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THE EFFECT OF FERMENTABLE DIETARY FIBERS ON COLORECTAL TUMORIGENESIS

A Thesis in

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by

Sangshan Tian

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The thesis of Sangshan Tian was reviewed and approved by the following:

Vishal Singh
Assistant Professor of Nutritional Sciences and Microbiome
Thesis Advisor

Connie Rogers
Flatt Professor of Nutritional Sciences
Department Head of Nutritional Sciences

Donna H. Korzick
Professor of Physiology and Kinesiology
Department Head, Chair of the Graduate Program

ABSTRACT

Colorectal cancer (CRC) is the second leading cause of cancer-related deaths in the United States. Individuals with inflammatory bowel disease (IBD) are at high risk of developing CRC. The diminished CRC occurrence in patients with IBD who received optimal treatment to manage active intestinal inflammation signifies the importance of controlling ongoing intestinal inflammation to reduce the incidence and prevalence of IBD-related CRC.

Dietary fibers (DFs) regulate many systems, including the gut microbiota and host intestinal immunity. DFs are metabolically inert to humans and must be fermented by gut microbes. Both immune and metabolic functions of the lower gastrointestinal (GI) tract could be altered substantially in response to various amounts and types of DFs, specifically by the presence or absence of fermentable DFs (FDFs) such as inulin and partially hydrolyzed guar gum (PHGG). Intriguingly, isolated dietary fiber supplementation is not found beneficial in all IBD patients and laboratory animal models. We theorize that in contrast to DFs naturally present (as a mixture of both fermentable and non-fermentable types) in fruits, vegetables, and whole grains, isolated FDFs fuel the growth of a selective group of bacteria (e.g., expansion of γ-proteobacteria) based on their specificity toward gut microbes. Such selective utilization of isolated FDFs promotes dysbiosis and susceptibility to colonic inflammation that can increase the risk of colorectal tumorigenesis. This study aimed to elucidate the effect of isolated FDFs (inulin and PHGG), commonly present in a wide range of processed foods, on colonic inflammation and colorectal tumorigenesis.

In this study, we found FDF supplementation (inulin and PHGG) did not alter intestinal inflammation markers in healthy mice. However, inulin and PHGG potentiated and prolonged colonic inflammation and delayed mucosal healing in mice with colitis [induced by dextran sulfate sodium salt (DSS)], suggesting that FDFs may fuel inflammation in the inflamed gut. Chronic intestinal inflammation, which fuels the continuous turnover of cells in the intestinal lining,

considerably increases the risk of colorectal tumorigenesis by increasing the chance of missense mutations that may lead to cancer. In line, we observed extensive colon tumorigenesis in both inulin and PHGG-containing diet-fed mice. Altogether, our experimental findings suggest that the consumption of isolated FDFs may increase the risk of colon cancer in patients with IBD by potentiating intestinal inflammation. Therefore, incorporating highly refined FDFs in processed foods should be cautiously approached as it may increase susceptibility to intestinal inflammation and CRC.

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List of Abbreviation

AJCC: American Joint Committee on Cancer
AOM: azoxymethane
Apc: adenomatous polyposis coli
αIL-10R: anti-interleukin-10 receptor antibody
CD: Crohn's disease
CDC: Centers for Disease Control and Prevention
COX-2: cyclooxygenase-2
CRC: colorectal cancer
CXCL1: chemokine (C-X-C motif) ligand 1
DAI: Mayo Score / Disease Activity Index
DBA: 3,3'-Diaminobenzidine
DF: dietary fiber
DR3: death domain-containing receptor
DSS: dextran sulfate sodium
FDF: fermentable dietary fiber
FODMAP: fermentable oligosaccharides, disaccharides, monosaccharides, and polyols
GI: gastrointestinal
H&E: hematoxylin and eosin
IBD: inflammatory bowel disease
IEC: intestinal epithelial cell
IL: interleukin
Lcn-2: lipocalin-2
LT: leukotriene
MK2: MAPK-activated protein kinase 2
MMP9: matrix metalloprotease-9
NBF: neutral buffered formalin
PHGG: partially hydrolyzed guar gum
SAA: serum amyloid A
TLR5: Toll-like receptor 5
TL1A: tumor necrosis factor-like cytokine 1A
UC: ulcerative colitis
VEGF: vascular endothelial growth factor

Chapter 1

Introduction

Fermentable dietary fibers (FDFs), which promote the growth of beneficial bacteria in the gut, are common prebiotics believed to have health-promoting functions. Accordingly, both preclinical and clinical studies suggest that inulin or partially hydrolyzed guar gum (PHGG) consumption exerts several physiologic beneficial effects, including improving gut barrier function and production of fermentation metabolites such as short chain fatty acids (SCFAs)[1, 2, 3, 4]. However, whether these highly-refined purified fibers hold physiological effects similar to their naturally occurring counterparts is largely unknown, particularly in patients with IBD and colon cancer. Opposingly, a subgroup of IBD patients, develop severe complications with FDF intake[5], and reduction of FDF intake improves abdominal symptoms in a subgroup of patients with IBD[6]. In addition, preclinical studies reported contrasting findings on the effects of fiber-enriched diets on IBD initiation and progression[7, 8, 9, 10, 11]. Collectively, FDFs improve or worsen intestinal inflammation and increase or decrease the risk of developing IBD-associated CRC remains elusive. (Fig. 1-1).

Due to limited scientific evidence on whether FDFs improve or aggravate ongoing inflammation, IBD patients are refrained from consuming FDFs. Therefore, we conducted a study using a mouse model to examine the effects of FDF: inulin and PHGG on the development of colitis and colitis-associated cancer. Moreover, inulin and PHGG are parts of our daily meals as they are widely present in processed foods. Inulin is approved by FDA as a food additive due to its low-degree sweetness and pleasing texture in processed food, such as cheese and pizza[12]. PHGG, with its suitable viscosity, has also been used as a food thickener and emulsifier, benefiting from its physio-chemical property[13].

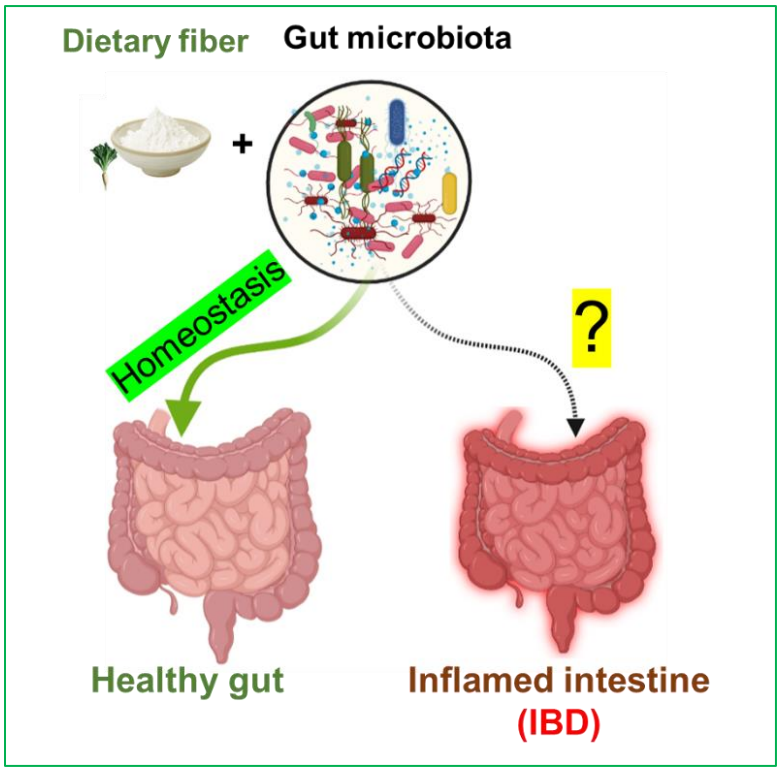

Figure 1 - 1 An illustrating model of the scientific question

A healthy gut contains a diverse microbiota community to efficiently process dietary fibers and generate metabolites to maintain intestinal immune homeostasis. However, to what extent compromised fiber fermentation in the inflamed gut influence dietary fibers' effect on intestinal health remain sparse.

Statement of Problems

Accumulating evidence suggests that not all isolated FDFs exert similar physiological effects, which may be due to differences in their solubility and chemical structures, which collectively influence their fermentability, capacity to nourish specific groups of gut microbes, and ability to generate microbial metabolites. These include deoxycholate, a secondary (2°) bile acid

implicated in promoting colon tumorigenesis. Moreover, the effect of FDFs on colon carcinogenesis also depends on their ability to regulate (attenuate or exacerbate) colonic inflammation. We speculated that two underlying reasons might explain the probable impacts of DFs on colitis. First, dietary fibers can be differentially metabolized in the healthy and inflamed gut. Second, structurally different dietary fibers may have a distinct effect on intestinal inflammation (Fig. 1-2).

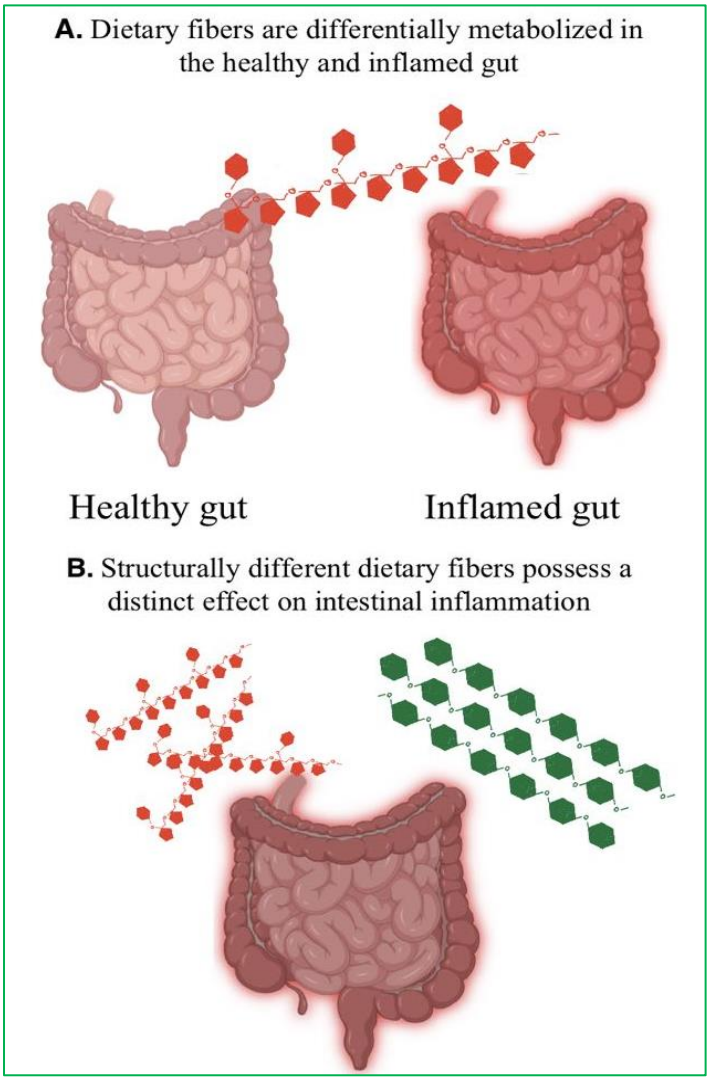

Figure 1 - 2: Schematic diagram showing two possible underlying reasons for the differential effects of dietary fibers on intestinal inflammation.

Scientific Aims and Hypotheses

Aim 1: Assess the effect of inulin on intestinal health in the non-inflamed and inflamed gut.

A large population-based case-control study suggests that consuming ultra-processed foods and drinks is associated with an increased risk of colorectal cancer[14, 15]. In contrast to inulin present naturally in whole fruits and foods, we propose that isolated purified inulin will fuel selective bacterial colonization (e.g., expansion of γ-proteobacteria), particularly in the inflamed gut.

Hypothesis: *Inulin will have a distinct impact on healthy and inflamed gut*

We aim to elucidate the effects of dietary inulin on the gut of healthy mice as well as on colitis development by using a well-established [dextran sulfate sodium (DSS)-induced] murine model of ulcerative colitis. Subsequently, we will evaluate the effect of inulin on mucosal healing and recovery from DSS-induced colitis. Such comprehensive analysis will allow us to deduce whether inulin supplementation accelerates or suppresses colon tumor progression.

Aim 2: Examine the effect of inulin on colitis-associated colorectal tumorigenesis

The development of colon cancers is intimately tied to inflammation, wherein a vicious cycle of injury and repair potentiates epithelial cells to undergo neoplastic transformation. Refined inulin is known to aggravate acute and chronic colitis[16, 17]; therefore, we surmise larger polyps and more tumor burden in mice fed inulin.

Hypothesis*: Inulin will impact, possibly promote, colon tumor development*

By employing the murine model (AOM/DSS) of colon cancer, we will elucidate the effect of dietary inulin on colorectal tumorigenesis.

Aim 3: Test whether structurally different fermentable fibers modulate intestinal inflammation and colon tumor development similarly

The use of dietary partially hydrolyzed guar gum (PHGG), another fermentable fiber, has been popular for its beneficial effect on obesity and metabolic health [18]. Since chronic intestinal inflammation highly coincides with tumorigenesis in the colon, we further planned to examine the impacts of PHGG on colonic inflammation and colitis-associated colon cancer development using the AOM/DSS model.

Hypothesis*: Since both inulin and PHGG are fermentable fibers, we surmise that PHGG will have a similar effect as inulin, particularly in the inflamed gut.*

Impact: Determining the effects of FDFs commonly present in processed foods on IBD-associated colon cancer will pave the way toward precision nutrition and help to develop recommendations for individuals with IBD about what types of fibers they should include in their diet. Additionally, identifying which specific FDFs attenuate colonic inflammation and which prolong inflammation and delay mucosal healing will establish a fertile ground for targeted research focusing on a novel dietary fiber-based therapeutic approach to reduce the prevalence of IBD-associated colon carcinogenesis.

Chapter 2

Literature Review

Overview of Colorectal Cancer

Prevalence of colorectal cancer

Up to date in 2019, the Centers for Disease Control and Prevention (CDC) had claimed colorectal cancer (CRC) as the third most common cancer among all cancer types in the United States and top forth worldwide[19]. In the year 2019, CDC reported 0.42‰ in males and 0.32‰ in females in the U.S. diagnosed with CRC[20]. Although a decreasing trend was observed over the last 20 years, it has been estimated that 147,000 new cases will be diagnosed by 2040 in the U.S.[21, 22]. The colorectal cancer screening program allows patients to receive the proper treatment at an earlier stage. Nevertheless, a trend of earlier onset age had been shown; primarily in the white and black American populations, and followed by Asian, Hispanic, and Native Americans[23]. Thus, there is an urgent need on understanding the contributing factors of colorectal carcinogenesis and investigate effective resolutions to lower the incidence of CRC.

Risk factors of CRC

Both genetic and environmental risk factors play a role in CRC development. Genome-wide association studies have identified more than 53 loci that are positively associated with CRC susceptibility, such as chromosome 10p14 and 8q23.3[24, 25]. Other than personal family history, two genetic syndromes were noticed that increases the risk of CRC: familial adenomatous polyposis

and Lynch Syndrome. Besides, aging and being male also contribute to the probability of CRC. Since these non-modifiable factors are hard to interfere with current techniques, more attention is directed toward modifiable risk factors which are certain lifestyles and health issues such as being obese[26], low physical activity[27], heavy alcohol drinking (>3 drinks/day)[28], and inadequate vegetable and fruit intake[29]. Among these modifiable risk factors, inflammatory bowel disease was reported as the most predominant factor [relative risk (RR)=2.93, 95 % CI 1.79-4.81], over genetic component [CRC history in a first-degree relative (RR = 1.80, 95 % CI 1.61-2.02)] by CM Johnson and the colleagues in a meta-analysis study[30]. Therefore, understanding and well-managing inflammatory bowel disease is important in preventing the development of CRC.

Stages of CRC

According to American Joint Committee on Cancer (AJCC) system, the colorectal cancer is classified into four stages: I, II, III, and IV. The classification is based on three parameters: tumor location (T), number of lymph nodes that cancers spread to (N), and number of distant metastatic sites (M). Prior to stage I, the stage 0 is considered as carcinoma *in situ*, describes the tumor only occurs at the mucosal layer with neither invasion of colon or rectal walls nor spreading out to other organs. While the cancer cells growing, it invades throughout the submucosal layer and maybe muscularis layer, which is known as stage I.

Stage II, III, and IV are subgrouped into three detailed categories: A, B, and C. Stage IIA illustrates the cancer only grows to sarcoma layer. IIB describes when the cancer is in touch with nearby lymph nodes but not spread to lymph nodes yet. When the cancer is growing to scramble the space of nearby lymph nodes, it is claimed as stage IIC. As the carcinoma keeps growing uncontrollably, stage IIIA is when it eventually spreads to 1-3 nearby lymph nodes or adipose tissue but not into the nodes themselves, or grows into 4-6 lymph nodes but not invades throughout the

colorectal wall yet. At IIIB, the cancer can be found in 1-7 lymph nodes and grown out of the colorectal wall. As keeping growing locally, IIIC is classified when the cancer spread into more than 4 nearby lymph nodes and out of the outermost layer of the colon. The last stage, IV, is still subclassified into three groups, but mainly announced when the cancer spread to more than 1 distant organs. The more detailed classification is included below from the website of American Cancer Society (Table 2-1)[31].

Table 2 - 1: The Table of CRC Classification.

American Joint Committee on Cancer stage	Stage grouping	Stage description
0	**Tis** **N0** **M0**	The cancer is in its earliest stage. This stage is also known as carcinoma in situ or intramucosal carcinoma (Tis). It has not grown beyond the inner layer (mucosa) of the colon or rectum.
I	**T1 or T2** **N0** **M0**	The cancer has grown through the muscularis mucosa into the submucosa (T1), and it may also have grown into the muscularis propria (T2). It has not spread to nearby lymph nodes (N0) or to distant sites (M0).
IIA	**T3** **N0** **M0**	The cancer has grown into the outermost layers of the colon or rectum but has not gone through them (T3). It has not reached nearby organs. It has not spread to nearby lymph nodes (N0) or to distant sites (M0).
IIB	**T4a** **N0**	The cancer has grown through the wall of the colon or rectum but has not grown into other nearby tissues or organs

	M0	(T4a). It has not yet spread to nearby lymph nodes (N0) or to distant sites (M0).
IIC	**T4b** **N0** **M0**	The cancer has grown through the wall of the colon or rectum and is attached to or has grown into other nearby tissues or organs (T4b). It has not yet spread to nearby lymph nodes (N0) or to distant sites (M0).
IIIA	**T1 or T2** **N1/N1c** **M0**	The cancer has grown through the mucosa into the submucosa (T1), and it may also have grown into the muscularis propria (T2). It has spread to 1 to 3 nearby lymph nodes (N1) or into areas of fat near the lymph nodes but not the nodes themselves (N1c). It has not spread to distant sites (M0).
Or:	**T1** **N2a** **M0**	The cancer has grown through the mucosa into the submucosa (T1). It has spread to 4 to 6 nearby lymph nodes (N2a). It has not spread to distant sites (M0).
IIIB	**T3 or T4a** **N1/N1c** **M0**	The cancer has grown into the outermost layers of the colon or rectum (T3) or through the visceral peritoneum (T4a) but has not reached nearby organs. It has spread to 1 to 3 nearby lymph nodes (N1a or N1b) or into areas of fat near the lymph nodes but not the nodes themselves (N1c). It has not spread to distant sites (M0).
Or:	**T2 or T3** **N2a** **M0**	The cancer has grown into the muscularis propria (T2) or into the outermost layers of the colon or rectum (T3). It has

		spread to 4 to 6 nearby lymph nodes (N2a). It has not spread to distant sites (M0).
Or:	**T1 or T2** **N2b** **M0**	The cancer has grown through the mucosa into the submucosa (T1), and it might also have grown into the muscularis propria (T2). It has spread to 7 or more nearby lymph nodes (N2b). It has not spread to distant sites (M0).
IIIC	**T4a** **N2a** **M0**	The cancer has grown through the wall of the colon or rectum (including the visceral peritoneum) but has not reached nearby organs (T4a). It has spread to 4 to 6 nearby lymph nodes (N2a). It has not spread to distant sites (M0).
Or:	**T3 or T4a** **N2b** **M0**	The cancer has grown into the outermost layers of the colon or rectum (T3) or through the visceral peritoneum (T4a) but has not reached nearby organs. It has spread to 7 or more nearby lymph nodes (N2b). It has not spread to distant sites (M0).
Or:	**T4b** **N1 or N2** **M0**	The cancer has grown through the wall of the colon or rectum and is attached to or has grown into other nearby tissues or organs (T4b). It has spread to at least one nearby lymph node or into areas of fat near the lymph nodes (N1 or N2). It has not spread to distant sites (M0).
IVA	**Any T** **Any N**	The cancer may or may not have grown through the wall of the colon or rectum (Any T). It might or might not have spread to nearby lymph nodes. (Any N). It has spread to 1

	M1a	distant organ (such as the liver or lung) or distant set of lymph nodes, but not to distant parts of the peritoneum (the lining of the abdominal cavity) (M1a).
IVB	**Any T** **Any N** **M1b**	The cancer might or might not have grown through the wall of the colon or rectum (Any T). It might or might not have spread to nearby lymph nodes (Any N). It has spread to more than 1 distant organ (such as the liver or lung) or distant set of lymph nodes, but not to distant parts of the peritoneum (the lining of the abdominal cavity) (M1b).
IVC	**Any T** **Any N** **M1c**	The cancer might or might not have grown through the wall of the colon or rectum (Any T). It might or might not have spread to nearby lymph nodes (Any N). It has spread to distant parts of the peritoneum (the lining of the abdominal cavity), and may or may not have spread to distant organs or lymph nodes (M1c).

Colorectal Cancer Stages Available: https://www.cancer.org/cancer/colon-rectal-cancer/detection-diagnosis-staging/staged.html, 2022

Inflammatory bowel disease as the trigger of CRC

Overview of IBD

The two clinical forms of human inflammatory bowel diseases, Crohn's disease (CD) and ulcerative colitis (UC), are chronic gastrointestinal (GI) inflammation that are believed to be immune-mediated disorders due to hyperactive immune responses to pathogenic and

nonpathogenic agents. Both histological and molecular analysis suggested these two types of disease have distinct characteristics. Clinically, UC mainly occurs from the rectum to the proximal colon, whereas CD can occur at any site in the gastrointestinal (GI) tract, even though the small intestine is more likely to undergo pathogenesis. Another difference is that UC is characterized by a continuous damaged region without clear segments in the colon, but CD usually has more than one focus[20]. Moreover, the histological phenotype has unique hallmarks in each type of IBD. Mucosal layer damages, crypt disruption, and neutrophil infiltration occur at UC's active sites. In contrast, the CD has the key feature of discrete granuloma surrounded by lymphoid, plasma, and immune cell infiltration in the GI tract[32]. However, unlike UC, the diagnosis of CD requires a comprehensive examination to exclude all other possible diseases that share at least one pathognomonic marker[33]. With the development of genetic analysis tools, there was an argument for subclassifying Crohn's disease into the young age of disease onset (age <17-year-old) and elder age (age >17-year-old)[34]. The young-age subclass of Crohn's disease is associated with particular genotype and serotype features. For example, the *IBD1* locus was found more in patients with young diagnosis age but not much in late-onset age[34, 35, 36].

Classification of severity and extent of IBD

There are more than ten clinical assessment tools for ulcerative colitis. The Mayo Clinic Score or the Disease Activity Index, created by Dr. Kenneth W. Schroeder in 1987, is one of the most commonly used clinical tools to assess the disease extent in patients with the UC (table 2-1)[37]. The disease extent is classified in three grades by the sum of four criteria: less than 4 listed in the table 2-1 is considered as mild; 4-6 as moderate; greater than 6 with other serological and physiological symptoms will be considered as severe disease (body temperature>37.8°C; pulse>90/min; hemoglobin<10.5g/dL; erythrocyte sedimentation rate>30mm/hour)[38]. However,

the Mayo disease index yielded a relatively high variance in endoscopy scores based on the overall disease assessment[39]. To overcome this, artificial intelligence and machine learning techniques are currently attempting to estimate disease severity with endoscopies[40, 41, 42].

Table 2 - 2: Mayo Score / Disease Activity Index (DAI) for Ulcerative Colitis

Stool frequency	Normal = 0 1-2 stool/day more than normal = 1 3-4 stools/day more than normal = 2 >4 stools/day more than normal = 3
Rectal bleeding	None = 0 Visible blood with stool less than half the time = 1 Visible blood with stool half of the time or more = 2 Passing blood alone = 3
Mucosal appearance at endoscopy	Normal or inactive disease = 0 Mild disease (erythema, decreased vascular pattern, mild friability) = 1 Moderate (marked erythema, absent vascular pattern, friability, erosions) = 2 Severe (spontaneous bleeding, ulceration = 3

Physician rating of disease activity	Normal = 0 Mild = 1 Moderate = 2 Severe = 3

The assessment tool for Crohn's disease has been developed based on a guideline from a National Cooperative Crohn's Disease Study in 1975[43]. Later versions included serum and fecal immunology markers, the patient's history, and endoscopic results to estimate the disease extent better. In 2011, a shortened CD severity assessment index was published and showed to have comparative power of estimation as the original version when body temperature and other general physiological markers are incorporated together[44].

Initiation and Progression of IBD and Potential Treatment

Early on, histological analysis of IBD patient biopsies indicated a large number of immune cell infiltration, which attracted the focus on pro-inflammatory markers. Before the era of genome-wide association studies, scientists first discovered several pivotal proinflammatory molecules that participated in IBD, including tissue necrosis factor alpha (TNFα) [45, 46] and nuclear factor-kappa b (NF-κB)[47].

With the continuous studying on UC and CD, people eventually noticed that these two diseases have their own immune pathways. TNFα was clarified as CD-mediating cytokine, but not in UC. As a result, drugs targeted at inhibiting these molecules had been developed. For instance, infliximab, also known as cA2, was genetically designed to block the TNFα internalization and DNA-binding[48, 49]. Clinical studies further confirmed the safety and efficacy of this drug in

eliminating the proinflammatory response in IBD patients, with mild adverse effects such as headache, nervousness, and fatigue[50, 51]. Other examples of anti-TNFα drugs are adalimumab and certolizumab pegol. Interestingly, TNFα-induced interleukin-1 (IL-1) family secretion was involved in many inflammatory diseases, IL-1β and IL-18 were reported to be significantly elevated in patients with both CD and UC[52, 53, 54, 55].

Several authors reported that the TNFα upstream cytokine IL-12 was upregulated in the lamina propria of both CD patients and the murine model of the disease[56, 57]. IL-12 is, a heterodimer of p40 and p35 subunits, synthesized by antigen-presenting cells, including monocytes, macrophages, and dendritic cells[58]. The elevated IL-12 level suggested an over-proliferation and maturation of effecter T cell specifically Th1 cell in the lamina propria that is involved in the pathogenesis of CD which was further confirmed by immunohistochemistry analysis showing a large number of lymphocytes infiltrated in the intestinal tissue[59, 60]. To exert the effect of IL-12, IL-12 receptors transcription were then found to increase in CD patients[61]. A drug targeted to IL-12, Ustekinumab, was further designed and shown to alleviate the symptoms of patients suffering from severe CD[62]. While naïve T lymphocytes are activated and maturated to Th1 cells via IL-12, its downstream molecule interferon-γ (INF-γ) was found to be upregulated via transcription factor for signal transducer and activator of transcription (STAT) -4 in CD murine models and human studies[63, 64]. The over-expression of STAT-4 in the mouse colitis model showed more severe disease[65]. Aside from STAT-4, T-bet was found as another transcription factor that is upregulated the INF-γ expression[66]. In turn, the anti-IFNγ was developed: Fontolizumab[67]. But unfortunately, phase 2 clinical studies on fontolizumab reported limited responses in the experimental group compared to the placebo group[68]. Although fontolizumab did not show a positive effect on the experimental group, anti-IFNγ was still believed to be the most possible treatment for CD by some researchers and doctors[69]. However, IL-12 solely cannot maintain the inflammatory response, the co-stimulators of IL-12 in CD patients have been under investigated.

Upregulation of IL-23/IL-17 axis was reported to be involved in many inflammatory autoimmune diseases[70, 71, 72, 73]. This drove a hypothesis that there are other effector T cells other than type 1 and 2. The answer to Th17 became described in 2005 by employing a disease model that IL-12 and IL-23 but no Th2 cytokines were detected[74]. Since IL-23 shares the same p40 subunit as IL-12, the genetic modified mouse model with IL-23/p19$^{-/-}$ was also tested with Ustekinumab on the colonic inflammation reduction effect. Interestingly, IL-23 and IL-17 were highly involved in disease progression even with the absence of IL-12 signals. This suggested that both Th1 and Th17 effector T cells are involved in human CD. This unique immune response pathway (through Th1 and Th17) was later confirmed to occur only in CD but not in UC patients[75]. The activated Th17 cells express cytokines in IL-17 family; IL-17A, IL-17F, and IL-22 are the main members. Therefore, drugs selectively inhibiting IL-23 p19, brazikumab and risankizumab, were designed and found to be effective for CD patients who displayed resistance to anti-TNFα previously[76, 77]. The dual activation of Th1 and Th17 suggested that CD resulted in both macrophage enhancement for intracellular killing and neutrophil recruitment to the mucosal layer for extracellular pathogen clearance.

A subgroup of CD patients remained non-responding to anti-IFNγ and anti-IL-12 drugs. More recently, genome-wide association studies discovered a high variance in *TNFSF15* gene in IBD population[78]. *TNFSF15* codes a member in TNF superfamily: tumor necrosis factor-like cytokine 1A (TL1A), which the expression was found to be upregulated in CD patients and the expression of its cognate death domain-containing receptor (DR3) was also upergulated[79]. TL1A is mainly expressed in epithelial cells, but in macrophages and lamina propria lymphocytes as well[79]. DR3 located in most of lymphocytes induces IFNγ and IL-23 over-expression when bind with TL1A in CD patients[80, 81]. Thus, drugs blocking TL1A signaling pathways could be possible solution for patients who are non-responsive to other drugs. C03V is a newly developed anti-TL1A molecule. Although more pre-clinical and clinical studies are needed to analyze the efficacy and

safety of C03V, it was successfully disrupting TL1A-DR3 combination in cell-based assays[82].

As CD and UC are the only two types of human IBD, these two diseases have been studied parallelly. A homozygous knockout mouse model of IL-2 unexpectedly noted that all the mice developed IBD with shared features of human UC which were later noticed to be potent Th2 cell inducers[83, 84]. Given that IL-5 and IL-13 are produced by Th2 cells, both were found to increase in the UC patients[85]. In addition, drugs interfering in IL-13 binding to its receptor on alpha subunit showed protective effects in DSS and anti-IL-10 receptor alpha subunit colitis murine model[86]. But unfortunately, the clinical trial studies on anrukinzumab, which is a monoclonal antibody to IL-13 receptor alpha2, did not show that the treatment group recovered better than the placebo group, but a fast systemic clearance of the drug indicated a higher dose might be necessary to exert its effect[87, 88]. The specific cytokine pattern in UC patients that elevated IL-5 and IL-13 expression via activated Th2 cells was not found in either adult or children CD patients[85, 89]. But the expression of IL-4 as a signature cytokine of Th2 cells was not altered in UC patients. This led more researches on IL-4 and IL-4 receptors in UC patients and with animal models. The fact of normal-range IL-4 expression was explained by Heller and the colleagues in details with oxazolone-colitis (OC) murine mode in 2002. OC was initially identified as a model that can better mimic the UC development over other models by increased Th2 cytokines expression, and IL-4 was believed to be the key modulator due to the fact that IL-4 neutralization ameliorated OC[90]. In the latter regard, Heller modified the oxazolone administration procedure by decreasing the dose of drug and prolonging the course of inflammation. Then he found that IL-4 is not expressed at the initiation stage of OC but gradually increases as the OC becomes more severe. The IL-4 receptors alpha (IL-4Rα) were found to increase in the colonic epithelial cells of UC patients, even though IL-4 was not increased. This upregulated IL-4Rα was explained by the finding that IL-13 receptor shares the alpha subunit as IL-4 but differed in the other subunit: IL-13Rα1 on the colonic epithelial cells[91]. The pivotal role of IL-13 was illustrated by diminished disease in the mice with IL-13Rα1

blockage[92].

Then, the next question raised to where the sources of IL-13 are if it is not secreted by Th2 cells, and natural killer T cells (NK-T cells) became the answer. NK-T cells were found to induce intestinal epithelium apoptosis via caspase-3 and tight junction damage on the gut barrier via claudin-2[93]. Moreover, the reason UC was named indicated the development of ulceration among the chronic intestinal inflammation. NK-T cells can be activated both directly via IEC or antigen-presenting cells, and indirectly via secreted IL-13 to expand NK-T cell population in the lamina propria[94]. Thus, UC is characterized as a Th2-like inflammatory response. Furthermore, IL-13 was reported to induce cell apoptosis and claudin-2 by activating the transcription factor STAT6 without activating NK-T cells[95, 96]. Later, non-classical NK-T cells, which the invariant NK-T cell receptors are not expressed, was identified to be involved in IL-13 secretion and colonic epithelial cytotoxicity [97].

Table 2 - 3: Comparisons of Mechanisms in CD and UC

	Chron's disease (CD)	**Ulcerative colitis (UC)**
Response immune cells	Th1 and Th17 effector T cells	Neutral killer T cells
Upregulated cytokines	IL-12, INFγ, IL-23, IL-17	IL-5, IL-13, IL-10

IBD to Colorectal Carcinogenesis

The concept of linkage between inflammation and cancer was hypothesized as early as in the middle nineteenth century: inflammation infiltration was observed in biopsies from cancer patients by Dr. Virchov[98]. The inflammation-associated cancers were estimated to contribute to 15-20% of all cancer types[99]. Inflammation mediating cancers include bacterial/viral infection, autoimmune disease, and spontaneous inflammatory response (unknown etiology). For example,

the human papillomavirus (HPV) infection is a predominant risk factor for cervical cancer[100]. Smoking and air pollution are positively associated with occurrence of lung cancer which is also mediated by lung inflammation[101]. Patients with prostatitis were three times more likely to develop prostate cancer than non-prostatitis group[102].

Correlation between IBD and colorectal cancer was defined as early as the late 1990s[103]. The relative risk of CRC in UC patients increased 1.4 folds compared to the general population. And the risk of CRC keeps accumulating at the rate of 0.5~1% annually after 8-10 years of having UC[104]. The strong coincidence between UC and colorectal cancer was a well-accepted concept, whereas there are still debates on whether CD has the similar effect on colorectal carcinogenesis initiation. The incidence rate of CRC increases with UC severity and associated with the sites of ulcerations[105]. Besides epidemiology results, the molecular pathways from UC to CRC were also highly investigated. There are two pre-requisites to initiate colon carcinogenesis and UC patients usually meet both of them. On one hand, the activated innate immune cells and injured epithelial cells keep secreting proinflammatory cytokines which will induce angiogenesis and create a microenvironment favoring tumor growth. On the other hand, mutations in the intestinal epithelial cells (IEC) are resulted from either frequent intestinal stromal cell differentiation and proliferation or, more likely, exposure to carcinogens in the lumen of gut. The carcinogenic process from CD to CRC was briefly presented in Fig. 2-2 below.

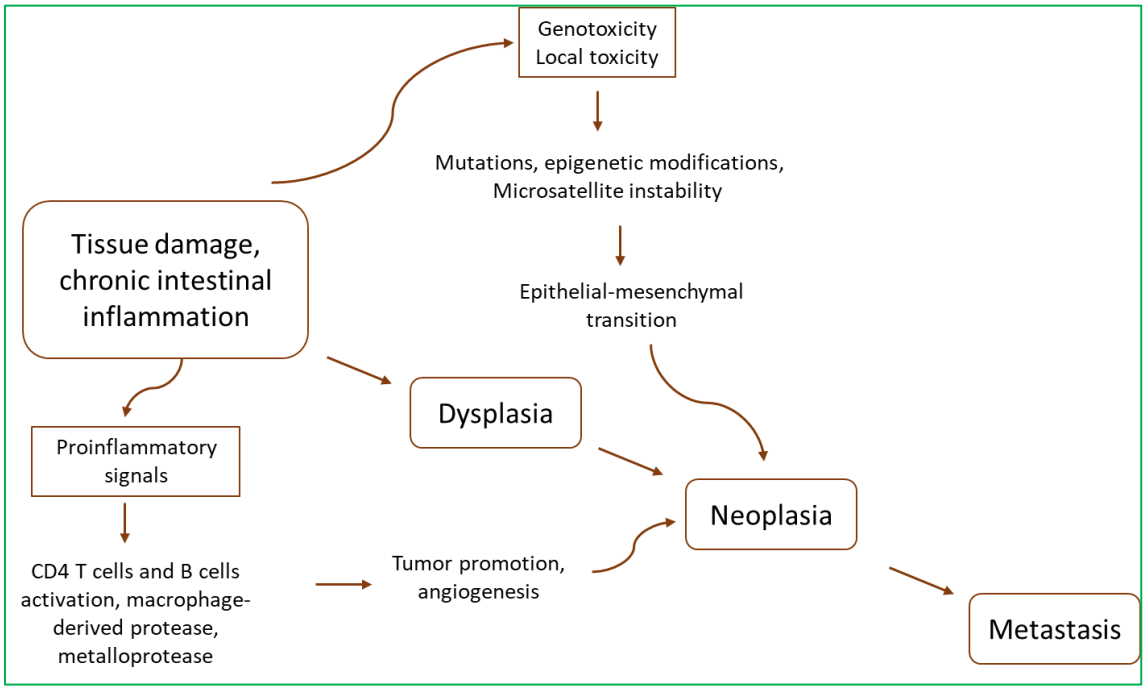

Figure 2 - 1: The pathogenesis from chronic colon inflammation to colorectal cancer

During chronic intestinal inflammation, the expression of multiple pro-inflammatory cytokines and chemokines are upregulated and accumulated at the inflammation sites. Mice lacking functional T and B cells in $Rag^{-/-}$ model developed colon cancer by bacterially-induced inflammation, but then $Rag^{-/-}$ with functional regulatory T (Treg) cells were successfully prevented from developing colonic tumors[106]. This finding demonstrated that immune cells which are promoting inflammation are necessary in colon cancer growth, and Treg cells usually exert antitumorigenic function. UC patients have elevated IL-13 and IL-5, while INFγ, IL-12, IL-22, and IL-17 are upregulated in CD patients. Other than disease-specific cytokines, NF-κB, TNF-α, IL-1β, IL-6, and IL-8 are involved in both forms of IBD. Inhibition of MAPK-activated protein kinase 2 (MK2) pathway hampering secretion of TNF-α IL-6, and IL-1β significantly reduced tumor growth in mouse model[107]. In the positive feedback loop, certain chemokines, such as CXCL1, attract immature innate cells to the inflammation sites, and then proinflammatory cytokines induce the

maturation. The mature immune cells further secrete proinflammatory cytokines to expand their population. Such innate immune cells are neutrophils, M2 macrophages, tumor-associated macrophages, and mast and effector T cells[108]. Among these upregulated proteins, NF-κB is believed to be the most potent tumorigenic molecule in UC-associated colon cancer in both animal models and clinical studies[109, 110]. It over-expresses vascular endothelial growth factor (VEGF) which plays a critical role in angiogenesis of colon tumors[111]. NF-κB also induces expression of matrix metalloprotease-9 (MMP9) which is involved in colonic tumor metastasis[112]. In addition to elevated pro-inflammatory cytokines, leukotrienes (LTs) and LT-producing enzyme, cyclooxygenase-2 (COX-2) was increased in colon from CRC patients[113]. LT participated in CRC via increased expression of COX-2 and B-cell lymphoma 2 (Bcl-2), and membrane accumulation o*f* β-catenin[114].

All of these proteins potently promote cell proliferation and suppress apoptosis. As increased induction of epithelial apoptosis, reactive oxygen species (ROS) and nitric oxide (NO) are produced. Not only can ROS lead to DNA damage, but the interaction between NO and ROS can dictate the type of gene mutation as NO suppresses DNA repair enzymes as well[115]. Patients with CRC are known with enhanced production of ROS and NO. But ROS in cancers act as a double-edged sword. Excess ROS production can induce mitochondria-mediated tumor cell death[116]. In addition to ROS-induced DNA repair, the IECs are exposed to gut bacteria and its metabolites which can also induce oncogene mutation in IBD patients.

The series of carcinogenic events occurs in a timely manner as illustrated in Fig. 2-3. At the early stage of CRC, p53 was found to be mutated in mucosal samples from UC patients. p53 is a critical tumor suppressor involved in many pathways to regulate the cell cycle and span. The mutation of p53 is found in a wide range of types of cancer in the brain, lungs, esophagus, liver, and colon[117]. Specifically, in IBD-associated colon cancer, the loss-of-function mutation on p53 happens at a very early stage of dysplasia and even in normal mucosal tissue from UC patients[118,

[119]. Microsatellite instability (MSI) as results of impaired DNA repairing function was found in human colon tumor biopsies prior to K-ras and adenomatous polyposis coli (APC) mutations which are well-known oncogenic mutations, evidenced by the fact that MSI were found mostly in regenerative (hyperplasia) mucosa samples, whereas K-ras and APC mutations were found in high-degree dysplasia and neoplasia mucosa predominately[120, 121]. Other than MSI, hypermethylation was found in dysplasia sites in UC patients compared to non-UC volunteers and normal mucosal tissues from UC patients[122]. The methylation status of *APC*, as a typical CRC-associated gene, was examined as soon as the hypermethylation was identified in CRC patients. Although majority highly methylated gene in CRC still remains to be understood, emerging reports showed altered protein translation of APC was due to hypermethylation and a trend of decreased methylation as CRC progression was observed[123]. Shao et al specifically revealed that the coding gene of one colon tumor-suppressing protein, *CPEB1*, was hypermethylated and its promotor region was highly methylated as well, then the subsequential transcription was diminished[124]. On the other hand, some genes transcription and translation were induced by methylation and found to be oncogenic genes, such as *PDX1*, *MSX1*, and *EN2*[125]. The timeline described above was illustrated in Fig. 2-2 below.

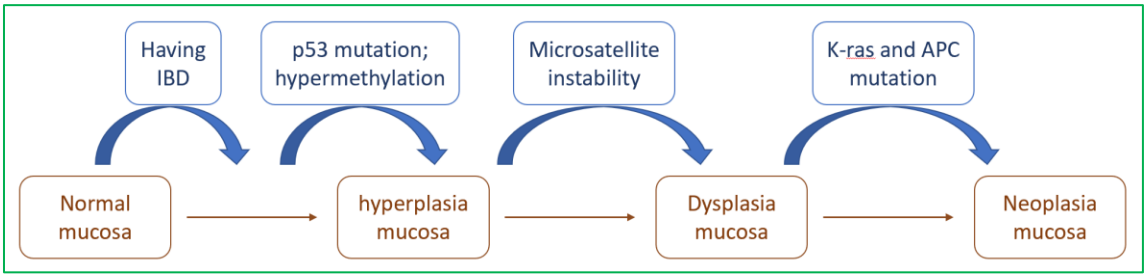

Figure 2 - 2: Simplified timeline of colon carcinogenic events

Since the gut microbiome are critical for initiating IBD and IBD-associated CRC, the means to establish a health-promoting composition of microbiome are pursued by many studies. Fermentable dietary fibers directly interplay with the gut microbiota, thus they are

closely discussed in this literature review and examined in this thesis project.

Dietary Fiber

History of dietary fiber

Hippocrates (ca. 460 BC-ca. 370 BC), "the father of Western medicine," claimed all disease begins in the gut, which pointed out the importance of the gastrointestinal (GI) system in regulating overall health. With the development of agriculture and the economy, people pay more attention to having "healthful" diets instead of just meeting their caloric needs. Because of this awareness among general public, many research works have been investigated individual nutrient components as well as overall dietary patterns. Dietary fibers are the key nutritional components of our daily diet and research has been conducted especially on the fermentable dietary fibers (DFs) in regulating GI health via both direct and indirect means, where gut microbiota serve as a connector between diet and the host health.

Dietary fibers are a nondigestible diet component from plants believed to have overall health-promoting effects. The term dietary fiber was first described in 1953 trying to understand a global increase in the incidence of pregnancy toxemia in urbanized regions. Although the report was mainly based on personal conversations, there were detailed comparisons of urbanized and indigenous dietary habits in Australia and Asia that showed a considerable number of fibers in the indigenous diets but not in urbanized diets[126]. From there, dietary fiber has been regarded as a new arm to resolve noninfectious diseases including IBD[127]**.** Physiologists and chemists had been cooperating to discover details of dietary fibers. The number of types of dietary fibers has been raised from 3 (cellulose, hemicellulose, and lignin) to a big family of compounds that include pectin, gums, and many other oligosaccharides[128]. The different chemical structure of dietary fibers

dictates their distinct properties and functions. For example, cellulose is composed of β-(1,4) linkages of glucose [129]. The β-(1,4)-linkage of cellulose allows the formation of a long and straight polymer that serves as the structure supporting material in plants. Although humans can apply glucose as an energy source, lacking of cellulase or β-(1,4) consuming bacteria stops absorption of glucose molecules through intestinal epithelial cells[130]. Thus, cellulose is classified as a non-fermentable fiber. In contrast, inulin is made of β-(2,1)-linked fructose which can be easily digested by human gut microbiota, mainly *Bifidobacteria* and *Bacteroides*[131], and fructose is released to be absorbed by the host. So, inulin is classified as fermentable fiber. Unlike cellulose or inulin that generally present in many plants, partially hydrolyzed guar gum (PHGG) is produced under controlled conditions to breakdown the guar gum that the extracted from the endosperm of the guar seeds. Its highly branched structure makes PHGG very viscose and reduces the nutrient absorption by trapping them with PHGG molecules so that it is considered as a calorie control material[13].

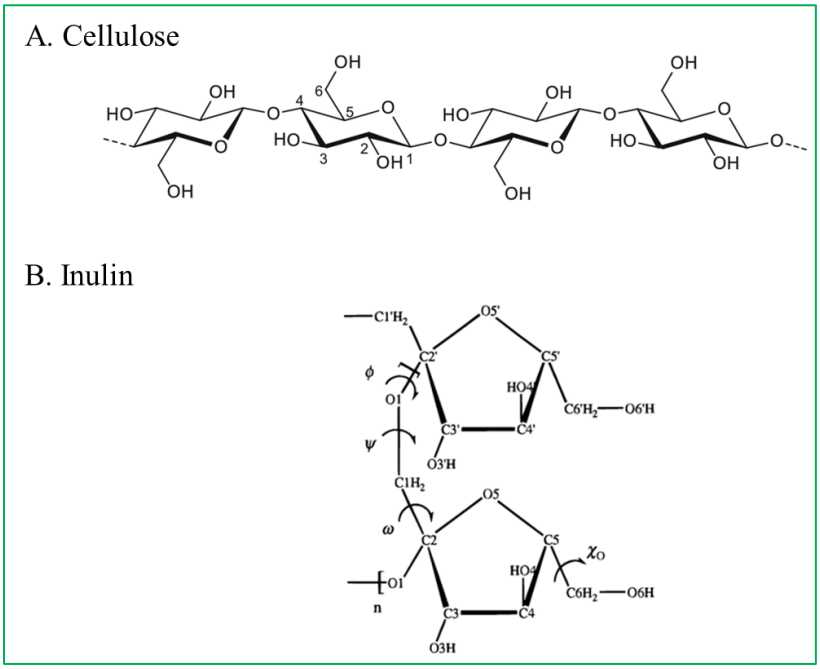

Figure 2 - 3 Chemical structure of cellulose (A) and inulin (B)

A. the chemical structure of cellulose [129], **B**. the chemical structure of inulin [132].

Dietary fiber estimation

An international-interlaboratory program was conducted to develop a methodology for accurately and precisely measuring dietary fibers. Beginning with a crude measurement in 1985 by Prosky et al, the total dietary fiber was estimated by dried food powder with blank samples as standard after enzymatic and dissolving removal of proteins and lipids[133]. The differences in water solubility among types of fibers were shortly described during the purifying process of classifying dietary fibers as soluble and insoluble ones and have been used until today[133, 134, 135, 136, 137]. The latest methodology employed the help of liquid-chromatography to separate each type of dietary fiber by the mass/charge ratio, then determined weight gravimetrically.

With improved techniques to measure the amount of water-soluble, insoluble, and total dietary fibers, people were able to conduct studies to explore the dose-response effect of dietary fibers on health and disease. The beneficial effects of dietary fibers have been vastly studied on cardiovascular disease, renal disease, and gastrointestinal (GI) tract-related diseases.

Dietary Fibers and Gut Microbiomes in IBD

The interplay between the gut microbiome and human immune system was first emphasized in 2001 by Hooper, et al[138]. Then Shanahan et al pointed out that gut microbiome may play a critical role in IBD pathology and can serve as a novel therapeutic target[139]. The commensal and symbiotic bacteria in the GI tract are considered an organ that participated in nutrient processing, maintaining immune system homeostasis, and many other health-related manners. The culture-dependent means to study gut microbiota are limited by the culturing environment since most of the gut bacteria are extremely anaerobic. The well-developed genetic sequencing

techniques resolve the difficulties in culturing-dependent methods and permitted researchers to comprehensively study the bacteria colonies in the GI tract in a culture-independent way.

Feces, as an outcome that is easy to access, was initially analyzed to compare the bacteria abundance difference in IBD patients and non-IBD control people. Distinct bacteria classes were found to be increased or decreased in IBD patients (Table 2-3-4). The findings that Toll-like receptor 5 (TLR5) was mediating the pathology of CD inferred a potential difference in bacteria composition in fecal samples and mucosal samples since TLR5 senses the antigens in the mucosal layer[140]. And this was further confirmed by Lo Presti et al by comparing the fecal and mucosal samples from the same patients[114]. At the same time, the reduction of mucosal bacteria diversity was reported by numerous studies[141, 142, 143, 144].

Table 2 - 4: Bacteria (class) Increased in IBD Patients and Experimental Models

Class of bacteria	**Reported paper**
Bifidobacterium	Wang, et al[145]; Manichanh, et al[146];
Bacteroides	Manichanh, et al[146]; Michail, et al[147]
Actinobacteria	Alam, et al[148]; Ogilvie, et al[149]; Bamola et al[150]
Prevotella	Manichanh, et al[146]
Clostridium	Marrinez-Medina, et al[151]; Sokol, et al[152]; Michail, et al[147]
Erysipelotrichi	Lo Presti, et al[141]; Salonen, et al[153]
Bacilli	Michail, et al[147]

Table 2 - 5: Bacteria (class) Decreased in IBD Patients and Experimental Models

Class of bacteria	**Reported paper**
Clostridium	Manichanh, et al[146]; Sokol, et al[152]; Issa, et al[154]
Firmicutes	Jones-Hall, et al[155]; Manichanh, et al[146]; Natividad, et al[156]
Faecalibacterium	Marrinez-Medina, et al[151]; Michail, et al[147]

Fusobacteria	Strauss, et al[157]; Michail, et al[147]; Han, et al[158]; Liu, et al[159]
Lactobacillus	Llopis, et al[160];
Spirochaetes	Xenoulis, et al[161]; Hampson, et al[162]; Michail, et al[147];

With a better understanding on the shift of gut microbiome in CD and UC patients and model animals by cross-sectional studies, the dynamics throughout the course of disease was investigated. Most pronounced, the long-term variance of microbiome diversity was significantly larger in IBD patients than health control[163, 164]. But whether this shifting is independent or coincided with the disease activity reminds debating[163, 165]. Among vast number of bacteria that is enriched or reduced in the disease condition, their metabolites and the meta-metabolomic map of IBD are not fully understood. So far, short-chain fatty acid (SCFA)-producing bacteria were one of predominant species that found to be negatively correlated with both forms of IBD[164, 166, 167].

In the last decade, many studies were conducted to investigate the efficacy of fecal transplantation as a treatment for human IBD. The gut microbiome alternation is known to be participated in IBD pathology and fecal transplantation in animal models showed effective to restore the donor's condition in the recipients. Both gnotobiotic or broad-spectrum antibiotics-induced microbiota-depleted mice resumed the disease as their colitis donors[168, 169]. Although fecal transplantation showed successful for treating acute *Clostridioides difficile* infection[170], results from clinical trial studies, unfortunately, were controversy[171, 172, 173]. It is hard to maintain certain bacteria composition as the donors. Diets can dramatically change the gut bacteria composition as short as in 4 days with a new dietary habit. Although the IBD patients who undergo fecal transplantation, it will be altered in a short period of time if the patients do not follow the diets as the donors. The dissimilarities of dose and frequency of administration in clinical trial studies could also cause the controversial results.

Since gut bacteria composition and load have been found to be associated with the pathogenesis of IBD, how to set the gut microbiome to a health-promoting position became to a question. Although anti-inflammatory drugs and hormones may alter the microbiota profile in IBD patients, diets are the most impactful modulator[174]. Thus, attentions have been paid to how dietary components can alleviate or aggravate the intestinal inflammation. The axis of diet to gut microbiota to immune response in IBD etiology has been confirmed by multiple studies conducted by various colitis models; however, gnotobiotic/pseudo-germ-free model animals were more susceptible to colitis induced by dextran sulfate sodium (DSS) administration than the group inoculated with commensal bacteria[175, 176, 177]. But mouse model (IL-10$^{-/-}$) lacking of immunosuppressing pathway spontaneously develop immune response to commensal bacteria, whereas gnotobiotic mice did not develop colitis and tumors[178]. This indicates that DSS-colitis model may not be a good model to study the gut microbiome-immune interaction in IBD.

Chapter 3

Fermentable Dietary Fibers Promote Inflammation Associated Colon Tumorigenesis

ABSTRACT

Background and Aim: Inflammatory bowel diseases (IBD) are a risk factor for colorectal cancer, a leading cause of death in the United States. The diet plays a significant role in IBD onset and progression by modulating the gut microbiota. Naturally occurring fermentable dietary fibers (FDFs) present in fruits and vegetables offer numerous beneficial effects on host health. However, whether the highly processed isolated FDFs hold similar beneficial effects on intestinal health is unknown. This study aimed to elucidate the impact of two FDFs: inulin and partially hydrolyzed guar gum (PHGG), which are commonly present in processed foods, on colonic inflammation and tumorigenesis.

Methods: Four-week-old WT C57BL/6J mice were fed diets with cellulose (control), inulin, or PHGG for four weeks. Subsequently, colitis was induced by adding dextran sodium sulfate (DSS) to the drinking water, which triggers colitis by disrupting the colonic epithelial cells. The body weight was monitored daily, and after seven days, the mice were euthanized and examined for colonic inflammation markers. Subsequently, the effect of the experimental diets on colon cancer development was assessed using a combination of DSS with the mutagenic substance azoxymethane (AOM). Post-seven weeks of AOM treatment, mice were euthanized, and the intestinal tumor burden was examined at a gross level and characterized by immunohistochemistry.

Results: Dietary intervention with inulin or PHGG potentiated DSS-induced colonic inflammation, as evidenced by more body weight loss, diarrhea, rectal bleeding, and local and systemic inflammation than the controls. At the histological level, we observed extensive disruption of colon architecture and massive infiltration of immune cells. In line, both inulin and PHGG-fed mice exhibited significantly greater sizes of colorectal tumors upon cotreatment with AOM/DSS (CRC

model). Immunohistochemical characterization of colon tumors showed comparatively high expression of cell proliferation marker Ki67 and activation of the Wnt signaling pathway as evidenced by increased intracellular accumulation and nuclear localization of β-catenin.

Conclusions: The FDFs inulin and PHGG augmented colon tumorigenesis by potentiating colonic inflammation. Therefore, fortification of these FDFs should be approached with caution as it may increase the risk of colorectal tumorigenesis in patients with IBD.

Key words: Inflammatory bowel disease, colon cancer, dietary fiber, colitis

Study Highlights

Chronic intestinal inflammation fuels the continuous turnover of cells in the intestinal lining, which increases the chance of irregularities (e.g., missense mutations) that may lead to cancer, thus considered a primary risk factor for colorectal tumorigenesis. Our study found that isolated soluble fibers inulin and PHGG potentiated and prolonged colonic inflammation and increased colitis-associated colorectal tumorigenesis. This data suggest that the consumption of these isolated dietary fibers may increase the risk of colon cancer in patients with IBD.

Introduction

Colorectal cancer (CRC) is the third most common cancer among all cancer types in the United States and the fourth top worldwide[19]. Both genetic (non-modifiable) and environmental (modifiable) risk factors play a role in CRC development. Among all the risk factors, inflammatory bowel disease (IBD) was reported as the most predominant factor [relative risk (RR)=2.93, 95 % CI 1.79-4.81], over genetic component [CRC history in a first-degree relative (RR = 1.80, 95 % CI 1.61-2.02)] in a meta-analysis study[30]. Therefore, understanding and well-managing IBD is necessary to prevent CRC. The two clinical forms of human IBD, Crohn's disease (CD) and ulcerative colitis (UC), which is resulted from chronic gastrointestinal (GI) inflammation and are initiated by dysregulated intestinal immune responses. IBD is a multifactorial pathology that develops due to interactions between genetic and environmental factors, including changes in the composition of the intestinal microbiota that has been reported to be a crucial factor in the pathogenesis of IBD.

Dietary fibers (DFs) are plant-derived complex carbohydrates believed to have overall health-promoting effects. Depending on their chemical structures and degree of fermentability, DFs are utilized by gut bacteria as the primary energy source; therefore, they influence the richness of the bacterial species and their metabolites in the gut[179]. Cellulose is an insoluble polysaccharide of D-glucose units[129] that resists intestinal bacterial fermentation in humans and mice[130]. Thus, cellulose is classified as a non-fermentable fiber. In contrast, inulin, made of β-(2,1)-linked fructose, can be easily degraded by human gut microbiota, mainly by Bifidobacteria and Bacteroides[131], releasing fructose further absorbed by the host. So, inulin is classified as a fermentable fiber (FDF), and it is widely used in the food industry due to its low-degree sweetness and pleasing texture in processed food[12]. Partially hydrolyzed guar gum (PHGG), which supports bifidogenic and lactogenic growth, is another example of an FDF that has been used as a food thickener and

emulsifier[13]. However, unlike inulin, which is generally present in many plants, PHGG is produced under controlled conditions to break down the guar gum extracted from the endosperm of the guar seeds[13].

Many studies have demonstrated that gut microbiota dysbiosis, largely defined by reduced microbial richness or expansion of certain species, negatively influences overall health. Thus, FDFs, as primary energy sources for gut microbes, may be employed to correct dysbiosis and associated health complications. The shift in gut microbiota composition due to diets low in FDFs has been linked to poor intestinal and overall metabolic health[180, 181]. Individuals who are tolerant of FDFs and consume an adequate amount of FDFs can be benefited from numerous health aspects. For example, clinical and pre-clinical studies suggest that inulin or PHGG consumption by healthy individuals improves the gut barrier function and production of beneficial fermentation metabolites such as short-chain fatty acids (SCFAs)[1, 2, 3, 4]. In this effort, food companies incorporate FDFs as prebiotics into food products during manufacturing to promote the growth of specific groups of bacteria, which are believed to be beneficial for human health. However, recent research cautions that FDFs might not be universally beneficial for health[11, 182], and whether such fiber-enriched food products deliver the expected health benefits during dysbiosis remains to be ascertained.

In the present study, we investigated the effect of inulin and PHGG, two widely used FDFs in food industries, on colonic inflammation and carcinogenesis. For that, we fed mice with diets containing inulin or PHGG or with a control diet containing cellulose. The effects of FDFs on intestinal inflammation were assessed by using the DSS model and on colon tumorigenesis by employing the azoxymethane (AOM)/DSS model of CRC. DSS is a classic colitogenic substance widely used that interacts with the colon tight junctions or mucus[177]. Our study showed that inulin and PHGG potentiated and prolonged colonic inflammation and correspondingly increased colitis-associated colon tumorigenesis, suggesting that the consumption of FDFs may increase the risk of colon cancer in patients with IBD.

Methods

Animal model and diets

Inbred four-week-old wild-type C57BL/6J mice were housed in a temperature- and humidity-controlled room with free access to water and to one of the following diets: standard chow diet (Laboratory Rodent Diet 5001) or isocaloric diets containing cellulose (10 % w/w, Control diet, Con), inulin (7.5% w/w inulin plus 2.5% w/w cellulose), or PHGG (7.5% w/w PHGG plus 2.5% w/w cellulose) (Research Diets, Inc., New Brunswick, NJ). The composition of the experimental diets is shown in appendix Table-1. Diets were replaced once a week. Every three days, the animals were weighed to determine body weight progression. All procedures were performed in compliance with the Institutional Animal Care and Use Committee of the Pennsylvania State University, which specifically approved this study. Both sexes were used in this study. Since there was no difference in colitis susceptibility, we combined the data from both sexes.

Dextran sulfate sodium-induced acute colitis: As outlined in appendix figure 1, wild-type C57BL/6J mice were weaned at day 22 (±1 day) and maintained on the standard chow diet. After one week, mice were switched to one of the experimental diets (n=4 in each group). After four weeks, 1.4% DSS was added to the drinking water. Fecal samples were collected on days 1, 5, and 7 of post-DSS intervention. Feces scoring was performed as described in appendix Table-2. Seven days post-DSS intervention, the animals were euthanized by carbon dioxide and blood, colon, and cecal samples were collected as described below and stored at -80°C until further analysis.

Mucosal healing progression after acute colitis: As illustrated in appendix figure 2, eight-week-old wild-type C57BL/6J mice were switched to drinking water containing DSS (3% w/v) for 7 days (n=6). During DSS intervention mice were continuously monitored for body weight. On day 7, feces and blood (through submandibular bleeding) were collected to assess the extent of colonic

and systemic inflammation. Mice exhibiting an equal inflammation status were randomly assigned to either Con or inulin diets and were on regular drinking water without DSS for 7 days until sacrifice. On the euthanasia day (7 days post-DSS withdrawal), blood, feces, and tissues were collected as described below, and stored at -80°C for further analysis.

Colitis-associated cancer induction: As outlined in appendix figure 3, four-week-old wild-type C57BL/6J mice were maintained on experimental diets for three weeks before cancer induction. Sequentially, colon tumorigenesis was induced by azoxymethane (AOM, Sigma-Aldrich)-DSS administration, as previously described in detail[183]. Briefly, AOM was injected intraperitoneally (7.5 mg/kg body weight). After 7 days, colonic inflammation was induced by the cycle of 7-day DSS water and 7-day regular water. In the first cycle the concentration of DSS in the drinking water was 1%, while in the second and third cycles was 0.75% DSS. The mice were sacrificed 7 days post last DSS/regular water cycle. The body weight of the animals was collected every three days.

Sample Collection and Preparation

Fecal content: Feces were collected for 10-30 minutes using an autoclaved box in the morning of the collection days. The fecal slurry was then prepared by making a 100 mg feces/mL solution in phosphate buffered saline (PBS) containing 0.1% Tween-20 (VWR Chemicals). The solutions were then centrifuged (2500 g, 15 minutes at 4°C), and the supernatants were stored at -80°C until further analysis.

Serum: Blood was collected from the portal vein of the euthanized mouse into serum-separation tubes (BD Microtainer). Serum was then obtained by centrifugation at 700 g for 8 minutes at 4°C, and stored at -80°C until further analysis.

Colon: Colons were dissected and flushed with ice-cold PBS. The proximal part (~1cm) was stored at -80°C until protein extraction. The rest of the tissue was fixed in 10% neutral buffered formalin

(NBF, Fisher Chemical) for 24 hours, then stored in 70% ethanol until embedded in paraffin for histology analysis.

Protein extraction and quantification: Colon proteins were extracted with RIPA Lysis and Extraction Buffer (Thermo Scientific) by adding 1% protease inhibitor (Thermo Scientific) and 1% phosphatase inhibitor (Thermo Scientific), and following the manufacturer's instructions. The total protein content was quantified by Pierce™ bicinchoninic acid protein assay kit (Thermo Scientific), following the manufacturer's instructions.

Inflammatory markers quantification

Serum, fecal, and colon Lcn2 levels and serum and colon SAA levels were measured by ELISA (R&D systems) as per the manufacturer's instructions.

Histologic Analysis

Several paraffinized sections (5 mm thickness) from the fixed colon tissue were prepared by Penn State Animal Diagnostic Laboratory, and one section was stained with hematoxylin and eosin (H&E). The remaining unstained sections of the fixed colonic tissue were then deparaffinized with the standard protocol, which immersed the section in xylene (Thermo Fisher), then rehydrate with 100%, 90%, 70%, 50% ethanol (Sigma-Aldrich), and PBS in series for 3 minutes each.

Picrosirius red staining: The deparaffinized sections were washed in the distilled water by putting the sections in plastic Coplin jars filled with distilled water for 3 minutes and refilling the jars with clean distilled water for 3 minutes again. Then, in a humidified chamber, clean and dry sections were stained with Sirius red 0.1% in saturated picric acid (Electron Microscopy Sciences) for 1 hour. The humidified chamber was prepared by putting wet tower tissue in a glass tray and sealed with sealing wrap. Next, the sections were rinsed in acidified water (0.5% glacial acetic acid in distilled water) twice for 1 minute and washed with distilled water twice. Sections were then dehydrated with 100% ethanol three times for three minutes each before being transported in

xylene. Finally, the xylene was cleaned by airdrying, and sections were mounted with DPX mounting medium (Sigma-Aldrich).

Immunohistochemistry staining: The procedure to visualize the colonic content of β-catenin and Ki67/MKi67 was performed on two consecutive days. On the first day, the deparaffinized colonic sections were immersed in pre-warmed sodium citrate buffer in a beaker (10mM tri-sodium citrate in distilled water, 0.05% Tween-20, pH 6.0) at 98°C for 20 minutes to retrieve antigens, then the beaker was put in ice for fast cooling. Subsequently, sections were rinsed in PBS twice every five minutes. The sections were then blocked by normal donkey serum (Sigma-Aldrich, 10%) containing 0.3% triton-100 (VWR) for 90 minutes, followed by two washes in PBS for eight minutes. Next, the sections were incubated with diluted primary antibody overnight at 4°C in dark. The antibody diluent was prepared by PBS containing 0.3% triton, 1% BSA, and 1& donkey serum. A negative control section was stained without incubating with the primary antibody. The primary antibodies were anti-β-catenin (Novus, NBP1-54467SS, 1:200), and anti-MKi67/Ki67 (Novus, NB500-170SS, 1:200).

For the β-catenin detection, on the second day, sections were rinsed in PBS four times every seven minutes with gentle rocking. Then sections were incubated with secondary antibodies for one hour at room temperature in the dark, followed by washing four times every ten minutes. The secondary antibody was anti-mouse (Abcam, ab269820, 1:400). Finally, the sections were mounted with a resinous mounting medium and visible under the fluorescence microscope.

For the Ki67/MKi67 visualization, endogenous peroxidase was blocked on the second day by incubating the sections in 30% hydrogen peroxide in PBS for 15 minutes followed by washing twice every 5 minutes. Then a universal secondary antibody (Vector Labs) was applied to the sections for 45 minutes, followed by washing eight times every seven minutes. Next, 3,3'-Diaminobenzidine (DBA) was dropped on the sections until the brown color was visible and quickly rinsed in running water for 20 minutes. The nuclei were stained with hematoxylin for 150

seconds followed by a 2-minute washing, and then stained with sodium bicarbonate (0.1% in distilled water) for one minute and washed in running water for five minutes. Finally, the sections were dehydrated with 50%, 70%, 90%, and 100% ethanol, and transferred in xylene to be mounted and examined under a brightfield microscope. All histology images were generated from Leica DMi8 through the LAS X software.

Image quantifications: The carcinogenic area was quantified by ImageJ. The whole colon area was automatically selected by the software via color-dependent selection. The tumors were manually selected by the predominant circular shape and brightness on the picture. All area was calibrated in centimeters by a ruler present aside in the picture of colon tissue.

Statistical Analysis

All data are represented as Mean ± SEM. The normality and equal variance were tested by the Shapiro-Wilk test and Bartlett test in RStudio. Statistical significance between two groups was calculated using an unpaired, two-tailed t-test. Data from more than two groups were compared using a one-way ANOVA followed by Tukey's multiple comparison tests (when to compare the mean of each column with the mean of every other column). The significance level of $*p < 0.05$, $**p<0.01$, and $***p<0.001$ were applied in this analysis to determine the difference by using GraphPad Prism 9. Sample sizes of more than three were considered for statistical analysis. For a sample size of less than 3, only the group average was calculated in both the experimental and control group.

Results

Inulin Exacerbated DSS-Induced Colitis

Inulin, a prebiotic FDF commonly present in processed foods, has been shown to have both beneficial and detrimental effects on intestinal inflammation [7, 184, 185]. Herein, we tested the effect of inulin consumption on colonic inflammation using DSS. Importantly, DSS-induced colitis in mice shares many characteristics with human ulcerative colitis (UC), including diarrhea, rectal bleeding, disrupted crypt structure, and neutrophil infiltration[186, 187], even though the underline cytokine pathway is not exactly the same as human UC [188]. Wild-type mice were fed with either a control diet (10% w/w cellulose, non-fermentable fibers; Con) or an inulin-containing diet (7.5% w/w inulin, 2.5% w/w cellulose; inulin) for 4 weeks, followed by sex and body-weight-matched splitting into two groups receiving drinking water without (no treatment, NT) or with DSS (1.4% w/v) (Fig. 3-1A, n=4 in each group). Body weight was not significantly different in the Con-NT and inulin-NT groups. However, the inulin-DSS group lost approximately 12.6% of the initial body weight on day 7, which was significantly more than the Con-DSS group (Fig. 3-1B, $p = 0.0006$). The change in body weight is a predominant sign of the disease extent in the DSS-induced colitis model, as the mice with severe colitis lose a substantial amount of blood and experience poor nutrient absorption. The daily body weight graph also showed that the disease initiated in inulin-DSS mice as early as day 4 on DSS administration, indicating the earlier onset of colitis in the inulin-DSS group than Con-DSS group. As described in the colitis severity rubric (Table 3-1), relatively more diarrhea and rectal bleeding were observed in the inulin-DSS group starting from day 4. Correspondingly, inulin-DSS mice displayed shortened colon and enlarged spleen upon euthanasia after 7 days of DSS intervention (Fig. 3-1C-E). The earlier loss in body weight and shortened colons indicated that the inulin-DSS mice were experiencing more severe colon

inflammation than con-DSS group. A trend of enlarged spleen in the Con-DSS group was found in comparison to the NT groups, but inulin-DSS mice displayed dramatically heavier spleen weight than all of other three groups (Fig. 3-1F). Lipocalin-2 (Lcn2) and serum amyloid A (SAA) levels were measured in the four groups of mice by ELISA to quantify inflammation. Lcn-2, a member of the lipocalin family, is secreted from epithelial cells and neutrophils in response to bacterial infection as an innate immune response, and is one of widely used biomarkers for colon inflammation[189, 190]. Similarly, SAA is another robust marker of both systemic and intestinal inflammation[191]. The levels of Lcn2 and SAA were not significantly different between the Con and inulin groups at the basal level (NT groups). However, both Lcn2 and SAA levels were significantly elevated in the inulin-DSS group compared to Con-DSS (Fig. 3-1F-H). Collectively, these data demonstrated that inulin aggravated colonic inflammation in the inflamed condition instigated by DSS administration. However, inulin consumption did not alter the immune response in NT groups, suggesting that inulin consumption does not exhibit adverse effects in the healthy gut.

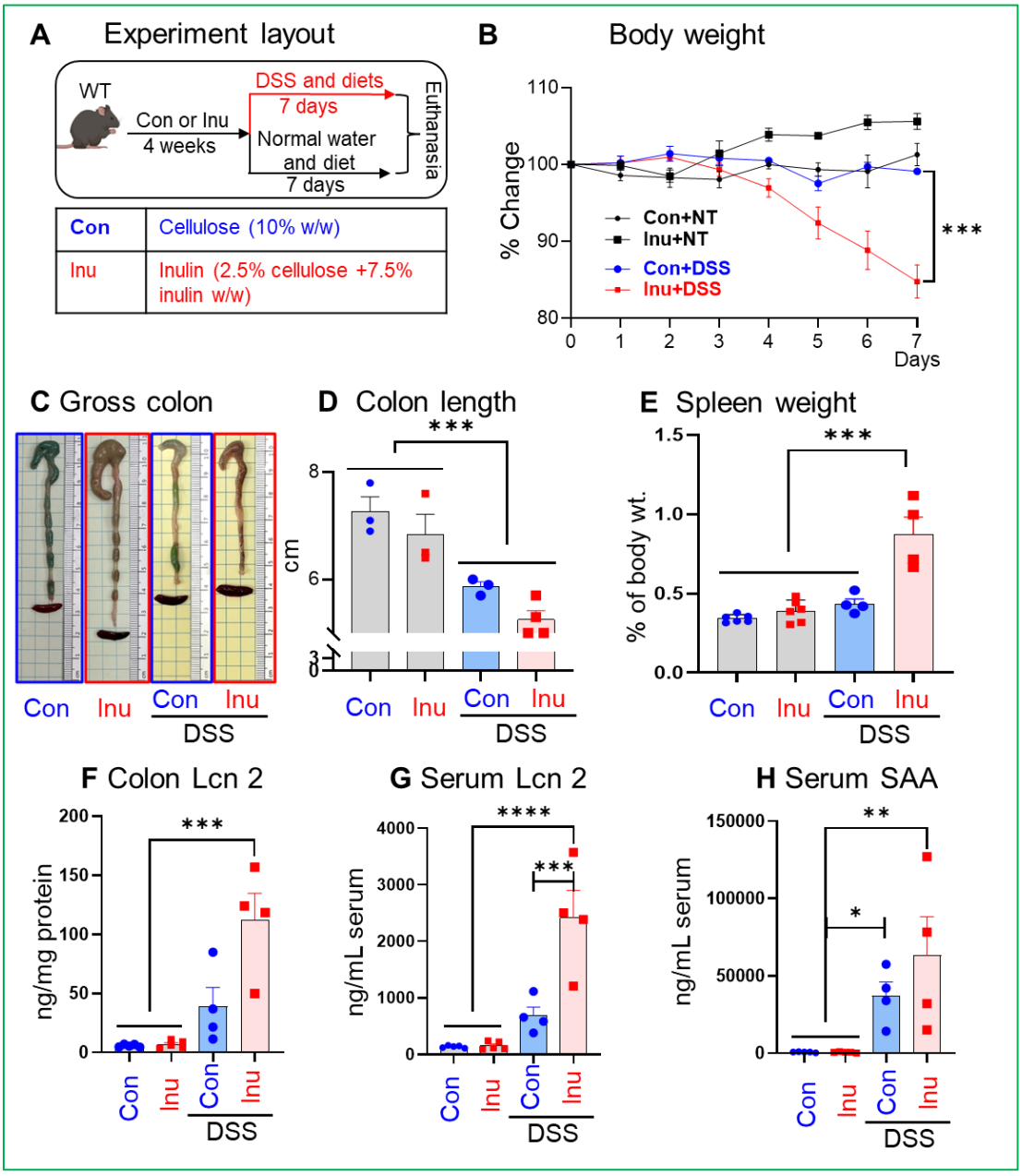

Figure 3 - 1: Inulin worsened dextran sulfate sodium (DSS)-induced colitis.

Wild-type mice were fed control (Con) or inulin (Inu) diets for four weeks followed by 1.4% DSS intervention for 7 days. **A**. experiment timeline, **B**. body weight change during DSS treatment and NT group at the same timeline as DSS group, **C**. gross colon pictures, **D**. colon length, **E**. spleen weight as percentage of whole-body weight on the same day, **F**. colon Lcn2, **G**. serum Lcn2, **H**. serum SAA. Values are shown as mean ± SEM. P <0.05 as *; <0.01 as **; <0.001 as ***

Inulin Delayed the Mucosal Healing Progress after Acute Colitis and Promoted Colonic Fibrosis

After characterizing the effect of inulin in inflamed intestines, we next asked whether inulin impacts the recovery from DSS-induced colitis. In mice fed with the chow diet, we did observe a substantial body weight recovery and normal stool consistency without a trace of blood on 7 days post-DSS withdrawal. Therefore, we employed a similar experimental timeline to assess the effect of inulin on recovery from DSS-induced injury (Fig. 3-2A). Body weight, fecal and serum Lcn2 and serum SAA on day 0 (basal level) and on day 7 (7 days post DSS intervention) were determined to randomize the mice for control and inulin diet feeding during the recovery phase. All mice displaying comparable levels of these markers at both time points were randomly assigned to either control or inulin diet (Fig 3-2B).

Both control and inulin-fed groups displayed comparable recovery in body weight in the first two days of the recovery phase (until day 9). However, on day 3 post-DSS withdrawal, the inulin group started showing a slower body weight regain, and a significant difference in body weight restoration was observed (Fig. 3-2Ci). After 7 days after DSS withdrawal, mice from the control group recovered to 99.6% of their initial body weight, whereas the inulin group only restored 90.7% on average (Fig. 3-2Ci, $p = 0.01$), suggesting a poorer recovery with inulin consumption. Strikingly, one mouse in the inulin experienced early mortality at day 11 (Fig. 3-2D), further confirming this dietary group's poor recovery from colonic inflammation. We further quantified the levels of the pro-inflammatory markers (Lcn2 and SAA) by ELISA in the feces, colon tissue, and serum after 7 days of DSS withdrawal. The inulin-fed group, when compared to the Con-mice, showed remarkably heightened systemic inflammation, which was in agreement with the observed poor recovery in this group of mice, evidenced by elevated levels of Lcn2 and SAA (Fig. 3-2Cii-v). However, fecal and colon Lcn2 and spleen weight were not statistically different between the Con and inulin groups, suggesting colonic inflammation was reduced to a

similar extent in both groups (Fig. 3-2F). The reasons for these discrepancies remain to be understood. Notably, H&E staining of the colon from these animals showed more disruption of the crypt structure and immune cell infiltration compared to the controls, which are histologic features of ongoing inflammation. We also observed thickened colon in inulin-fed mice (Fig. 3-3A). Interestingly, the better convalesced control mice had a short colon length and comparable spleen weight, which led us to further investigate the impact of inulin consumption on tissue regrowth and fibrosis (Fig. 3-2E-F). We theorize that the lengthened and thickened colons in the inulin-fed mice could be resulted from colitis-related intestinal fibrosis due to excessive deposition of collagenous scar tissue. We performed Picrosirius red staining in the colonic sections to visualize collagen fibers and collagen accumulation. A substantially high collagen accumulation was observed in the inulin-fed group, particularly in the submucosa (Fig. 3-3B-C), affirming that the thickening of the colon in this group of mice was largely due to fibrous tissue accumulation.

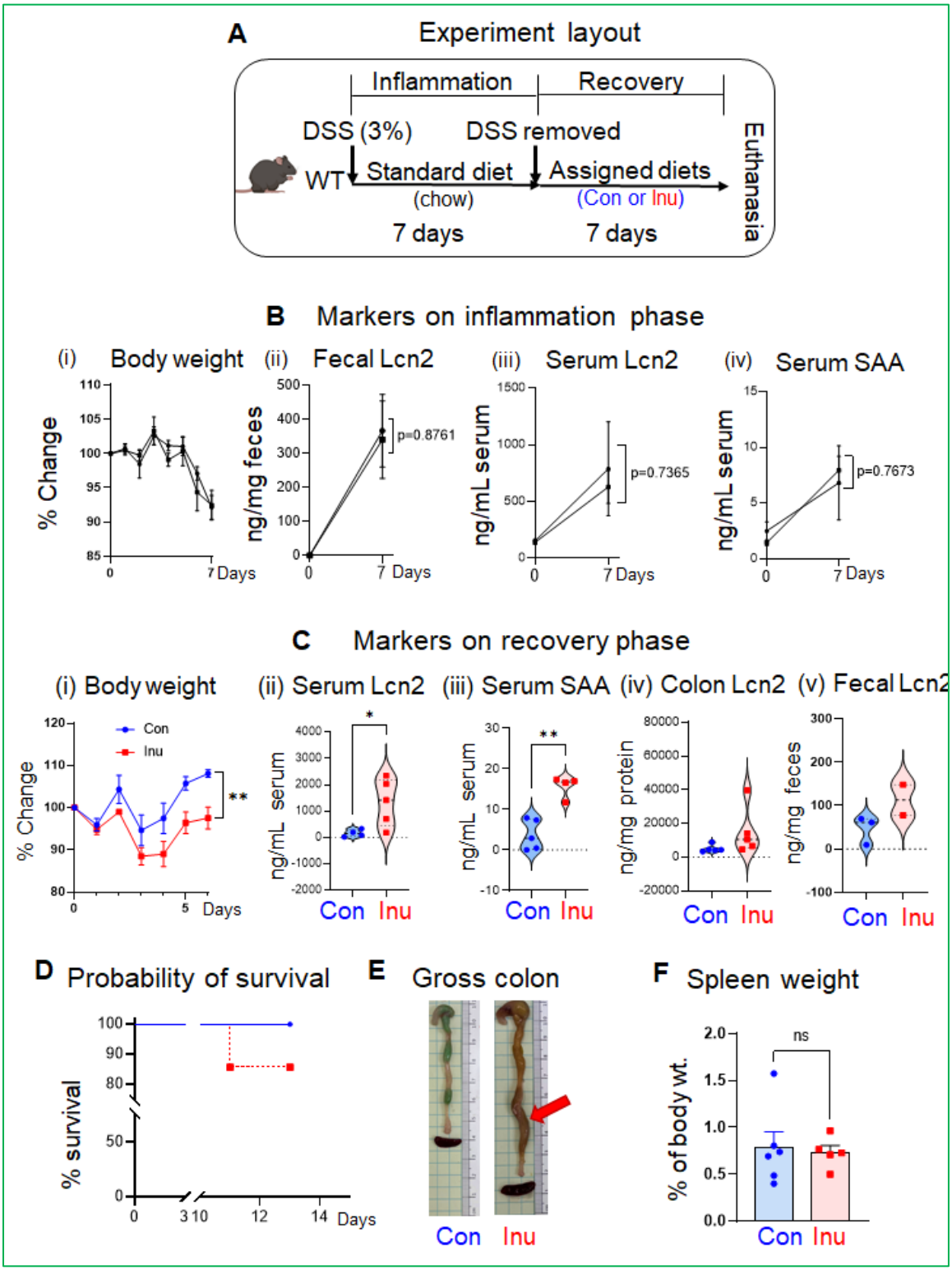

Figure 3 - 2: Inulin-fed mice displayed poor recovery after DSS withdrawal.

A. experiment timeline, **B**. body weight, fecal Lcn2, serum Lcn2, SAA during inflammation phase (on DSS water), **C**. body weight and inflammation markers during recovery phase (day 7 post DSS withdrawal), **D**. probability of survival, **E**. representative gross colon pictures from each dietary group (the red arrow indicating colon thickening), **F**. spleen weight in percentage of whole-body weight on the same day. Values are shown as mean ± SEM. P value <0.05 as *; <0.01 as **; <0.001 as ***

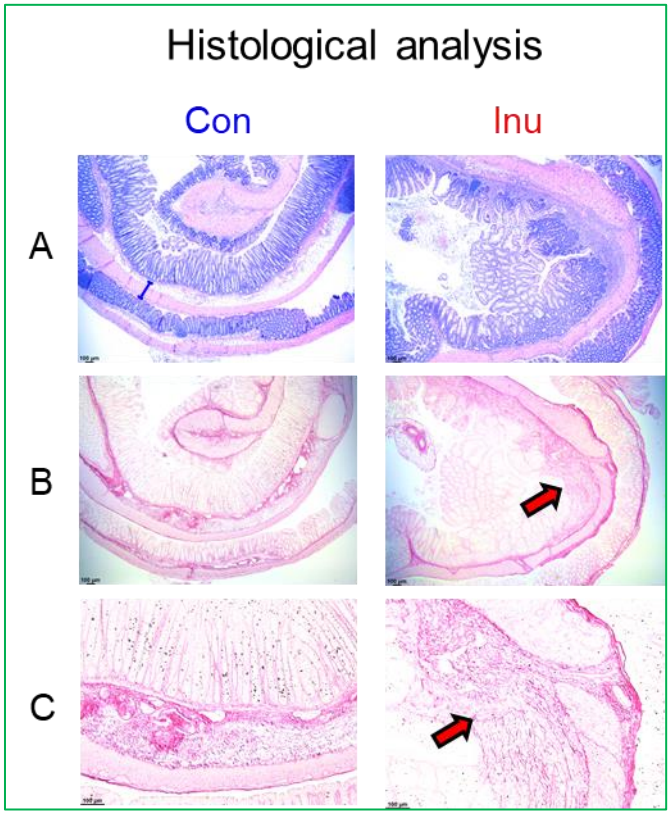

Figure 3 - 3: Inulin promoted intestinal fibrosis

Histological analysis of colonic sections from control and inulin groups. **A**. H&E staining at 16 magnification factors, **B**. Picrosirius red staining at 16x magnification factors, **C**. Picrosirius red staining at 50x magnification factor. Red arrows indicate collagen accumulation in the colon mucosa area.

Inulin Worsened IBD-Associated Colorectal Tumorigenesis in AOM/DSS Model

Prolonged intestinal inflammation is the top risk factor for the incidence of CRC. To expand our understanding of the impact of inulin on intestinal health, specifically inulin-induced exacerbation of intestinal inflammation on the risk of developing CRC, we used the azoxymethane (AOM)/DSS model. The combination of DSS with AOM is a well-established model for colitis-associated cancer due to its reproducibility, potency, and induction of tumors that closely resembles human colorectal cancers[192]. As outlined in Fig. 3-4A, one-week post-AOM injection, the mice received 1 % (1 cycle) or 0.75% (two cycles) DSS in drinking water every other week and consumed regular water in the weeks between each DSS week. The mice were sacrificed two weeks after the last DSS cycle (day 56). Remarkably, a substantial mortality (~50%) during the DSS intervention period was observed in the inulin-fed group, supporting our observation that inulin consumption delays the recovery from DSS-induced colon epithelial injury (Fig. 3-4 B). This early death was caused by severe intestinal inflammation and blood loss, by the observation that dead mice were very lean, undergone severe diarrhea; however, no colon tumor developed. Relative to the control mice, the survived inulin-fed animals experienced more body weight loss at both the DSS intervention period and the normal water break period (Fig. 3-4 C). Inulin-fed group developed extensive tumors. On average, 23.3% of the colon area in these mice was covered by tumors, while only one control mouse developed a colon tumor. Moreover, the tumor size (7.8% of colon area) was substantially smaller in control than in any mouse in the inulin-fed group (Fig. 3-4E). We also compared the inflammation levels in both groups and found that the inulin group had significantly higher levels of colon inflammation marker, Lcn2 (Fig. 3-4F). Although SAA was not significantly different between two groups, but the spleen from the inulin-fed mice was almost 4-fold heavier than the controls, indicating an active immune response and immune cell proliferation in the inulin group (Fig. 3-4G-H). Collectively, the inulin-fed group exhibited

significantly higher inflammation, greater morbidity, and extensive colon tumorigenesis, showing that inulin consumption predisposes mice to develop CRC.

Histological analysis of H&E-stained colon tissues revealed infiltration of immune cells in both control and inulin-fed groups (Fig. 3-5A). To characterize the colon tumors in the inulin group, we next performed immunostaining for ß-catenin and Ki67, which are widely used markers for CRC development. The Wnt/ß-catenin signaling pathway plays a critical role in cell proliferation and homeostasis of intestinal epithelial cells; thus, it is widely used as a marker of colon tumorigenesis [193, 194]. The internalization of ß-catenin, from the cell membrane to cytosol and to nucleus, is linearly correlated with the progression stages of colon carcinoma [195]. Herein, immunostaining showed that ß-catenin was largely located on the epithelial cell membrane in the control group. In contrast, nuclear and cytosolic ß-catenin were extensively detected in the inulin group, suggesting reduced degradation of ß-catenin and upregulation of the Wnt signaling pathway, which in turn leads to dysplasia (Fig. 3-5Bi). Besides ß-catenin, Ki67 is another strong prognostic marker in colon cancer that is highly expressed in proliferating cells in the cell cycle of G1-M phases[196]. Immunohistochemical analysis in the colon tissues from both control and inulin groups showed that Ki-67 was primarily expressed in the tumor region as well as in the non-tumor areas of inulin-fed colons (Fig. 3-5Bii). The histological analysis revealed that the colon tumors in the inulin group accumulated ß-catenin in the nuclei of intestinal epithelial cells and exhibited upregulation of Ki67 expression. The overall evidence demonstrated that dietary inulin, compared to the consumption of a non-fermentable fiber, promotes colon tumorigenesis in mice.

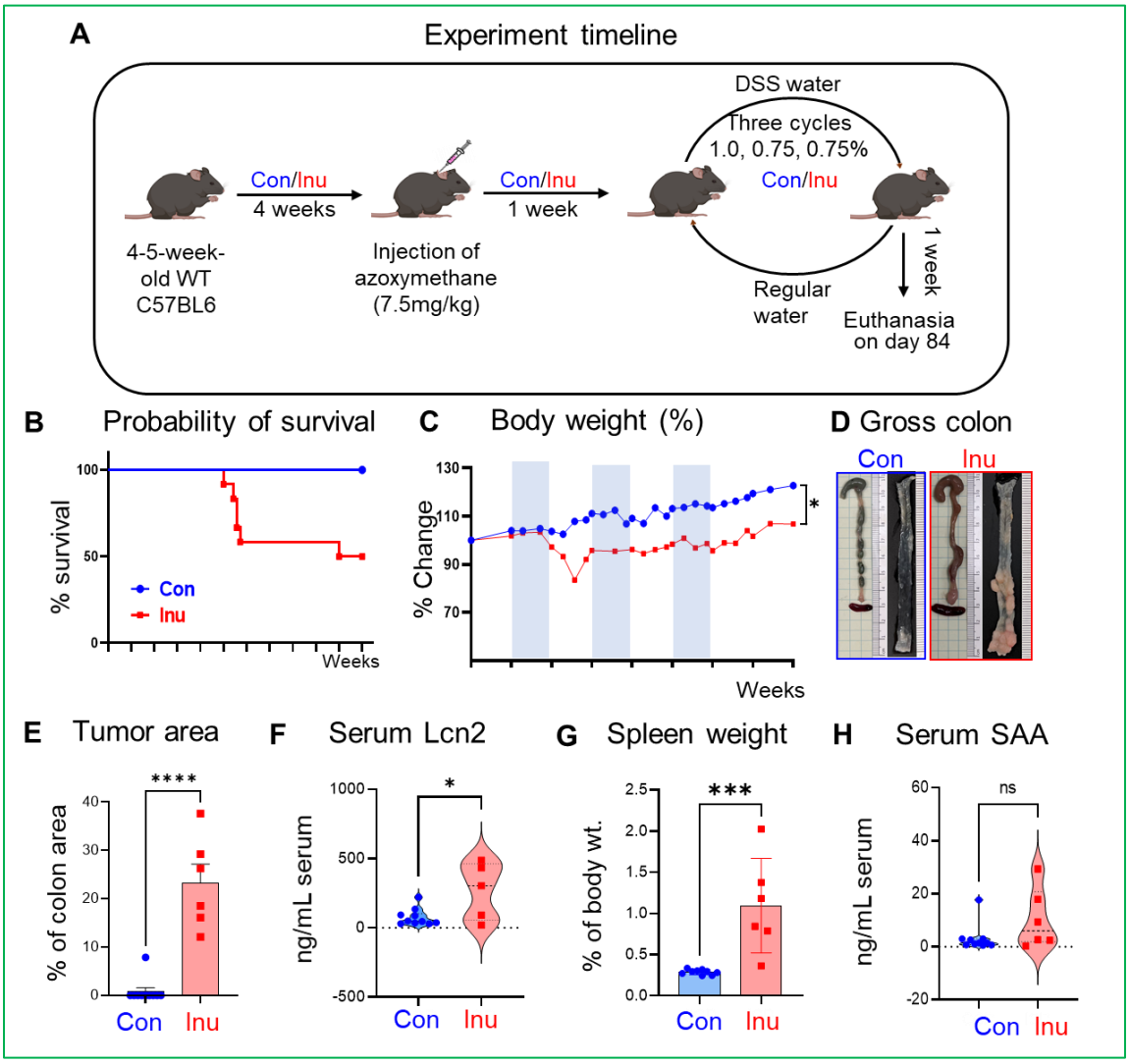

Figure 3 - 4: Inulin consumption exacerbated colitis-associated CRC progression.

Mice were maintained on control (Con) or inulin (Inu) diet for four weeks before azoxymethane (AOM) administration. One-week post-AOM injection, DSS water was provided to all the mice for seven days followed by seven days of regular water. The cycle of DSS/normal water was continued for six weeks, three cycles in total. Mice were sacrificed one week after last (third) cycle of DSS. **A**. experiment layout, **B.** probability of survival of AOM-treatment mice during DSS/water cycles, **C**. body weight change in percentage of the initial weight on AOM injection day, **D**. representative pictures of gross colons and longitudinally opened colons

illustrating gross appearance of tumors, **E**. tumor area in percent of the total colon area, **F**. spleen weight represented as percentage of whole-body weight on the day of euthanasia. G-H. Violin plots show serum level of Lcn2 (**G**) and serum amyloid A (**H**). Values are presented as mean ±SEM. P value <0.05 as *; <0.01 as **; <0.001 as ***.

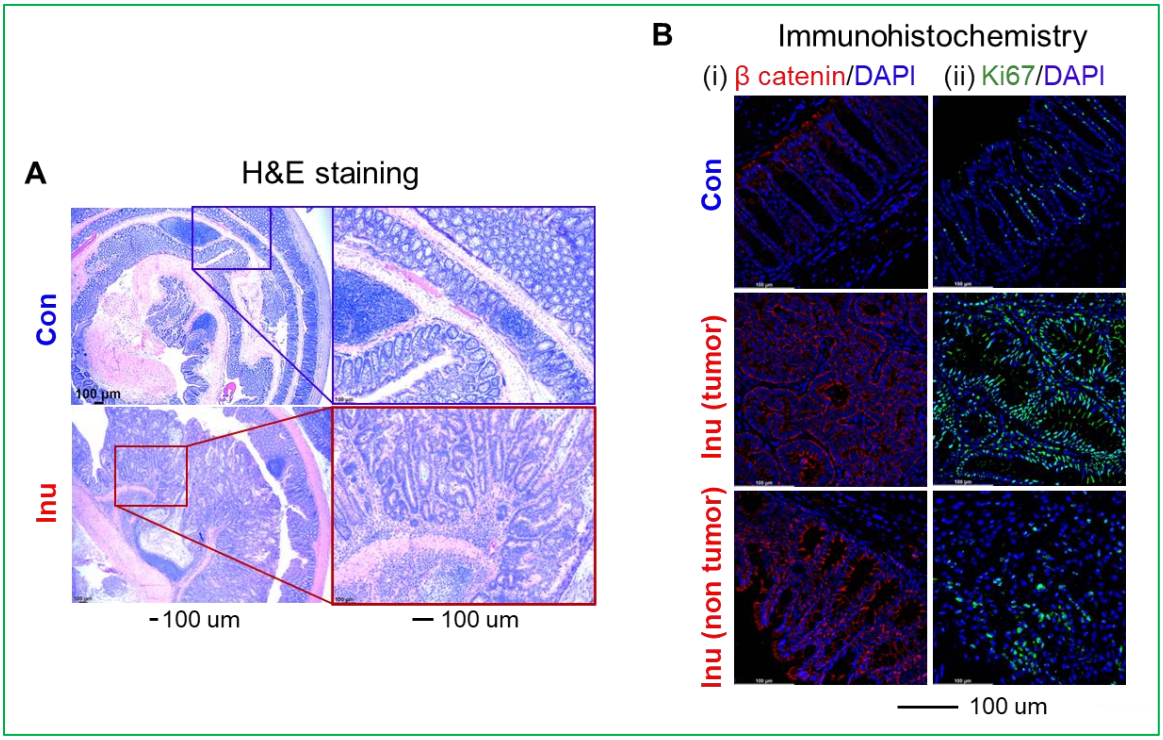

Figure 3 - 5: immune cell infiltration, Wnt/ ß-catenin activation, and high MKi67 expression was observed in inulin group.

Representative histology of a tumor in the distal colon resulting from AOM/DSS administration. **A**. H&E staining of colon sections at 16 (left) and 50 (right) magnification factors (red arrow indicating the immune cell infiltration), **B**. immunohistochemistry staining of ß-catenin (red) and Ki67 (green) at 400 magnification factors.

Inulin-Fed Mice Displayed Upregulated Proinflammatory Cytokines and Chemokines in AOM/DSS Colorectal Cancer Model

Chronic colonic inflammation is an important contributor to colon tumorigenesis. Therefore, we analyzed the levels of inflammation markers in colon tissue derived from control and inulin-fed mice. IL-1β, a member of the IL-1 family, was significantly elevated in the inulin group (Fig. 3-6A). Since the H&E staining indicated the infiltrations of immune cells, CXCL1 was also measured due to its chemoattractant property to neutrophils in actively inflamed colons[197], and found to be elevated in the inulin-fed mice (Fig. 3-6B). In addition, serum CXCL1 was also increased in this group of animals, indicating a systemically hyperactive innate immune response compared to the control group (Fig. 3-6C). Collectively, the data suggest that the heightened colon tumorigenesis in the inulin-fed group positively correlates with elevated levels of the pro-inflammatory cytokine IL-1β and the chemokine CXCL1.

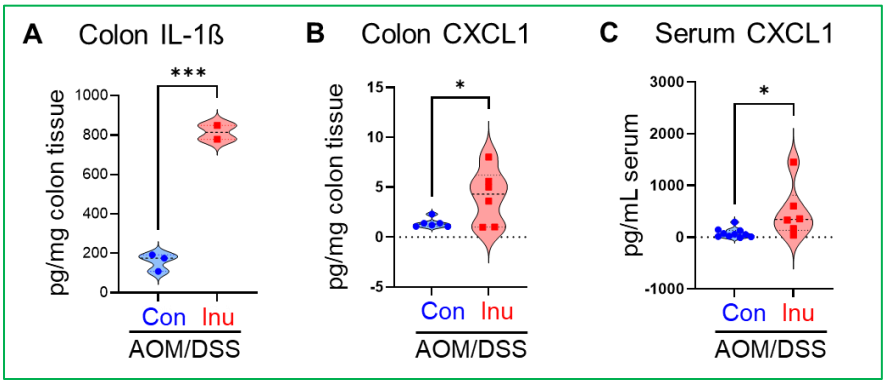

Figure 3 - 6: Inulin-fed mice displayed increased levels of proinflammatory cytokines and chemokines

Violin plots represent colonic level of **A**. interleukin-1ß (IL-1ß) and **B**. chemokine (C-X-C motif) ligand 1 (CXCL1), and **C**. serum level of CXCL1. Values are presented as mean ±SEM. $p<0.05$ as *; <0.01 as **; <0.001 as ***.

Partially Hydrolyzed Guar Gum-Fed Mice also Exhibited Severe Colitis and High Colon Tumorigenesis

After characterizing the effect of inulin on DSS-induced colitis and on colon tumorigenesis, we further tested another FDF, PHGG, which is also widely used as an additive in processed food by food industries. After four weeks on Con or PHGG diets, we implemented a similar experimental design to instigate colitis and colon cancer in these animals (Fig. 3-7A). Upon DSS administration, a significant reduction in body weight, and a trend of decrease in colon length and higher spleen weight were observed in the PHGG group than in the Con mice (Fig. 3-7B-H). Moreover, PHGG-fed group showed elevated levels of inflammation markers in both colon and serum, suggesting exacerbation of colonic inflammation in response to PHGG feeding. However, no difference in body weight and markers of inflammation was found in Con or PHGG groups at basal level.

Based on the previous observation that the inulin-fed group showed heightened colonic inflammation positively associated with an increased incidence of CRC, we next tested the effect of PHGG on colitis-associated CRC. Surprisingly, the PHGG-fed group experienced a dramatically higher mortality rate than the one previously observed in the inulin group; specifically, 5 out of 7 mice (~70% mortality) died after the first cycle of DSS (Fig. 8B). No mortality was observed in the control group (Fig. 8B), which reinforced our previous observation and the notion that the FDF-fed mice exhibited poor recovery from DSS-induced injury. The two survived mice in the PHGG-fed group exhibited low body weight throughout DSS/water cycles, and an extensive colorectal tumorigenesis (Fig. 8C). Moreover, spleen weight and serum proinflammatory cytokines were also elevated in PHGG-fed mice compared to the control group (Fig. 3-8F-H). The histological analysis revealed immune cell infiltration and tumor growth in the PHGG group (Fig. 3-8I). Next, we performed immunostaining for Ki67 and β-catenin to characterize the colon tumors. Relative to control, an increased colonic expression of Ki67 and β-catenin in PHGG-fed mice (Fig. 8J) suggested heightened proliferation and activation of Wnt/β-catenin signaling in these mice.

Collectively, we found that PHGG consumption predisposed mice to colonic inflammation and colitis-associated CRC development.

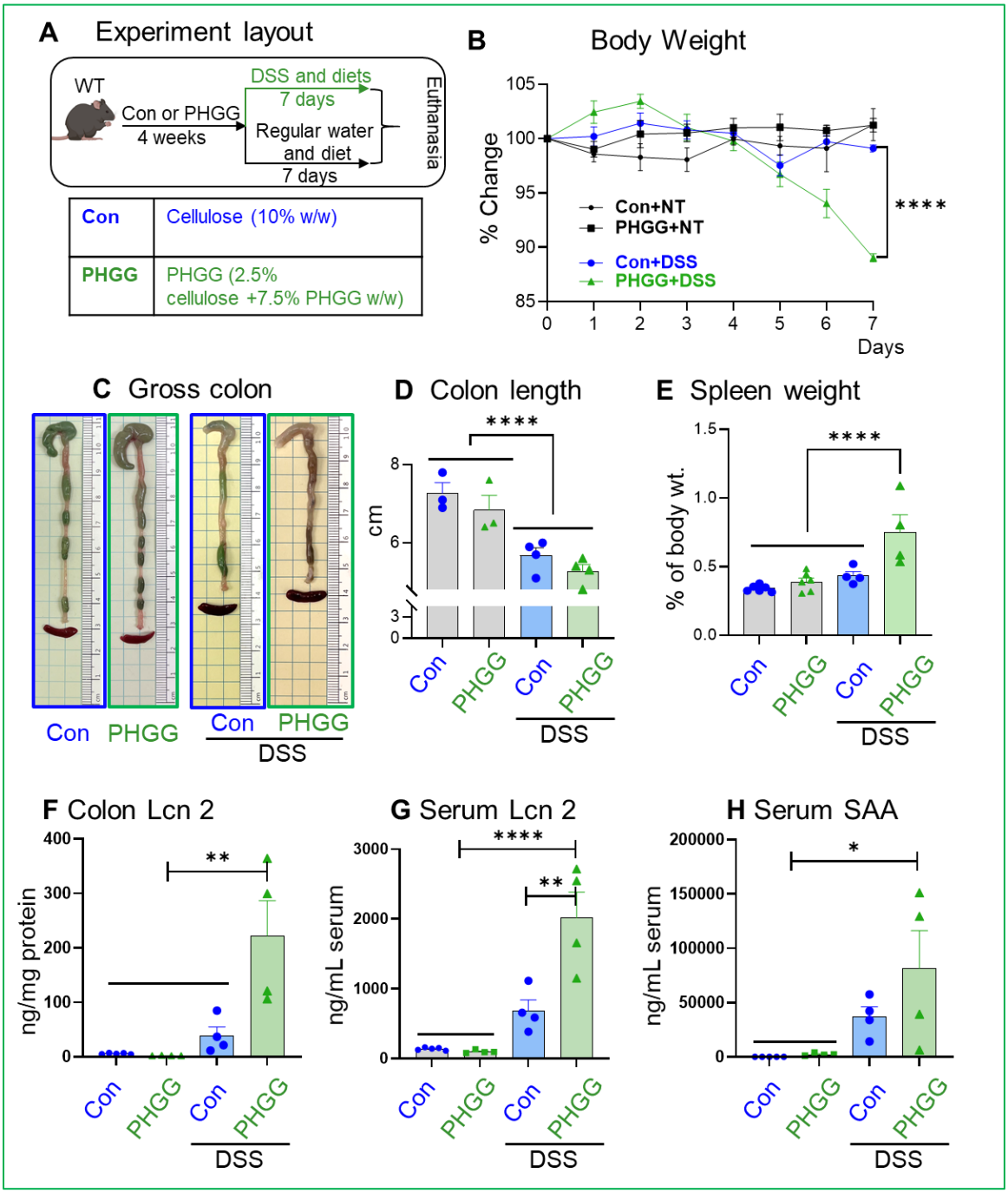

Figure 3 - 7: PHGG-fed mice exhibited severe colitis upon DSS administration.

A. experiment layout to study the impact of PHGG consumption on DSS-induced colitis, **B**. body weight change in percent of initial body weight on the same day of starting DSS, **C**. representative gross colon pictures, **D**. colon length in cm, **E**. spleen weight as percentage of whole-body weight on euthanasia day, **F**. colon Lcn2, **G**. serum Lcn2, **H**. serum SAA. Values are presented as mean ±SEM. P value <0.05 as *; <0.01 as **; <0.001 as ***.

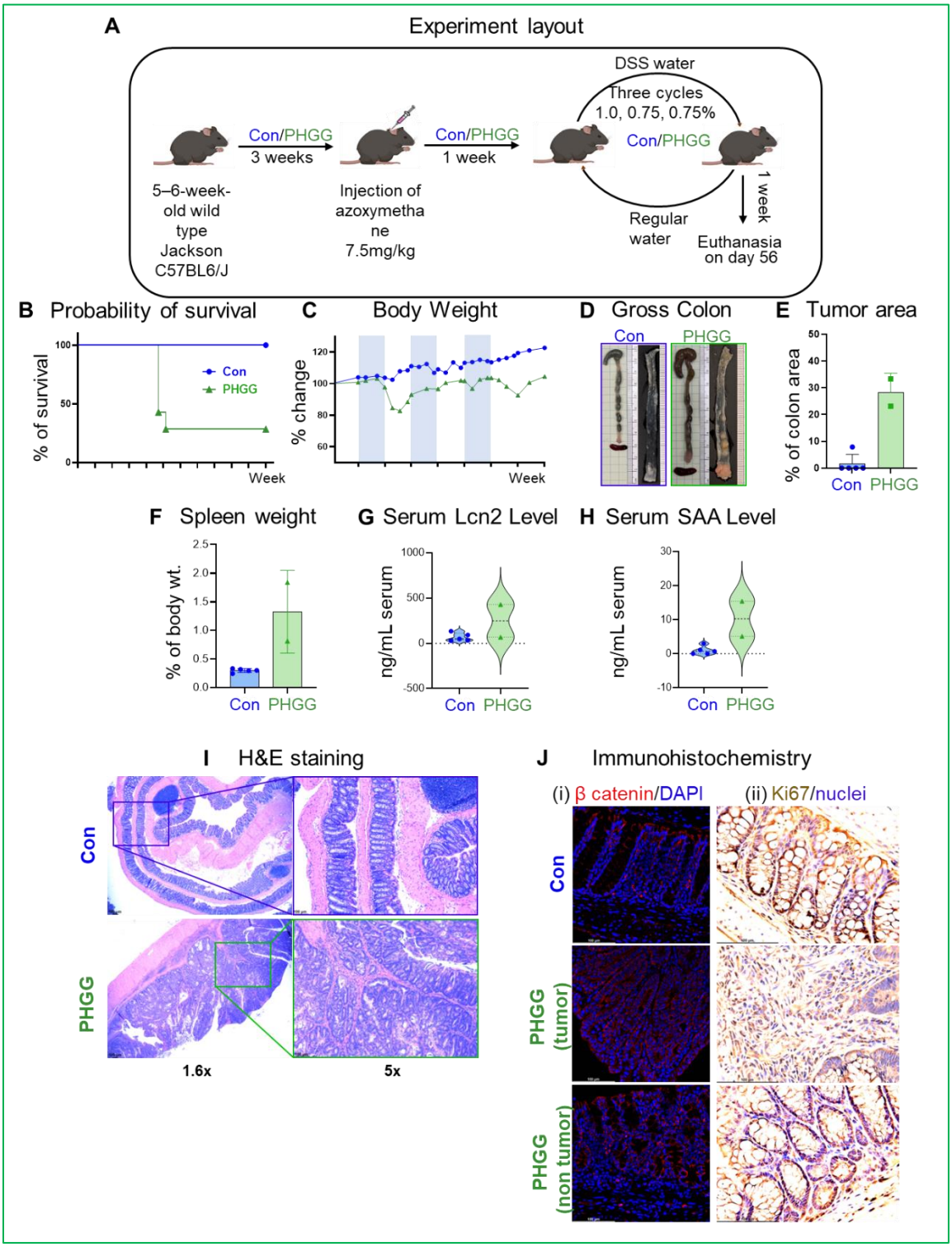

Figure 3 - 8: Similar to inulin, PHGG promoted CRC development in the AOM/DSS model

A. experiment timeline, B. probability of survival from AOM injection to euthanasia, C. change of body weight in percent of the initial body weight, D. representative pictures of gross colon and longitudinally opened colon displaying tumor load, E. tumor area in percentage of the whole colon area, F. spleen weight in percent of body weight, G. serum Lcn2 concentration, H. serum amyloid A concentration, I-J representative histology of tumor in distal colon from Con and PHGG groups, I. H&E staining of colon sections, J. immunohistochemical staining for ß-catenin (red) and Ki67 (brown). Values are presented as mean ±SEM. P value <0.05 as *; <0.01 as **; <0.001 as ***

Discussion

This study investigated the effect of FDFs on colitis progression, mucosal healing, and colitis-associated CRC development. We found two structurally distinct FDFs, inulin, and PHGG, predisposed mice to colonic inflammation and delayed mucosal healing. Approximately ~50% of the inulin-fed and ~70% of the PHGG-fed group succumbed after the first cycle of DSS in the colitis-associated CRC model (AOM/DSS), showing that these FDFs impaired recoveries from DSS-induced injury by decelerating mucosal healing. Notably, inulin and PHGG-fed groups exhibited extensive colon tumor load, whereas the control group had minimal apparent colon tumors. More importantly, inulin or PHGG consumption did not alter the markers of colonic inflammation in the healthy mice but exacerbated epithelial injury-induced colitis and exhibited extensive colorectal tumorigenesis upon co-administration of mutagenic substance (AOM) with DSS.

IBD is a significant risk factor for the development of colorectal tumorigenesis. As the third most prevalent malignancy, the incidence rates of both IBD and CRC are markedly higher in high-income countries and also are increasing in developing countries [198, 199]. Most CRCs are observed in adults after 50 years old in the United States, but alarmingly, early-onset CRC in youth is emerging[200]. A global epidemiology study reported that in 2020 CRC accounted for 10% of global cancer incidence and 9.4% of cancer deaths. Moreover, based on the projection of aging, population growth, and human development, the authors predicted that the global number of new CRC cases would reach approximately 3.2 million in 2040[198]. Such escalating trends of CRC, particularly among younger generations, pose a heavy financial burden and a substantial public health challenge and signify the need for the prevention of risk factors associated with CRC and novel strategies for CRC management, including healthy diet choices.

FDFs found naturally in fruits and vegetables benefit intestinal health, including maintaining microbial diversity in healthy individuals[201, 202]. Based on this long-standing notion that naturally occurring dietary fibers offer numerous beneficial effects on gut health, food companies advocate consuming processed FDFs such as inulin and PHGG regularly. However, it remains unclear whether such highly processed FDFs hold physiological effects similar to their naturally occurring counterparts. Studies have yielded conflicting evidence on whether refined FDFs attenuate or deteriorate IBD development[11, 203, 204]. For example, fermentable oligosaccharides, disaccharides, monosaccharides, and polyols (FODMAPs) were shown to exacerbate clinical complications in a subgroup of IBD patients[205, 206]. Such inconsistencies observed in human and animal studies examining the effect of FDFs on IBD are reminiscent of human IBD, where the intake of DF is linked with reduced flares in patients with Crohn's disease but not in patients with UC[207]. The effects of FDFs in the context of IBD-associated colorectal cancer are also poorly understood. DFs naturally present in whole grains but not in other sources, such as in packaged food in processed forms, were found protective against CRC in the NIH-AARP Diet and Health Study cohort[8]. The beneficial potential of dietary fibers is primarily deduced from studies involving healthy individuals; we propose that the response of FDFs on host intestinal health is impacted by pre-existing dysbiosis, as in the inflamed gut. In support, we found no protection with isolated FDFs inulin and PHGG in the present study. In fact, both FDFs worsened colonic inflammation and augmented colon tumorigenesis.

An intricate relationship between FDFs, gut bacteria, and intestinal immune cells helps to fine-tune intestinal immune response and prevent chronic intestinal inflammation. We theorize that regular consumption of an ultra-processed single type of DF affects such complex networks of interactions detrimentally by promoting the growth of a selective group of bacteria. For example, supplementation of inulin preferentially increases the proportion of Bifidobacterium [208]. A recent study by Wei et al. [145] found that *Bifidobacterium* was significantly increased in the colon biopsy

specimens of active UC patients compared to those in healthy controls. In this study, the authors suggested a cautious use of probiotics containing *Bifidobacterium* in IBD patients during the active phase of the disease as such a disproportionate increase of mucosal *Bifidobacterium* could contribute to the IBD flare-up. Similarly, we found that both FDFs, inulin and PHGG, potentiated DSS-induced colitis, delayed mucosal healing, and promoted colonic fibrosis. Strikingly, more than ~50% of inulin or PHGG -fed mice succumbed to death in AOM/DSS treatment group due to severe illness and impaired recovery. Notably, no death or a worsened colitis phenotype (abrupt loss in body weight and severe bloody diarrhea) was observed in the control (non-fermentable fiber) group. More importantly, all the survived mice from the inulin or PHGG groups, but not in the control group, exhibited extensive colorectal tumorigenesis. We surmise that in contrast to inulin or PHGG present in whole fruits and foods, processed forms of inulin or PHGG fuel selective bacterial colonization (e.g., expansion of *Gammaproteobacteria*, *Bifidobacterium,* and secondary bile acid producers) that exaggerates colonic inflammation and increases colon tumorigenesis in our study. However, these speculations need to be tested through detailed characterization of gut microbiota and their metabolites in samples derived from both healthy and inflamed gut. Another possible mechanism for why FDFs potentiated inflammation is the presence of a high amount of unfermented fiber in the inflamed gut. This inference is supported by an elegant study that showed that patients with IBD have a high amount of unfermented β-glucan, and this undigested β-glucan contributes to the exacerbation of colon inflammation[209].

Summary and Conclusion

Colorectal cancer (CRC) is the third most prevalent cancer worldwide. About a 3-5-fold increase in the risk of developing CRC is estimated in patients with IBD[210]. Since gut microbiota plays a critical role in initiating IBD and CRC, dietary habits and dietary components are studied to explore the mechanisms of how they can affect the gut microbiome and then interfere with IBD and CRC. The gut bacteria can ferment the DF, but minimal digestion by humans, so it is considered the feast of the gut microbiome. Inulin, as a widely present fermentable DF, has been assessed on its impact on IBD. To date, several carcinogens are identified to be produced by the gut microbiomes, such as *Bacteroides fragilis* toxins, to set the host IECs at the initiation stage of CRC [211]. The chronic inflammation further creates a tumorigenesis-favored environment, and a high rate of IEC regeneration and proliferation helps the immortalized cells expand their colony and, finally, colorectal tumor progression.

In the present study, we tested how colitis and tumorigenesis are impacted by the addition of isolated FDFs, inulin, or PHGG, to the diet in the mouse model. Our study suggests that consuming an isolated processed FDF may predispose individuals to colonic inflammation, delay mucosal healing, and promote colon tumorigenesis. Therefore, adding highly processed FDFs to packaged foods or recommending FDF regular consumption as supplements should be approached with caution as it may increase the risk of colitis and colitis-associated colon cancer in the long run.

Study limitations and Future Plans

The limitation of the study includes a small sample size, specifically in the PHGG-intervention group, which exhibited high mortality due to poor recovery from DSS-induced epithelial injury. The impact of sex differences (disease phenotype in male vs. female mice) in the disease was also not evaluated extensively. We plan to perform future studies using male and female mice to circumvent these limitations.

The AOM/DSS model indeed enabled us to evaluate the effect of DFs on colitis-associated CRC. Colon tumorigenesis in AOM/DSS model is inflammation dependent. Since both inulin and PHGG exacerbated colonic inflammation, so the overserved extensive tumorigenesis in both groups was possibly due to heightened colonic inflammation. Such inflammation dependency of the AOM/DSS model limits us from identifying whether FDF-gut microbiota axis-derived products have an independent effect on colon cancer development. To surmount this limitation, we plan to employ genetically susceptible strains of mice ($Apc^{Min/+}$ mice) to study the impact of FDFs on intestinal neoplasia in our future studies. Furthermore, although this study characterized the effects of DFs on the inflamed intestine, it did not elucidate the mechanism behind the mucosal healing process and the CRC initiation and progression. Therefore, in the future, we plan to characterize the impact of fiber-related gut microbiota-derived metabolites on colon cancer development. Lastly, in this study, mice were exclusively exposed to a single type of refined FDFs with a marginally higher amount of recommended FDF for human consumption. Thus, our further studies will use lower doses of fiber intervention to match human dietary intake.

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Appendix: supplementary figures

Table appendix - 1: Diet Composition

Diet Formulas	Cellulose containing diet (Con)		Inulin containing diet (Inulin)		PHGG containing diet (PHGG)	
Product #	D12081401		D12081402		D22030208	
	gm%	kcal%	gm%	kcal%	gm%	kcal%
Protein	18.5	20	19	20	19	20
Carbohydrate	61.8	65	67.8	65	60.8	63
Fat	6.4	15	6.5	15	6.5	15
Total		100		100		100
Kcal/gm		3.78		3.88		3.88
Ingredient	g	Kcal	g	Kcal	g	Kcal
Casein, 30 Mesh	200	800	200	800	200	800
L-cysteine	3	12	3	12	3	12
Corn starch	409	1636	381	1524	381	1524
Maltodextrin 10	110	440	110	440	110	440
Sucrose	150	600	150	600	150	600
Cellulose, BW200	100	0	25	0	25	0
Inulin (Orafti® HP)	0	0	75	113	0	0
PHGG	0	0	0	0	75	113
Soybean oil	70	630	70	630	70	630
Lard	0	0	0	0	0	0
Mineral Mix S10026	10	0	10	0	10	0
DiCalcium PO4	13	0	13	0	13	0
Calcium Carbonate	5.5	0	5.5	0	5.5	0
Potassium Citrate	16.5	0	16.5	0	16.5	0
Vitamin Mix V10001	10	40	10	40	10	40
Choline Bitartrate	2	0	2	0	2	0

FD&C Red dye #40	0	0	0.05	0	0	0
FD&C Blue dye #1	0.05	0	0	0	0.025	0
FD&C Yellow dye#5	0	0	0	0	0.025	0

Table appendix - 2: Rubric to Assess Feces Condition in DSS-Colitis Models

Stool frequency	Normal = 0 1-2 stool/day more than normal = 1 3-4 stools/day more than normal = 2 >4 stools/day more than normal = 3
Rectal bleeding	None = 0 Visible blood with stool less than half the time = 1 Visible blood with stool half of the time or more = 2 Passing blood alone = 3

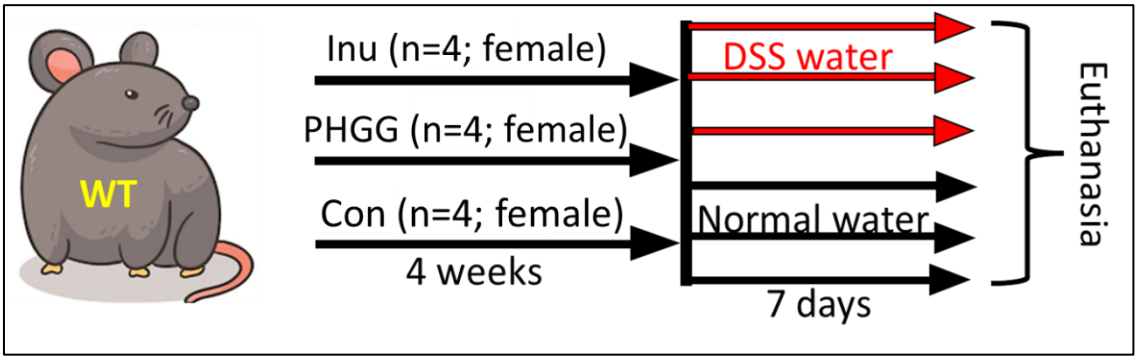

Figure Appendix- 1: Experimental timeline of DSS-induced colitis

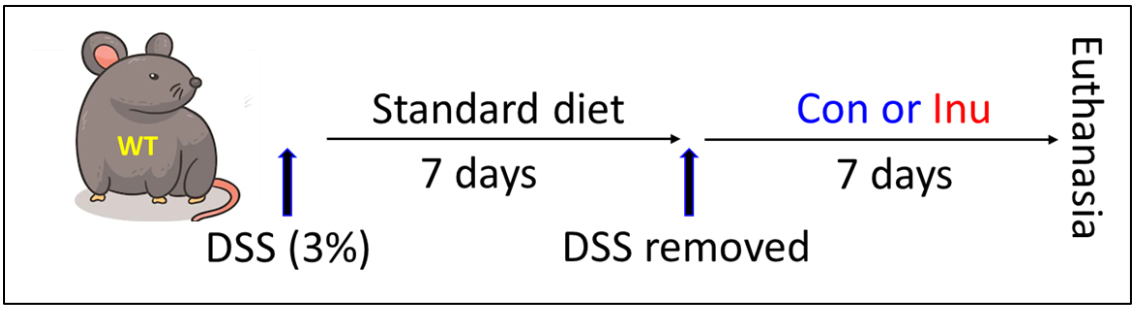

Figure Appendix- 2: Experimental timeline for mucosal healing experiment. Six male mice were included in each group

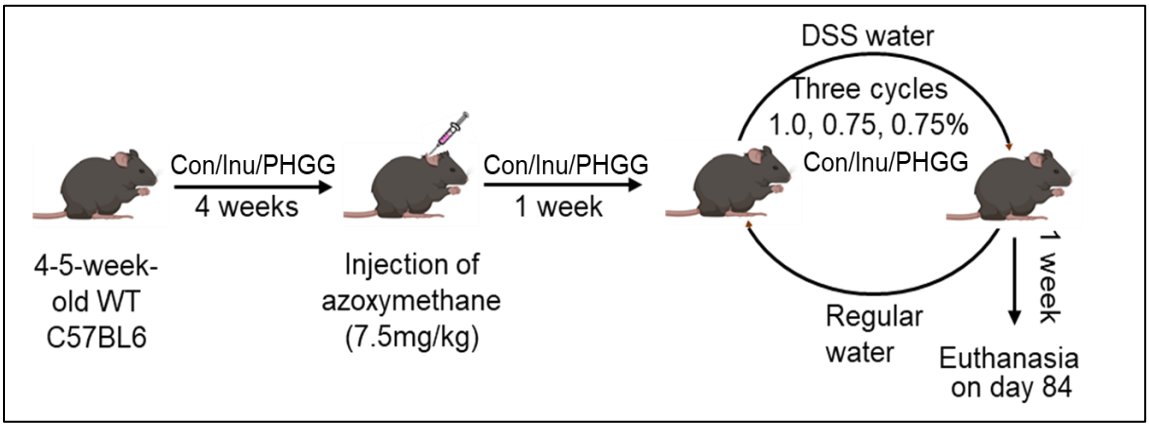

Figure Appendix- 3: Experimental timeline for IBD-associated CRC experiment. Survived mice from each group (ncon=10; ninu=6; nPHGG=2) were analyzed in this experiment.