, food Disappearance and Liver Weight. No significant differences were seen in the body weight gain or food disappearance over the period of 8 weeks. An average body weight gain was 175 ± 6 g, and the average daily food disappearance of rats was 15.2±1.4 g/day. No effects of diet or AOM were observed on rat liver weight.

Cecum Weight and Ph. Results of total cecal weight with content, cecal tissue weight and cecal content weight were consistent in that rats fed Guat always had significantly elevated weights compared with the rats fed AR or ARXGuat within either AOM-treated groups or saline-treated groups. Significantly higher cecal tissue weight was also seen in AOM-injected rats fed ARXGuat compared with those fed AR. Other than these, no
differences of cecal weight were observed between AR and ARXGuat fed rats (Figure 1B, C and D).

Cecal content pH was significantly decreased in the cecum of rats fed Guat compared with the rats fed the other 2 diets whether AOM or saline were injected. Azoxymethane-treated rats fed ARXGuat had a statistically significant decreased cecal content pH compared with those fed AR, but the same trend of decreased cecal content pH in the ARXGuat fed rats compared with the AR fed rats was not seen in the saline-injected groups (Figure 1A).

**Aberrant Crypt Foci and Mucin Depleted Foci.** ACF and MDF were only seen in the colons of AOM-treated rats. Rats fed Guat showed a small but statistically significant increase in average crypts/foci in their colon samples compared with those fed the other 2 diets (Figure 2B). A 37% decrease of ACF number was observed in the colon sample from AOM-treated Guat rat group when compared to the AR-fed rats, and a 26% of decrease trend was also seen in the ARXGuat group compared to the AR group, yet neither of these decreases were significant (Figure 2A and 2B). The total AC number, which is a multiple of ACF number and average crypts/foci, did not show significant differences between the rats fed 3 diets, although the total AC number in the AR group was 30-40% higher than in the ARXGuat or Guat group (Figure 2D). No differences in the MDF number were observed (Figure C).

**Discussion**

The results of this study showed that feeding rats with ARXGuat did not significantly alter the yield of ACF or MDF numbers, and only the average number of
crypts/foci was somewhat elevated in the Guat-fed rat colon. Since there was actually a reduced trend of ACF number in the Guat group compared with the other two diet groups, the total AC number was not elevated in the Guat group against AR or ARXGuat groups. On the other hand, MDF was considered a subgroup of ACF that may predict tumor outcome better than ACF (Caderni et al., 2003; Femia et al., 2003). In our study, although a decreased trend of the numbers of ACF, MDF and AC was seen with the increased resistant starch content in the diets, no significant differences were observed, suggesting that the preneoplastic lesion rate was not altered by Guat or ARXGuat starch in AOM-induced Fisher 344 rats compared with the normal control cornstarch AR. Meanwhile, Crypts/foci is a measurement of the ACF size, which was considered more related to the malignant potential of CRC than ACF number (Osawa et al., 2002; Pierre et al., 2003). A study of the predictable feature of ACF size on polyp histopathology was conducted on patients undergoing routine colorectal cancer screening over a 31-month period (20 crypts/ACF as small ACF; 20-100 crypts/ACF as medium; >100 crypts/ACF as large). The study showed that small flat ACFs correlated with the occurrence of distal adenomas, and large raised ACFs were more of a precursor of hyperplastic polyps (Kim et al., 2008). In our study, although the ACF size slightly elevated in the Guat-fed rat group after AOM treatment, ACF sizes assessed from the rats fed all of the 3 diets fell in the range of small ACFs. We thus believe ACF size in rats fed either Guat or the other two diets prone to present the potential of these preneoplastic lesions to form distal adenomas.

Rats fed Guat starch diet had increased cecal tissue and content weight, and decreased cecal content pH compared with those fed AR or ARXGuat starch diets. The increased cecal weight and decreased cecal pH were previously reported as a result of the fermentation of RS and production of SCFAs in the rat colon by gut bacteria. In a previous study, a diet containing 16.7% potato starch (RS 1) or 66.7% modified potato
starch (RS 2) to male Wistar rats increased rat cecal and fecal SCFA, and stimulated the growth of bifidobacteria compared with basal diet (60% waxy maize). A significant increase in total culturable bacteria, lactobacilli, streptococci, and enterobacteria was also observed in RS2 treated animals (Kleessen et al., 1997). Cecal weight gain was also observed in Sprague-Dawley rats after they were fed high amylose resistant cornstarch (Keenan et al., 2006). In our study, Guat maintains statistically higher resistant starch content than the other two starches, thus a stronger fermentation may occur in the Guat-fed rats and caused a decreased cecal pH and increased cecal weights. This high fermentation may relate to increased SCFAs. Meanwhile, SCFA, especially butyrate, was reported as an inhibitor of CRC through the regulation of proliferative, differentiation and apoptosis (Inagaki and Sakata, 2005; Kruh, 1982; Smith, 1986), but this link between the increased SCFA accumulation and reduced preneoplastic lesions was not seen in our study. It might be because the resistant starch content is not high enough to produce sufficient SCFA that can inhibit carcinogenesis, but is this assumption correct, or what is a ‘sufficient’ amount of resistant starch content or SCFA content is not known yet. In our study, the resistant starch content of water-boiled Guat diet was $18.6 \pm 0.9\%$, which was similar to the resistant starch content of water-boiled HA diet in the last study (see Chapter 3) where the resistant starch content of the w-HA was $16.4 \pm 1.2\%$. Comparing with their respective normal starch diet controls, rats fed either diet had an increased cecal weight, but neither of the groups of rats fed these diets experienced a decrease in the ACF/MDF numbers. This provided some evidence for our assumption that diets much contain a certain amount of resistant starch in order to inhibit colon carcinogenesis.

In conclusion, Guat did not show inhibitory abilities on ACF and MDF, the two colorectal preneoplastic lesions, although Guat contains significantly higher resistant starch content than either ARXGuat or AR starch. Not surprisingly, rats fed ARXGuat did
not show any inhibition of preneoplastic lesions against rats fed AR, which might be related to the fact that the hybrid cornline ARXGuat did not increase the resistant starch content compared with either of its parent cornlines, either as raw starch or after cooked and made into diets. Guat diet feeding to the rats resulted in a decreased cecal content pH and increased cecal weights (cecal tissue weight, cecal weigh with content and cecal content weight), suggesting a fermentation process undergoing by gut microflora. This feature of Guat gives us a hint that Guat might be able to bring benefits to human by fighting disease such as diabetes and obesity. In a human study, dietary fibre was found to produce a SCFA profile that could have anti-inflammatory effects in the body (Roelofsen et al., 2010). In another human study with 23 hypertriglyceridemic subjects who were overweight mostly because of abdominal adiposity, subjects fed a high-amylose starch diet showed a lower fecal pH and increased short-chain fatty acid concentrations in fecal water. A significant reduction in the overall postprandial plasma insulin concentration relative to the low-amylose diet was also observed (Noakes et al., 1996). Long-term consumption of high-amylose starch on insulin and glucose response were investigated in another human study. Chronic consumption of high-amylose foods normalized the insulin response of hyperinsulinemic subjects. All these evidences led to a potential benefit of high resistant starch for diabetic subjects (Behall and Howe, 1995). There are also reports showing that RS can effectively control obesity in rats mediated by the high resistant starch content of the starch (Shimotoyodome et al., 2009; Aziz et al., 2009).

**Abbreviations**

ACF, Aberrant Crypt Foci

AOM, Azoxymethane
AR, AR cornstarch diet

ARXGuat, Hybrid resistant starch diet came from the parental lines AR and Guat

CRC, Colorectal cancer

Guat, Guat cornstarch diet

MDF, Mucin Depleted Foci

**Safety**

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

**Acknowledgements**

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**References**


Reeves PG. Components of the AIN-93 diets as improvements in the AIN-76A diet. J Nutr. 1997;127(Suppl. 5): 838S-841S.


Figure Captions

Figure 1. Cecal endpoints collected after rats were fed water-boiled AR, Guat or ARXGuat diet and killed (9 weeks after the first AOM injection): (A) cecal content pH; (B) Cecal weight with content (g); (C) Cecal tissue weight (g); (D) Cecal content weight (g).

Values are means ±SD. a,b,c bars with different letters in each panel are significantly different P-value < 0.05. Each diet was fed to 15 rats (10 rats were injected AOM, and the other 5 were injected saline).

Figure 2. Lesions observed in rat fed water-boiled AR, Guat or ARXGuat diet (rats were killed 9 weeks after the first AOM injection): (A) average ACF number; (B) average crypts/foci in ACF; (C) average MDF number; (D) average AC number. The ACF and MDF were measured in the distal 75mm of the colon.

ACF were only seen in AOM-treated rats. Values are means ±SD. a,b bars with different letters in each panel are significantly different P-value < 0.05. Each diet was fed to 15 rats (10 rats were injected AOM, and the other 5 were injected saline).
Figures

Fig. 1

A. Cecal Weight with Content (g)

B. Cecal Content Weight (g)

C. Cecal pH

D. Cecal Tissue Weight (g)
Table 1: Diet Ingredients

<table>
<thead>
<tr>
<th>Diet Ingredient</th>
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<tr>
<td>starch</td>
<td>55.0%</td>
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<tr>
<td>casein</td>
<td>20.0%</td>
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<tr>
<td>Dextrose</td>
<td>15.0%</td>
</tr>
<tr>
<td>cellulose (insoluble fiber)</td>
<td>0.0%</td>
</tr>
<tr>
<td>mineral mix (AIN-93)</td>
<td>3.5%</td>
</tr>
<tr>
<td>choline</td>
<td>0.2%</td>
</tr>
<tr>
<td>methionine</td>
<td>0.3%</td>
</tr>
<tr>
<td>vitamin mix (AIN-93)</td>
<td>1.0%</td>
</tr>
<tr>
<td>corn oil</td>
<td>5.0%</td>
</tr>
</tbody>
</table>

1. Starches were cooked as described in the text. All non-starch diet ingredients were purchased from Harland Teklad (Madison, WI) or Spectrum (Gardena, CA).
Table 2: Resistant starch contents (RC) of the three starches and three diets, and water content (WC) of the three diets.

<table>
<thead>
<tr>
<th>Starch Groups</th>
<th>Resistant Starch Content of the Diets on Dry Diet Basis (n=3) (% ± sd)</th>
<th>Water Content of the Diets (n=3) (% ± sd)</th>
<th>Resistant Starch Content of the Starches on Dry Diet Basis (n=2) (% ± sd)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AR</td>
<td>5.41±0.53%</td>
<td>20.01±0.92%</td>
<td>1.2 ± 0.1</td>
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<tr>
<td>GUAT</td>
<td>18.58±0.90%</td>
<td>14.12±0.89%</td>
<td>34.5 ± 2.0</td>
</tr>
<tr>
<td>AR X GUAT</td>
<td>8.57±0.59%</td>
<td>12.99±0.98%</td>
<td>1.9 ± 0.1</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD. Resistant starch contents of diets were measured by AOAC method 991.43.
Chapter 5: General Conclusions

General Discussion

Resistant starches are natural food ingredients obtained from many plants. In western countries, the intake of high RS diet is on average poor, impeding humans from the benefits in intestinal/colonic health. Studies showed that 28 grams of resistant starch per day was well tolerated in healthy humans, which is 6 times more than the average daily intake in a western diet (Van Munster et al., 1994).

In our studies, two types of RS were tested for their inhibition of colorectal preneoplastic lesions: RS obtained from a combination of amylose and lipid (SAC), and RS from inbred or hybrid of corn lines (Guat and ARXCuat). We measured the incidence and multiplicity of ACF and the incidence of MDF in male Fisher 344 rats 8 weeks after AOM injections. We provided clear evidence that consumption of a diet rich in cooked SAC suppressed AOM-induced colon carcinogenesis, but water-boiled Guat and ARXGuat did not show ability as colon preneoplastic lesion suppressors. The results have important implications for humans because starchy food is usually consumed by humans in a cooked form. I believe our research on the inhibitory effect of cooked SAC is the first to study the impact of RS on colorectal carcinogenesis when the RS was cooked by different methods. There has been a single report of the impact of cooking dietary beans and peas fed to Wistar rats after these diets were cooked in a microwave (Krupa-Kozak et al., 2009). A reduction of glucose and total cholesterol concentration in rat blood serum and induced SCFA in the cecal digesta was observed, but no effect on CRC incidence was tested.

Our data showed decreased ACF or MDF numbers and increased ACF multiplicity in the distal colon section of rats fed SAC when compared to their respective controls. The
distal colon section was collected and observed because reports have shown greater CRC occurrence in the distal colon than in the proximal colon in men and in women (Distler and Holt, 1997). The incidences of ACF were also reported to be higher in the middle and distal colon than in the proximal colon in Wistar rats treated with AOM and fed RS diet (Liu and Xu, 2008). In our study testing the inhibitory effect of SAC, a global gene expression analysis was conducted on the colon mucosa samples collected from the distal colon or the adjacent colon. The genetic analysis was focused on the more distal part of the colon because the genetic changes occurring in our carcinogen-induced sporadic CRC were expected to be more frequent in the distal section of the colon as was reported by other studies. Furthermore, a bioinformatics analysis of a large amount of published papers revealed that proximal and distal colons may reflect different colonic neoplastic transformations. Genetic alteration in the proximal tumors appear to be more stable and may relate more to the inherited nonpolyposis colon cancer, while distal tumors showed greater genetic instability and were more similar to the polyposis-associated colorectal cancer syndromes (Bufill, 1990).

It is notable that in our study, the rats were injected AOM twice before their 8-week feeding of RS. This order of RS diet administration after AOM exposure can be pivotal for the inhibition of carcinogenesis. Actually, the differences in the order of treatments indicated the impact of RS in the different tumorigenesis stages. Carcinogenesis occurs in three different stages: initiation, promotion, and progression. Initiation is caused by an irreversible damage to DNA by viral, chemical or physical agents and promotion occurs when the initiated cells are transformed into a population of preneoplastic cells (Thangapazham et al., 2006). In our study on cooked SAC, RS obviously showed its effect in the promotion stage. In fact, inhibitory effects of RS on CRC or on its pre-cancerous lesions in the promotion stage and an opposite impact in the pre-initiation stage
were shown in some previous researches. Liu and Xu reported that feeding male Wistar rats with dietary RS after they were treated with AOM significantly suppressed ACF and total AC formation, yet an increased ACF number was observed when the RS diet was fed prior to AOM exposure (Liu and Xu, 2008). One of the explanations for the opposite impacts of RS in the different carcinogenesis stages is that RS enhances carcinogen absorption during the initiation stage, thus promoting the formation of preneoplastic lesions and increasing the incidence of CRC. On the other hand, the RS may dilute potential toxins and carcinogens and shorten their contact time with the colonic epithelium by increasing fecal bulk and reducing transit time at the promotion stage. The main increase in the fecal bulk comes from the SCFA induction during the fermentation of prebiotic RS by gut bacteria. The RS impact on the carcinogen uptake and disposition in a pre-initiation stage was revealed in a study on female Wistar rats (Kestell et al., 2004). The rats were first fed 35% maize starch (negative control), 35% hi-maize, or 35% potato starch, and then gavaged with [2-14C] 2-amino-3- methylimidazo [4,5-f]quinoline (IQ). Both plasma and urinary metabolites were tested. Plasma contained markedly higher level of intact IQ and lower level of major IQ metabolites in rats fed 35% hi-maize or 35% potato starch than those fed negative control. A significantly slower metabolite excretion in urine was also revealed in rats fed these two diets. A similar study by the same group of scientists also revealed that RS enhanced carcinogen bioavailability in both colonic mucosa and bloodstream in the pre-initiation stage (Ferguson et al., 2003). Meanwhile, the suppression effect of RS in the promotion stage was reported on rats fed potato starch, high-amylose maize starch, or an alpha-amylase-treated high-amylose maize starch, where increased fecal and cecal weight and a slightly shortened transit time were observed (Ferguson et al., 2000). These increased fecal bulk and reduced transit time may imply a cause for CRC inhibition when RS was administrated in the promotion
stage. Previously there was controversy regarding the relationships between dietary RS and CRC. Studies on the impact of RS at the different carcinogenesis stages obviously helped to clear up the arguments, and our study provided powerful evidence that cooked RS had an inhibitive role in the promotion stage.

Aberrant crypt foci were one of the first observed biomarkers for CRC (Bird, 1987; McLellan and Bird, 1988), but some more recent studies suggested that MDF predicts tumor outcome better than ACF in rodent models (Femia et al., 2003; Caderni et al., 2003; Pierre et al., 2010). In our first study to assess the inhibitory effect of water-boiled resistant starch or raw resistant starch in AOM treated rats, no effect of either raw or water-boiled SAC diet on the total number of ACF at 8 weeks after initiation with AOM was detected compared with their respective controls, yet a reduction in the total number of MDF were seen in the rats fed water-boiled SAC compared with rats fed water-boiled CS. It was also suggested that ACF with multiple crypts are better predictors of tumor incidence since they were considered more prone to progress into cancer (Magnuson, 1993; McLellan and Bird, 1991). In my study assessing the impact of SAC prepared by different cooking methods, large ACF numbers decreased dramatically with the increase of resistant starch content in the diets, and this trend was consistent with what we observed in the total ACF number.

Our data demonstrated increased cecal weights and decreased cecal content pH with the increase of resistant starch content in both the SAC starch studies and the Guat and ARXGuat starch study. Reports suggested these changes are due to an increased fermentation by colonic bacteria using RS as substrate to produce SCFA (Gibson, 1996). This assumption was consistent with our findings in the cooked SAC studies that the total amount of SCFA, as well as the amount of butyrate and acetate, increased significantly with the increase of the resistant starch content of the diets. Resistant starch was
considered to achieve its anti-carcinogenic effect through increased SCFA from the anaerobic fermentation of RS in the colonic lumen. It was assumed that rapidly fermented fibers leave no substrate for fermentation in the distal colon since they can be completely broken down, while less well fermented fibers produce butyrate all along the large bowel (Scharlau et al., 2009; McIntyre et al., 1993). Butyrate was thought to be the main component in SCFA to promote colonic health and protect against CRC by inhibiting cell proliferation, inducing differentiation, and enhancing apoptosis of CRC cells (Sengupta et al., 2006; Scheppach et al., 1995; Whitehead et al., 1986). Markedly, the role of butyrate on the prevention of CRC incidence is still not clear since variable results have been discovered. Although some negative correlation of butyrate concentration and tumor mass were reported, some opposite observations were also revealed. Rats treated with 1,2-dimethylhydrazine and given 1% or 2% sodium butyrate in drinking water actually experienced enhanced development of colonic neoplasia with increased fecal butyric acid concentrations (Freeman, 1986). Another rat study showed no inhibitive effect of resistant starch against colon carcinogenesis although the butyrate concentration was increased in the large intestine of the rats (Sakamoto et al., 1996).

In the cooked Guat and ARXGuat maize starch study, the rats fed Guat starch obtained reduced cecal pH and enhanced cecal weights, but did not show any suppression of the pre-cancerous lesion incidence. One of the explanations is that a sufficient amount of resistant starch content is required to show the inhibitory impact of a starch on colon lesions. As was reported, the suppressive effect of dietary RS on AOM-induced ACF formation was dose-dependent (Liu and Xu, 2008). It was also reported that the positive correlation between higher cecal pH and more ACF numbers was dose-dependent (Verghese et al., 2002). In our studies, SAC diet contained 32.7% and 27.2% of resistant starch content in the bread-baked and water-boiled starch diets respectively, while the
resistant starch content of water-boiled Guat diet was only 18.6%. It is possible that the Guat starch diet did not have enough resistant starch content to prevent the colon lesion formation, although this amount was already enough to show obvious fermentation differences in the rat large bowel. On the other hand, although resistant starch content is frequently used to measure the potential of RS to promote intestinal fermentation and suppress colon lesions, it is not the only indicator since other factors, such as starch type, efficiency of starch absorption, and differences of SCFA metabolism, can also impact the results. For example, different starches may have different fermentation efficiencies and impact SCFA metabolism in different ways even when they have the same resistant starch content. In a study feeding high amylose maize starch or hydrothermal treated high amylose maize starch to young Large White crossbred pigs, although both starches contained 85% amylose and both were mixed in the diet by 50%, profoundly more resistance to small intestinal digestion was shown in hydrothermal treated high amylose maize starch. Consequently, hydrothermal treated high amylose maize starch promoted more fermentation in the distal colon (Bird et al., 2007). In this study by Bird et al., hydrothermal treated high amylose maize starch was observed to deliver more fermentable carbohydrate to the large bowel than high amylose maize starch although both diets had the same amylose content and the higher amount of fermentable carbohydrate in hydrothermal treated high amylose maize starch might connect to the more active fermentation of hydrothermal treated high amylose maize starch. In our study, we assessed the resistant starch content of each diet, but the amount of fermentable carbohydrate in the large bowel or differences of SCFA metabolism by each starch diet were not measured, which might also be determinant factors on the inhibitory effect of different RS to the intestinal fermentation and ACF or MDF formation. Meanwhile, resistant starch content or the fermentation ability of a RS are not the only factors
impacting the formation of preneoplastic lesions. In a study on 1,2-dimethylhydrazine-treated Sprague-Dawley rats, a less fermentable fiber, wheat bran was found to result in significantly higher fecal butyrate concentration and fewer malignant tumors than those fed highly fermentable fibers, guar and oat bran (McIntyre et al., 1993). In this study, although wheat bran was less fermentable than Guar gum and oat bran, fermentation of wheat bran continued in the distal colon, while fermentation of the other two fibers did not.

In our studies, a short feeding period with cooked RS of 8 weeks was conducted with AOM-induced rats. Although a reduced number of AOM-induced ACF and MDF were observed, these studies need to be followed up in a long-term tumor experiment to provide a more complete measurement of the anti-carcinogenesis potential of the RS. Previously, a long-term study feeding high amylose starch to male Sprague-Dawley rats revealed a significant reduction in the incidence and multiplicity of adenocarcinomas in the rat colon compared with the control normal starch diet (Le Leu et al., 2007). A long-term intake of raw potato starch also showed an improved colonic mucosal integrity and reduced gut apoptosis and blood immune cells in pigs compared with those in normal corn starch fed pigs (Nofrarias et al., 2007). But none of the publications we found have done the long-term assessment of tumorigenesis by cooked starches, nor did any studies show this long-term relationship in humans.

Our studies used a rodent model where Fisher 344 rats were injected with AOM twice with an interval of one week, and then RS or control diets were fed to them for 8 weeks before the preneoplastic lesion assessment. Effective as this rodent model is to induce the sporadic form of rat large bowl lesions in the distal colons and to associate with human sporadic colorectal cancer (Shamsuddin and Trump, 1981; Druckery, 1972; Papanikolaou et al., 1998), there are limitations of this experimental system for studying the molecular
and pathological changes of human CRC. First of all, this animal model is not associated with the inherited forms of human CRC such as human FAP and HNPCC since the process of carcinogenesis and gene alteration in FAP or HNPCC are comparatively different from the ones in sporadic CRC. Secondly, marked differences are considered between rodent and human sporadic CRC at the cellular and molecular levels. Not a lot of studies have been reported on the differences in tumorigenesis between Fisher 344 rat and human, but the differences have been reported on many other rodent models. Generally, the basal metabolic rate is 7 times higher in mice than in humans, which may lead to a higher level of endogenous oxidants causing DNA damage by oxidizing the bases (Ames et al., 1993; Adelma et al., 1988). At the genetic level, fewer genetic changes were required in a mouse model than in human CRC tumorigenesis. CRC is known as a stepwise accumulation of genetic changes in humans including key gene mutations such as APC, K-ras, p53, SMAD4, and DCC. In AOM-treated rodents, APC mutations or p53 allelic loss were rarely seen in their invasive colonic carcinomas (Okamoto et al., 1993). A comparison of 4 rat models (Apc\textsuperscript{Min/+} mice model, AOM carcinogen model, Tgf\textbeta1\textsuperscript{-/-}Rag2\textsuperscript{-/-} mice model, Smad3\textsuperscript{-/-} mice model) and 100 human CRC samples revealed that human CRC adenocarcinomas lost additional suppressor modules (IGFBP4, MAP4K1, PDGFRA, STAB1 and WNT4), and gained expression of modules associated with advanced malignancy (ABCC1, FOXO3A, LIF, PIK3R1, PRNP, TNC, TIMP3 and VEGF) compared with the rodent models (Kaiser et al., 2007). Furthermore, arguments exist in that laboratory rodents are exposed to high levels of dietary carcinogens or toxicants that are usually rare in humans. All these reports indicated a gap between a rodent model and a human application, thus human studies will be necessary after the results are gained and concluded from rodent experiments.
In our study, the global view of gene expression during the early phase of colonic carcinogenesis revealed for the first time the genetic alterations by cooked resistant starches in the SAC starch studies. Microarray technique provided powerful analysis of a large number of different mRNAs, identified genes and gene families whose expressions were altered by diet in previous studies (Kato et al., 2004; Park et al., 2008). Most cancer related signalling pathways in the process of AOM-induced gene expression focused on the up-regulated pathways including Jak-STAT signalling pathway, apoptosis pathway, Alzheimer’s disease related pathway, natural killer cell mediated cytotoxicity pathway, focal adhesion pathway, cancer-related pathway, and so on. Genes such as JAK1, STAT1, CASP3, CASP8, CD9, CD38, CD48, MAPK1, IL1a, Kras, Braf, and RAF1 were shared by these signalling pathways and up-regulated in rats treated AOM compared to saline treated control rats. These genes and pathways have been reported to involve in immune function, cell growth, cell apoptosis, proliferation, migration, tumorigenesis in the colon cancer cell lines, rodent models or human CRC patients, and crosstalk with pathways such as TGF-β-signaling pathway, ERK1/2/NF-κB signal transduction pathway, TF/FVIIa/PAR2 pathway, RAF/MEK/ERK pathway (Slattery et al., 2011; Zugowski et al., 2011; Sreevalsan et al., 2011; Lin et al., 2011; Li et al., 2011; Kim and Hong, 2011; Guo et al., 2011; Slattery et al., 2010; Behl et al., 2011; Wilhelm et al., 2004; Benvenuti et al., 2007). When comparing the gene expression of SAC or control diet treatment, four down-regulated pathways, DNA replication, cell cycle, RNA degradation, and ABC transporters were most connected with colon cancer incidence. Of these pathways, DNA replication stress relates to some oncogene-induced precancerous DNA lesions (Petermann and Helleday, 2007; Ichijima et al., 2010). Abnormal cell cycle reduces sensitivity of signals to adhere, differentiate, or apoptosis thus causes inappropriate proliferation and is associated with cancer (Hartwell and Kastan, 1994). Improper
regulation of RNA degradation can lead to cancer and other diseases (Ross et al., 2001). ABC transporters are one of the largest transportome in the human genome and are overexpressed in several cancers (Fojo et al., 1987). Common genes, like CD55, RAC1, TFRC, HSPD1, HSPH1, and MCM6, were up-regulated by AOM induction and down-regulated by SAC feeding. These genes have been reported to regulate cell development, growth and proliferation, or enhance cancer cell migration and invasion (Zhu et al., 2011; Li et al., 2008; Bjørge et al., 1996; Nioi et al., 2008; Jung et al., 2011; Muchemwa et al., 2006). The up-regulated by AOM induction and down-regulated by SAC feeding of these genes indicated an inhibitory effect of SAC on AOM induced carcinogenesis in the genetic level. CD48, an immune response gene in the NF-kappa B gene family was found to be down-regulated by AOM treatment and up-regulated by SAC feeding. The differentially expressed genes, both induced by AOM and reduced by SAC, can be used as potential target genes for the intervention of CRC.

In conclusion, our studies suggest that the cooked resistant starch SAC, an amylose-lipid complex, inhibited preneoplastic lesions in the AOM-induced rat. The cooked Guat or ARXGuat starches did not show these inhibitory effects in a same rat model. Different cooking methods were also compared in the SAC study, and bread-baked method gained a more effective inhibitory impact than the water-boiled method by a dramatic reduction of ACF and MDF. It was the first time cooking procedures were involved and compared in the consideration of inhibition on preneoplastic colonic lesions by novel RS gained from different methods. Our studies suggested that enhanced intake of high content RS with appropriate cooking method will help to provide health benefits of RS to the maximum extent. The differentially expressed genes selected by the genome-wide gene expression screening on the water-boiled rat colon samples are potentially target genes for the prevention of CRC.
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