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DIETARY PHYTOCHEMICALS AS POTENTIAL INTERVENTIONS FOR INFLAMMATORY BOWEL DISEASE

A Dissertation in
Food Science

by
Zachary T. Bitzer

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The dissertation of Zachary T. Bitzer was reviewed and approved* by the following:

Joshua D. Lambert  
Associate Professor of Food Science  
Dissertation Co-Advisor  
Co-Chair of Committee

Ryan J. Elias  
Associate Professor of Food Science  
Dissertation Co-Advisor  
Co-Chair of Committee

Andrew D. Patterson  
Assistant Professor of Molecular Toxicology

Robert F. Roberts  
Professor  
Head of the Department of Food Science

*Signatures are on file in the Graduate School
ABSTRACT

Inflammatory bowel disease (IBD) afflicts nearly 2.2 million people worldwide. IBD is composed of two main groups, ulcerative colitis (UC) and Crohn’s disease. Nearly 20% of those who suffer from IBD will develop colorectal cancer and 50% will die as a result. In addition to the decrease in quality of life and increases in colorectal cancer risk, IBD also presents a significant economic burden. The total economic burden of ulcerative colitis (UC) and Crohn’s disease in the United States is estimated to be US$19 – 30 billion annually. IBD patients suffer from increased inflammatory gene expression, cytokine production, oxidative stress, and decreased gut barrier function. This decrease in gut barrier function is a result of modulation of tight junction proteins responsible for modulating paracellular transport in the epithelium. Decreased gut barrier function allows for intestinal endotoxins, such as lipopolysaccharide (LPS), to pass from the lumina into the systemic circulation causing further inflammation. Dietary intervention is of particular interest when studying IBD as many phytochemicals have biological activity and some have been shown to decrease inflammation. Though some may are not very bioavailable, they may have activity through direct interaction with epithelial cells. The overall goal of this project was to investigate the beneficial effects of dietary supplementation with dietary phytochemicals on dextran sulfate sodium (DSS)-induced colonic inflammation and gastrointestinal permeability. The specific objectives were: a) to evaluate the effect of epigallocatechin-3-gallate (EGCG) on markers of inflammation, oxidative stress, gut barrier function, and nutrition in mice with DSS-induced ulcerative colitis and in DSS-treated Caco-2 cell monolayers in culture, b)
evaluate the effect of an isoflavone-free soy protein concentrate on markers of inflammation, oxidative stress, gut barrier function, and inflammasome formation in mice with DSS-induced ulcerative colitis and in DSS-treated Caco-2 cell monolayers in culture.

EGCG, the most abundant green tea polyphenol, has been shown to inhibit oxidative stress in animal and cell studies. We examined the effect of EGCG on DSS-induced permeability in Caco-2 cell monolayers and found that EGCG treatment significantly reduced permeability. We examined the anti-inflammatory, anti-nutritional, and intestinal permeability effects of EGCG in the DSS-induced mouse model of ulcerative colitis. Mice received DSS (1.5% w/v) for 7 days to induce ulcerative colitis and then received 0.32% EGCG as their sole source of drinking water for an additional 3 days. Colonic levels of the inflammatory markers, interleukin (IL)-1β, IL-6, and tumor necrosis factor alpha (TNF-α), were increased in DSS-treated mice, but the effect was mitigated as a result of EGCG administration. Gut barrier improvements were seen in the mice that received EGCG intervention as measured by a multi-sugar permeability assay. These results indicate that EGCG decreases the inflammatory response of the intestine and modulates intestinal permeability. In spite of these anti-inflammatory effects, we observed that EGCG decreased feeding efficiency and enhanced body weight loss compared to DSS-treated control mice. These changes were related to increases in fecal proteins and lipids and were observed in EGCG-treated mice suggesting that EGCG exerted anti-nutritional effects and indicating a potential dose-limiting toxicity.

Soy (*Glycine max*), a widely consumed dietary component, has been reported to have beneficial effects against a number of chronic diseases. We examined the ability of
soy protein concentrate (SPC) to suppress inflammation and oxidative stress, and improve gut barrier function both in vivo and in a cell culture model. In vitro, SPC exhibited radical scavenging activity. This activity was further supported by an in vivo experiment where SPC protected Caco-2 human colon cells from H₂O₂-induced cytotoxicity. DSS-induced increases in Caco-2 monolayer permeability were also decreased as a result of SPC treatment. In a DSS-induced ulcerative colitis CF-1 mouse model, mice received 1.5% DSS for 7 days and consumed dietary SPC (6% or 12%) throughout the course of the study. Dietary SPC reduced colonic cytokine levels of IL-1β, IL-6, and monocyte chemotactic protein-1 (MCP-1). Gene expression of IL-1β, EGF-like Module-containing Mucin-like Hormone Receptor-Like 1 (EMR-1), nucleotide-binding oligomerization domain receptor-3 (NLRP3), and toll-like receptor 4 (TLR-4) were also observed. SPC decreased Caspase-1 activity, suggesting inhibition of inflammasome formation. In the mice, protein levels of glucagon-like peptide 2 (GLP-2) increased and mRNA expression of tight junction proteins, claudin-1 and occludin, tended to normalize as compared to the negative control. Our results suggest that SPC attenuates colonic inflammation in part by suppressing oxidative stress and intestinal permeability.

Collectively, we have demonstrated that dietary treatment with EGCG and SPC can reduce inflammation and improve gut barrier function in DSS-induced ulcerative colitis in mice. These results are supported by studies of antioxidant and epithelial protective effects in cell culture. While the exact mechanism of each of the treatments remains to be investigated further, it suggests that dietary intervention with plant based
phytochemicals may be a viable route to reducing inflammation and improving intestinal barrier function in the context of IBD.
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Chapter 1
Literature Review

1.1 Colorectal Cancer

1.1.1 Incidence, Risk Factors, and Financial Impact

Colorectal cancer is the second leading cause of cancer-related deaths in the United States and Europe.[1] It is estimated that nearly 1.4 million people worldwide are affected by colorectal cancer.[2] The National Cancer Institute estimates that 136,830 men and women will be diagnosed with colorectal cancer in the United States in 2014 and that 50,310 will die.[3] Colorectal cancer accounts for approximately 10% of all cancer deaths.[2]

Colorectal cancer risk is strongly influenced by genetics. Incidence of colorectal cancer development among those with a first-degree family member with colon cancer is 20–30%.[4] Genetic conditions like hereditary non-polyposis colorectal cancer or familial adenomatous polyposis (FAP) are the two main forms.[5] Subjects with FAP have a nearly 100% risk of developing colorectal cancer. Individuals with inflammatory bowel diseases such as Crohn’s disease and ulcerative colitis are also at a significantly higher risk of developing colorectal cancer. Inflammatory bowel disease is estimated to affect nearly 2.2 million people worldwide and those suffering from it are nearly 3 times more likely to develop colorectal cancer.[6, 7]

Sex, age, and race are also factors affecting colorectal cancer risk. Men are more prone to developing colorectal cancer than women. The risk of developing colorectal cancer increases with age with the majority of all colorectal cancer cases diagnosed after age 50. Incidence of colorectal cancer are higher in African American populations and lower in Asian American
populations compared to non-Hispanic white populations.[3] These racial disparities often are the result of an underlying historical or socioeconomic problem such as access to high-quality healthcare.[8]

Studies of immigrants in numerous countries have shown that, after accounting for hereditary factors, their rate of colorectal cancer incidence become the same as the country to which they have immigrated.[9] This demonstrates a role for environmental factors in the development of colorectal cancer. Smoking, obesity, physical inactivity, excessive alcohol consumption, and diet have all been identified as risk factors for the development of colorectal cancer.[10, 11] Increased risks for colorectal cancer are also present for those who have received abdominal surgery, radiotherapy, or have had occupational exposure to asbestos.[5] Most of these environmental factors are avoidable or can be modified in order to lower colon cancer risk.

Many epidemiological studies have found correlations between specific foods and colorectal cancer risk. Studies have shown that diets high in cholesterol and diets low in folic acid and vitamin B6 are associated with a high risk of colon cancer.[12, 13] Increased consumption of brown rice, cooked green vegetables, dairy products, dried fruit, calcium, nuts, and decreased consumption of red meat correlate with a lower risk of colorectal polyp formation.[14-16]

In addition to premature death due to colon cancer, it is estimated that nearly US$14.14 billion per year is spent on colorectal cancer treatment in the US.[17] This includes doctor visits, surgeries, medication, and continued screenings until death. This number is expected to increase to over US$17.41 billion by the year 2020 due to the aging population.[17]
1.1.2 Pathogenesis, Progression, and Stages

Cancer progression is typically summarized in a three-stage model: initiation, promotion, and progression. During the initiation phase, mutations in DNA occur resulting in the inactivation of tumor-suppressor genes and the activation of oncogenes.[18] If these damaged cells are not repaired, they will begin to replicate in the promotion phase. This stage is still reversible as these proliferating cells can be eliminated by apoptosis or programmed cell death. If these damaged proliferating cells are not eliminated, the third stage, progression, will begin. During this stage, tumor size will increase, tumor cells gain an invasive phenotype, and eventually metastasis to other locations can occur.[19, 20]

Colorectal cancers typically start with the formation of polyps in the epithelium. The epithelium is composed of a roughly $10^7$ crypts, and each crypt contains thousands of differentiated cells and between 1-10 stem cells. These stem cells are found at the bottom of the crypts and divide asymmetrically allowing the differentiated cells to travel to the tops of the crypts (Figure 1-1). Each division represents a potential cancer risk because of mutational events that can occur during normal DNA replication.[21] Adenomatous polyposis coli (APC) is a tumor suppressor gene that is inactivated in almost 85% of all colorectal cases while β-catenin gene mutations account for half of the remaining cases.[21] Silencing of APC also disrupts β-catenin protein regulation. In a crypt with an APC-mutant cell, dysplasia, an expansion of immature cells and decrease in mature epithelial cells, can occur resulting in a polyp forming (Figure 1-1). The formation of a larger polyp requires further mutations of oncogenes such as Kirsten rat sarcoma viral oncogene homolog (KRAS) or B-Raf proto-oncogene, serine/threonine kinase (BRAF). These larger polyps still require further mutations in order to become carcinomas. Modifications to cell monitoring and cell death pathways, such as TNF-β and p53,
result in these cells not undergoing apoptosis and continuing to grow until they begin to metastasize (Figure 1-1).

All polyps begin as non-cancerous growths but a subpopulation can develop into cancer.[5] Polyp formation increases over time and is why frequent screening is recommended for those over the age of 40 years old. Polyps are classified into three categories: inflammatory, hyperplastic, or adenomatous. The likelihood of cancer progression increases with each respective category. Adenocarcinomas and adenomatous polyps account for the majority of colorectal cancers.[2] Polyp formation and dysplasia are of paramount importance because of their ability to become cancerous over time. Dysplasia is very common in those with inflammatory bowel disease such as ulcerative colitis or Crohn’s disease due to the chronic inflammatory state of their intestinal tract.[6]
At early stages, the tumor is localized to the epithelium, however, as the tumor continues to grow, it will begin to invade the basal layer of the colon (Figure 1-2). Tumors are typically characterized by the depth and extent of the growth (T), the extent of lymph node spread (N), and if it has metastasized (M). Using these three factors, physicians can determine the stage of a particular cancer. These stages range from no growth beyond the inner lining (0) to metastasis with spread to one or more organs (IV).[5]

In general, 40% of those diagnosed with colorectal cancer will die from the disease.[24] Detection at early stages is important for improving a patient’s chances of survival. For example, the five-year survival rate for people diagnosed when the cancer is at a localized state is 90%. 

Figure 1-1. Genetic instability and polyp formation. Adapted from Humphries et al. [22]

Figure 1-2. Stages of colorectal cancer. Source: National Cancer Institute [23]
However, if diagnosed at a later stage when metastasis to distal organs has occurred, the five-year survival rate drops to 13%. [25] The high rate of survival at the early stages is also why yearly screenings are recommended for those over age of 40.
1.2 Inflammatory Bowel Disease

1.2.1 Prevalence, Symptoms, and Colorectal Cancer Development

Inflammatory bowel disease (IBD), a chronic inflammation of the gastrointestinal tract, affects nearly 2.2 million people globally.\[7\] Nearly 20% of IBD patients will develop colorectal cancer and of those, 50% will die as a result.\[26\] In addition to increases in colorectal cancer risk, IBD causes a dramatic decrease in quality of life as well as a significant economic burden. In the US, the total economic burden of ulcerative colitis and Crohn’s disease is estimated to be US$19-30 billion annually.\[27, 28\]

Crohn and Rosenberg first characterized IBD and its correlation with colorectal cancer in patients in 1925.\[29\] In the ulcerative colitic colon, chronic inflammation causes a series of ulcers or open sores to form that can release blood into the stool. Individuals with IBD suffer from increased inflammatory gene expression, cytokine production, oxidative stress, and decreased gut barrier function.\[30-32\] This chronic inflammatory state can lead to loss of tumor suppressor genes such as APC, the “gatekeeper” gene of the colon.\[5\] Though medications such as anti-inflammatory, immune suppressants, and anti-diarrheal drugs can be used to control the symptoms, those with IBD are still at an increased risk of developing colorectal cancer.

Risk factors for IBD are similar to those of colorectal cancer. As with colorectal cancer, there have been a few susceptible genes, such as interleukin (IL)23R, IL12B, IL18RAP, and tyrosine kinase 2 (TYK2), associated with IBD.\[33, 34\] Interestingly, while genetics may play a role in IBD, a recent study focused on the inheritance of IBD found a rate of concordance between monozygotic twins to be only 27% for Crohn’s disease and 15% for ulcerative colitis.\[35\] Environmental factors, however, appear to have a more significant role. For
example, family member or spouses with shared living environments have similar risk for IBD.\cite{36, 37}

A number of environmental factors such as air pollution and living in an urban environment have been reported to influence IBD risk.\cite{38} While these environmental factors may be out of the control of a patient, other risk factors, such as diet, can be modulated. Other environmental factors studied in epidemiological studies have yielded variable results (Figure 1-3). For example, a number of studies have examined the impact of breast-feeding on IBD risk in offspring. It is hypothesized that breast-feeding provides beneficial gut microflora that prevent inflammation and the progression of IBD. This hypothesis is not supported in other studies that found breast-feeding had no effect.\cite{38} As with colorectal cancer, diet plays a significant role in the development and severity of IBD. While specific therapeutic dietary interventions will be discussed later, it is clear that high carbohydrate and high fat diets are positively associated with IBD.\cite{39} Diets high in linoleic acid, which can increase arachidonic acid levels in adipose tissue, have been linked to ulcerative colitis prevalence.\cite{40, 41}
It should also be noted that while the term IBD encompasses both Crohn’s disease and ulcerative colitis, they do not always respond to the same treatments, nor do they share the same risk factors. For example, smoking increases the risk of Crohn’s disease but decreases the risk of ulcerative colitis.[38] Though not fully understood, it has been proposed that this effect is due to nicotine and modulations in oxidative stress, though further research is needed to fully understand this effect.[42]

IBD is a leading risk factor for colorectal cancer after hereditary nonpolyposis and familial adenomatous polyposis.[2] A 2001 epidemiological study found that patients with IBD were at a greater than 2.5-fold increased risk of developing colon carcinomas compared to the general population.[6]
1.2.2 Inflammation and Oxidative Stress

Inflammation is an important area of concern as it has been directly linked to colorectal cancer risk.[43] Inflammation is an immune response to infection or trauma involving host cells, proteins, and other molecules with the purpose of eliminating the root cause of the injury and initiating the repair process. Leukocytes normally reside in the blood and travel to areas of inflammation where they can act as phagocytes and ingest cellular debris and foreign pathogens. They can also release secretory vesicles that can damage the pathogens. Leukocytes also release inflammatory stimuli that maintain the inflammatory response within the tissue until the insult has subsided. Typically granulocytes mediate acute inflammation while chronic inflammation is mediated by monocytes and lymphocytes.[44]

Chronic inflammation, however, can result in DNA damage, cell death, and cancer promotion (Figure 1-4).[45] Unchecked inflammatory cytokines released by monocytes and reactive oxygen species (ROS) generation will begin to damage DNA bases and cause genomic instability. Proliferation and fixed DNA mutations, if left uncorrected, will promote tumorsgenesis. The duration of chronic inflammation correlates strongly with the development and progression of cancer.[46] Genomic instability as a result of chronic inflammation and ROS can cause a loss of function in tumor suppressor genes such as APC and TP53, resulting in carcinoma development.[43] Epidemiological and laboratory studies all support the hypothesis that chronic inflammation contributes largely to increased colorectal cancer development.[47] For example, significant associations between histological inflammatory scores and advanced neoplasia were shown in a 2007 cohort study yielding a hazard risk of 3.0.[48] In another case-controlled study, an odds ratio of 5.1 was found between histological inflammatory scores and colorectal neoplasia.[49] Experimentally, inflammation and colon cancer progression correlates with a shortening of the colon and enlarging of the spleen in mice.[45]
1.2.2.1 Oxidative Stress

Oxidative stress has also been strongly linked to carcinogenesis due to its ability to damage DNA. This stress comes from free radicals, which are molecules that contain an unpaired valence electron and are extremely reactive. Free radical ROS contain at least one oxygen with an unpaired valence electron such as superoxide and hydroxyl radicals.\[50\]

ROS have been shown to play a role in a number of important physiological processes as secondary messengers and as immune effector molecules.\[50-52\] Usually, levels of ROS within a healthy cell are very low and are modulated through the use of enzymes and radical scavenging molecules. Enzymes, such as superoxide dismutase (SOD) and catalase, and free radical scavenging molecules, like reduced glutathione and antioxidant molecules, help to modulate free radicals and prevent damage.\[46\]
When ROS formation exceeds the elimination rate of the cell’s baseline processes, oxidative stress will occur (**Figure 1-5**). During these stages of oxidative stress, ROS can oxidize proteins, DNA, and lipids thus severely impacting the cell’s normal functions. Free radical induced DNA damage includes strand breaks, adducts, base dimerization, base modifications, and crosslinking.[53] This type of damage can lead to cellular initiation. If this damage is not repaired or additional oxidative stress has caused damage to the repair mechanisms, the cell will then continue on in the promotion phase. If the ROS levels are sufficiently high, they can act as a promoting agent by killing off cells and allowing the proliferation of initiated cells. Aside from the direct effects of ROS, oxidative stress can also activate other carcinogens that can damage the DNA.[46, 51]

**Figure 1-5.** The effects of ROS on carcinogenesis. Source: Valko *et al.*[20]

All cells produce free radicals during normal metabolism and also as secondary messenger molecules; however, inflammatory cells such as macrophages produce them in much
higher concentrations to serve as mediators of immune response.[54] This production of ROS and reactive nitrogen species (RNS) will typically occur in response to inflammatory cytokines and chemokines, or immunogenic stimuli.[53] ROS and RNS can recruit other inflammatory cells and lead to increased levels of oxidative stress. In an acute phase this could be beneficial as excess free radicals can cause damaged or foreign cells to be killed through apoptosis. For chronic inflammation, a constant buildup of free radicals will increase the chances of unrepaired DNA damage and carcinogenesis.[32, 55]

1.2.2.2 Cytokines

Macrophages are an essential part of maintaining the body’s immunity but also play a large role in maintaining tissue homeostasis.[56] Macrophages start out as monocytes derived from the bone marrow where they will eventually enter the blood stream and begin to differentiate into one of two main types: highly microbicidal (M1) and immunosuppressive (M2a, M2b, and M2c).[44]

This differentiation allows the macrophage to respond to specific foreign or inflammatory stimuli and secrete cytokines and chemokines that serve as immune signaling molecules. Over 100 cytokines and chemokines have been identified and range in function throughout the body from local and systemic inflammation regulation to metabolism and cellular proliferation.[57] Cytokines in particular are mainly responsible for regulating inflammation and the immune response. These cytokines can be classified by their area of action: acting within the area of its release (paracrine), acting on the cell that secretes it (autocrine), and acting on remote regions (endocrine).[44]

The formation of cytokines starts with pattern recognition receptors (PRRs) on the surface of the macrophage. The four major PPR classes involved in IBD are: nucleotide-binding
domain and leucine-rich repeat-containing (NLR) family, Toll-like receptors (TLRs), RIGG-I-like Helicase receptors, and C-type Lectin receptors.[58] PRRs can directly or indirectly modulate inflammatory signaling cascades and regulate cell proliferation, survival, remodeling, and repair, and can lead to production of ROS.[59] These receptor classes are responsible for detecting pathogens, damage, and stress through the recognition of pathogen-associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs).[60]

Each cytokine is detected by specific receptors located on the cell surface that set off a signaling cascade within the cell when the cytokine is detected. For example, TLR4 is able to bind to lipopolysaccharides (LPS), an endotoxin derived from the outer membrane of Gram-negative bacteria. LPS initiates recruitment of Mal and MyD88 adaptors to TLR4 where they serve as the scaffold for interleukin-1 receptor-associated kinases (IRAKs). IRAK1 then activates TRAF6, by an unknown mechanism, which links itself to K63-linked polyubiquitin. This triggers a signaling cascade that activates the inhibitor of nuclear factor kappa-B kinase subunit beta (IKK-β).[61] IKK-β phosphorylates nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα) resulting in its ultimate proteasomal degradation thus allowing the p65-p50 NFκB dimer to move into the nucleus and initiate transcription of inflammatory cytokines (Figure 1-6). [19, 61, 62]
Expression of NFκB has also been shown to influence the progression of cancer through its control of cell-cycle progression, proliferation, differentiation, and apoptosis.\[63\] This is thought to be due to inflammatory cytokines released by NFκB indirectly triggering ROS and eventually a loss of APC and p53 as a result of CD24+ cell-derived cytokines (Figure 1-7).\[64\]
Inflammatory cytokines are also produced by endothelial cells, activated lymphocytes, and fibroblasts mostly as a result of NFκB initiated transcription. These inflammatory cytokines are generally beneficial at normal levels, however, excessive production can result in an acute inflammatory response and cause extensive damage. Two major inflammatory cytokines that have been studied extensively in relationship to IBD and colorectal cancer have been tumor necrosis factor alpha (TNF-α) and interleukin-1 (IL-1).

TNF-α was first described for its ability to induce necrosis in transplanted tumors in mice and is typically one of the first cytokines to be released in response to a pathogen. It is an
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endocrine cytokine capable of acting on a number of different distant organs. TNF-α is able to modulate vascular permeability enabling lymphocyte, neutrophil, and monocyte infiltration to the affected area.[44] This cytokine is of particular importance when discussing inflammation in the intestine and IBD in particular as elevated levels of TNF-α have been found in the stool, mucosa, and blood of IBD patients.[66, 67] As a result of these findings, TNF-α was one of the first pharmacological targets for treatment of IBD patients. Anti-TNF antibody therapy has been successfully used to reduce the clinical severity of ulcerative colitis and Crohn’s disease.[68]

TNF-α trafficking within the cell increases upon the cell’s exposure to LPS.[69-71] LPS can also stimulate cells to release IL-1, another inflammatory cytokine family.[71] IL-1 consists of three main forms, IL-1α, IL-1β, and IL-1Ra. IL-1α and IL-1β are the primary pro-inflammatory cytokines. IL-1β, in particular, is similar to TNF-α in that it is released during the early stages of inflammation and is secreted by a number of different cell types including epithelial cells. Both human and animal studies of IBD show a strong correlation between IL-1β and inflammation. [62, 72-75] As with TNF-α, elevated concentrations of IL-1β are closely related to carcinogenesis. High levels of IL-1β have been found in cancerous pancreatic, breast, stomach, and colonic tissue suggesting that excess amounts of this cytokine correlates to localized cancer progression.[76-80] Numerous transgenic animal studies have demonstrated the ability of IL-1β to suppress immunosurveillance and drive oncogenesis.[76]

1.2.2.3 Inflammasome and Caspase-1 Activation

Within the cytosol, a multiple protein complex called an inflammasome forms in response to PAMPs and DAMPs and is responsible for the activation of inflammation processes related to innate immune function.[81] This activation includes the proteolytic conversion of pro-IL-1β to mature and active IL-1β (Figure 1-8).
Figure 1-8. Activation of IL-1β by caspase-1 as a result of activation of the inflammasome.

The major components of an inflammasome include: nucleotide-binding domain and leucine-rich repeat-containing proteins (NLRs), adaptor protein apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain (ASC), and caspase-1. A number of different inflammasome complexes can be formed based on the NLR isoform included. NLR isoforms contain differing PAMP and DAMP sensing domains.[82] The mechanisms by which NLRs are activated are currently unknown. It has been suggested that some act through direct ligand binding while others are activated through an indirect sensing mechanism for both formation and activation.[60] For example, NLRP3 inflammasome formation may be occurring as a result of K+ efflux, generation of mitochondrial/intracellular ROS, or damage to the lysosome.[83]

The NLRP3 inflammasome is the most well-studied and there is strong data to support its activation by ROS. Inhibition of ROS generation or inclusion of ROS scavengers have been
shown to inhibit the formation and function of the NLRP3 inflammasome.[84] Not all sources of ROS appear to activate NLRP3 and it has been suggested that additional factors are needed in order to elicit caspase-1 activation.[83] For example, inhibition of the NADPH oxidase pathway has been shown to decrease the activation of NLRP3 inflammasomes suggesting that ROS may be a requirement.[85-87] Furthermore, inhibition of mitochondrial voltage-dependent anion channels, causing a reduction in mitochondrial ROS formation, suppresses inflammasome formation.[60, 87]

Thioredoxin interacting protein (TXNIP) represents a link between cellular ROS and NLRP3 inflammasome assembly. In the unstimulated condition, TXNIP is bound to thrioredoxin. Upon ROS generation, TXNIP dissociates from thrioredoxin and binds to NLRP3 facilitating the formation of the inflammasome through an unknown mechanism.[88]

NLRP3 has been examined extensively in IBD patients and a number of single nucleotide polymorphisms (SNPs) in the NLRP3 gene are highly correlated with susceptibility to Crohn’s disease.[89] Conflicting data on the impact of NLRP3, IL-1β, and caspase-1 inhibition in clinical studies have been noted and, as a result, it has been hypothesized that the microbiome may be playing a factor.[60] In animal studies, both exacerbation and attenuation of IBD symptoms have been associated with decreases in NLRP3 expression.[90-92]

TLRs have also been tied to the modulation of NLRP3. While the transcriptional factors of NLRP3 are not clearly defined, one study found that the TLR4 agonist, LPS, significantly increased expression of NLRP3 mRNA and protein in peritoneal murine macrophages.[93] As discussed earlier, TLR4 stimulation with LPS initiates NFκB activation which is responsible for generating a number of different inflammatory processes including the production of IL-1β. Interestingly, it was found that LPS stimulated TLR4 induced NFκB binding to the NLRP3 promoter gene, suggesting that TLR4-induced NFκB activation regulates NLRP3 expression in murine macrophages.[93]
1.2.2.4 Epithelial Barrier and Intestinal Permeability

The gastrointestinal epithelium is covered with a mucous layer that creates a barrier and prevents many large molecules and most bacteria from coming into direct contact with the epithelial cells.[94] Defective mucus production has been linked to intestinal diseases and mice lacking mucin genes develop spontaneous ulcerative colitis.[95, 96]

The next line of defense is the intestinal epithelial cells themselves. The plasma membranes of the cells prevent transcellular movement of most large or hydrophilic molecules through the cell, and the junction proteins between the epithelial cells control the paracellular transport of molecules. These barriers are selectively permeable and allow fluid and nutrient exchange, but this permeability changes with location in the gastrointestinal tract.[97] For example, the permeability of larger molecules decreases as you move from the crypt to the villus.[98]

An apical junctional complex containing a tight junction and adherens junction is held together by a perijunctional ring of actin and myosin and can modulate intestinal permeability (Figure 1-9).[94, 99] Current evidence suggests that this ring is modulated by myosin light-chain kinase (MLCK), however there is evidence to suggest that this is not the only control mechanism.[100]
The adherens junctions are responsible for maintaining cellular proximity and intracellular communication. They are composed of a family of transmembrane proteins, the cadherins, and form homotypic interactions with other cadherins on adjacent cells. [97] These adherens junctions are essential for the formation of the tight junctions responsible for sealing the paracellular space.

These tight junctions are complexes composed of transmembrane tight junction proteins, membrane scaffolding proteins, and regulatory molecules. Claudins, a family of tight junction proteins, are tissue specific and mutation or deletion has a substantial impact on an organ’s function. [97] Another important tight junction protein, occludin, is responsible for interacting with claudins and actin. Junctional adhesion molecule (JAM) proteins are another family of this tight junction complex. JAMs function to assemble the complex and are involved in leukocyte transmission, platelet activity, angiogenesis, and virus binding. [101] Zonula occludins (ZO) assist in the tight junction assembly and maintenance by providing multiple domains for claudins, occludin, and actin to interact with. The extracellular domains of these transmembrane tight
junction proteins interact with one another and are able to adapt to a number of different stimuli including nutrients, cytokines, and bacteria.[97] Increased intestinal permeability has also been linked to the development of colon cancer.[102, 103]

The intestinal microflora have been shown to modulate tight junctions and mucosal barrier function through direct interaction or through the production of a secreted product.[101, 104]. Direct adhesion of Escherichia coli (E. coli) to epithelial cells has been shown to alter permeability by causing the dissociation of tight junction proteins from one another.[105, 106] Similar results have been reported for Clostridium perfringens.[107] Disruption of these junctions allow for paracellular transport of endotoxins, such as LPS, from the lumina into systemic circulation.[108] Conversely, probiotics and beneficial bacteria appear to enhance tight junction function by acting directly on tight junctions proteins and stabilizing the perijunctional actin ring.[109-111] In a mouse model of colitis, pretreatment with a putative probiotic microorganism, E. coli Nissle, reduced inflammatory symptoms, up-regulated ZO-1 gene expression, and decreased intestinal permeability.[112]

Many of these tight junction proteins respond to inflammatory cytokines and are significantly down-regulated in patients who suffer from IBD.[30, 31, 101, 113] Oxidative stress and inflammation have both been linked to increases in intestinal permeability.[114] TLR4, in addition to its role in the formation of the inflammasome and IL-1β, has also recently been linked to changes in intestinal permeability.[101] A study in rats found that oral administration of methotrexate, which induced colonic mucositis, increased TLR4 and inflammatory cytokine expression and decreased occludin, claudin-2, and claudin-4 mRNA expression.[115] TLR4, aside from being stimulated by LPS, is also stimulated by IL-1β suggesting another possible trigger involved in permeability regulation. A recent study demonstrated that IL-1β-mediated activation of NFκB induced a decrease in gut barrier function and tight junction expression in human epithelial cell culture.[116] Use of an NFκB translocation inhibitor
ameliorated the increase. The specific mechanism by which this occurs is currently under investigation, but data suggests a role for NFκB-mediated modulation of MLCK.[117, 118]

1.2.2.5 Experimental Models of Inflammatory Bowel Disease

A number of different laboratory models have been developed to study IBD. Although a number of different animal models have been used since the first rabbit model in 1957, for the purposes of this chapter, only mouse models will be discussed.[119] Mouse IBD models fall into two broad categories: genetically engineered and chemically induced. A number of conventional and conditional transgenic and knockout mice have been created to look at the effects of specific proteins and genes such as TGFβ KO and NFκB1 KO. While these genetic modifications provide a valuable tool for investigating specific pathways, their effects are often not localized to only the particular area of study and could cause developmental changes introducing potentially confounding variables.[120]

There are three compounds used for the chemical induction of colitis: trinitrobenzene sulfonic acid (TNBS), 4-ethoxymethylene-2-phenyloxazol-5-one (oxazolone), and dextran sulfate sodium (DSS). TNBS is a contact-sensitizing allergen that is delivered via enema where it causes acute colitis by an immune response to “hapten-modified self-antigen” and is thought to closely resemble the symptoms of Crohn’s disease.[119, 121] Oxazolone is also administered intrarectally and induces inflammation in the distal colon. It is characterized by hemorrhagic inflammation and submucosal edema, more closely resembling symptoms of ulcerative colitis.[119]

DSS is a polymer of sulfated glucose monomers produced by *Leuconostoc spp.* that is produced in a variety of molecular weights and induces acute colitis in a molecular weight and concentration-dependent manner. The use of 1.5% DSS (w/v, mw = 36 – 50 kDa) in drinking
water for 7 days has been shown to induce inflammation to the entire colon and leads to increased incidences of metaplasia and tumor formation in both rats and mice.[122, 123] Unlike TNBS and oxazolone, mice treated with DSS recover quickly allowing researchers to examine intestinal repair and the effects of therapeutic agents on that repair.[119] DSS can be used to induce chronic, cyclic colitis, which recapitulates human disease, by repeated periods of DSS treatment followed by periods of recovery with water. Unlike TNBS and oxazolone, DSS is administered in the drinking water.[119] DSS is also unique in that the gut microflora are required for modulating the inflammation caused by DSS. Germ-free mice and mice treated with wide spectrum antibiotics typically develop lethal colitis as a result of DSS.[124, 125]

Typically, the severity of colitis is evaluated by histological scoring, inflammatory cytokine production, and intestinal permeability. Histological scoring can be done through sectioning, staining, and scoring, and cytokine production can be measured through mRNA and protein expression. Intestinal permeability can be assessed by measuring tight junction mRNA and protein expression. There are many conflicting studies describing the trends seen in mRNA and protein expression in mice.[100, 116, 126, 127]

In human and animal studies, a multi-sugar assay has been used to assess the relative permeability of the intestinal tract.[128-130] In these multi-sugar experiments, subjects are typically given a mixture of L-rhamnose, lactulose, erythritol, and sucralose. The monosaccharaide, L-rhamnose, and the sugar alcohol, erythritol, are absorbed transcellularly, whereas the disaccharides, lactulose and sucralose, enter the blood stream via paracellular transport. In a healthy colon, paracellular absorption of sugars is minimal. Lactulose and sucralose are not metabolized and their presence in the blood and urine indicates increased gastrointestinal permeability.[128, 129] Since L-rhamnose and erythritol are absorbed transcellularly, they can be used to normalize permeability to baseline gastrointestinal function. This assay also allows selective interrogation of gastrointestinal permeability.
L-rhamnose and lactulose are absorbed to a greater extent in the small intestine while erythritol and sucralose are absorbed in the colon. When combined, it provides a quantifiable picture not just of the colon, but of the overall permeability deficits of most of the intestinal track. Typically this is monitored in the plasma and can be analyzed via gas chromatography-mass spectrometry (GCMS).[131, 132] This method is useful as a non-invasive means of assessing permeability.

*In vitro* assessments of gut permeability usually uses the Caco-2 human colorectal adenocarcinoma cell line. These cells undergo differentiation to an intestinal epithelium-like monolayer 21 days after reaching confluence.[133] This differentiated monolayer resembles mature intestinal absorptive cells both in morphological polarity and levels of brush border hydrolases.[134] Permeability and absorption assays are typically conducted by growing the monolayer on a semipermeable well insert, known as a transwell, and suspending it in a well with media. The transwell separates the apical and basolateral compartments with the monolayer as the barrier between the two areas. The integrity of these barriers can be monitored via transepithelial electrical resistance (TEER).[135] TEER measures the electrical resistance of a monolayer by passing an alternating current across the membrane without damaging it. This can be used to ensure that monolayers have fully differentiated. The permeability of the monolayer can be increased by the addition of DSS to the apical compartment.[136] The effects seen by doing this are similar to those observed in ulcerative colitis patients. Permeability can be assessed quantitatively by measuring the apical to basolateral flux of fluorescein isothiocyanate–conjugated dextrans (FITC-D).[136, 137] The flux of FITC-D (MW 30-50 kDa) increases as a function of monolayer permeability (*Figure 1-10*).
Figure 1-10. Caco-2 human colon cell monolayer transwell model.
1.3 Dietary Phytochemicals for Prevention and Treatment of Inflammatory Bowel Disease

1.3.1 Types and Mechanisms of Dietary Treatment

As discussed above, diet has a strong influence on the development of IBD. High intake of mono- and disaccharides, and triglycerides strongly correlates with both forms of IBD.[138] This is of particular interest as this diet is tied closely with metabolic syndrome which has also been associated with chronic intestinal inflammation.[139] The issue with studying the influence of diet is that there a number of factors that cannot be controlled for such as underreporting of food questionnaires, dietary habits changing as a result of symptom onset, or as a result of disease activity as patients attempt to self-medicate.[38] As a result, much of the research on IBD has been done in cell and animal models using inflammation and intestinal permeability markers as a measure of IBD severity.

Numerous cell and animal studies have examined specific dietary components and their effect on inflammation in models of IBD. Many studies have focused on plant derived bioactive components such as terpenes, lignans, and other polyphenolic compounds. Many of these bioactive components decreased the production of IL-1β and TNFα, and inhibited NFκB signaling in animal and cell-line models.[140] For example, dietary supplementation of rats with proanthocyanidin (PAC)-rich grape seed extracts increased occludin levels and reduced intestinal permeability.[141, 142] Another study found that polyphenol-rich blueberry powder supplemented into the diet of obese rats decreased TNFα and NFκB mRNA expression in the plasma.[143] As will be discussed in subsequent sections, polyphenol-rich green tea has also been found to decrease inflammation and has been linked to lower incidents of colorectal
Collectively, polyphenols have been shown to inhibit NFκB signaling and thus cytokine production.[146-149]

The precise mechanisms underlying the anti-inflammatory effects of polyphenols is unclear. Early studies involving polyphenols focused mostly on their ability to quench free radicals. However, more recent studies have found that their anti-inflammatory effects cannot be explained solely by their antioxidant abilities.[150] The two main signaling pathways that appear to be influenced by polyphenols are the NFκB pathway and the mitogen activated protein kinase (MAPK) pathway.[151] These activities seem linked to individual molecules and structures rather than their classification as polyphenols. For example, (-)-epigallocatechin-3-gallate (EGCG) strongly inhibited IKK activity while other antioxidants had a little effect.[150]

Polyphenols are not the only bioactive food components that have been linked to decreases in inflammation and symptoms of IBD. Omega-3 fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been shown to suppress inflammatory cytokine production in DSS treated mice.[152, 153] Dietary fiber has also been shown to have positive effects on IBD and inflammation. The mechanism behind it is thought to be due to fiber being fermented by microbiota into short-chain fatty acids like butyrate.[154] Butyrate is not only a major energy source for colonocytes but has also been shown to inhibit NFκB and decrease intestinal permeability.[155] Dietary animal proteins appear to correlate with an increased risk of IBD whereas vegetable proteins, though not significantly, appear to decrease the risk.[156] Recently, the therapeutic effects of bioactive soy components and soy protein isolate have been investigated and will be discussed later.[157] Collectively, dietary intervention to mitigate inflammation in the context of IBD has the potential to directly improve symptoms related to IBD and reduce colon carcinogenesis.
1.3.2 Green Tea

1.3.2.1 Components of Green Tea and Antioxidant Mechanisms

Tea (Camellia sinensis, Theaceae) is the second most popular beverage in the world and an important agricultural crop for many countries with over 6.5 million tons produced in 2012.[158, 159] Green, black, and oolong tea are made from the same plant, but differ in terms of processing and chemical composition. Green tea is typically dried soon after harvesting to avoid oxidation of the polyphenols. Oolong and black teas are allowed to ferment before they are dried. The degree of fermentation leads to changes in the appearance, flavor, and chemical components of the tea.[160] Polyphenol oxidase is primarily responsible for catalyzing the conversion of catechins to theaflavins and thearubigins (Figure 1-11).[144] The former are characteristic of green tea, whereas the latter are characteristic of black tea. On a dry weight percentage, polyphenols may account for as much as 25% of a tea leaf, more than cellulose and protein combined.[158] These polyphenols as well as the purine alkaloids in tea have been studied extensively for their cancer preventive and anti-inflammatory effects.[161] The most abundant polyphenol in green tea is (−)-epigallocatechin-3-gallate (EGCG) which can account for as much as 50% of the tea catechins by dry weight.[158]
Free radicals, as discussed previously, can disrupt a number of different cellular processes. Polyphenols have the ability to absorb these radicals in a resonance-stabilized fashion (Figure 1-12). The antioxidant activity of polyphenols may also be due to their ability to chelate and form complexes with transition metals, which are important catalysts of oxidation reactions.

Based on different binding motifs, polyphenols have been shown to complex ferric (Fe$^{3+}$) ions, thereby disrupting their ability to catalyze redox reactions.[162] Binding motifs in quercetin and a number of other flavonoids have also been shown to have a strong affinity for ferrous (Fe$^{2+}$) ions. [163] Quercetin has been shown to be a strong ferrous chelator and is capable of suppressing hydroxyl radical generation as a result of the Fenton reaction.[164]
1.3.2.2 Anti-Carcinogenic Studies of Tea in Human Subjects

Numerous epidemiological studies have examined the relationship between green tea consumption and cancer risk, but have yielded mixed results. This lack of consistent results may be due to confounding variables such as underlying genetics, inaccurate measures of tea consumption, or unaccounted differences in diet.[160, 166] There have been some recent clinical intervention studies in prostate and oral cancer patients which have suggested that treatment with green tea extract may reduce carcinogenesis in human subjects.[167-169] While these results are promising, clinical studies in esophageal, gastric, liver, and lung cancers have all yielded less convincing results.[160, 166] For example, intervention with decaffeinated green tea (4 cups/day) by smokers was found to reduce urinary concentrations of 8-hydroxy-2'-deoxyguanosine (8-OHdG), a marker of oxidative stress.[170] This effect was not observed in groups that consumed black tea suggesting that green tea is better equipped to reduce oxidative stress within the body than black tea. Conversely, green tea extract (0.5-3 g/m²) given to patients with advanced stage lung cancer yielded no response in cancer severity.[171]

The potential beneficial effects of tea on colorectal cancer were found in a recent meta-analysis of 25 epidemiological studies from 11 countries. The researchers found that an inverse relationship existed between green tea consumption and colon cancer risk; however, this was only observed in case-controlled studies and not prospective cohort studies.[172] In a prospective cohort study of Chinese men and women between the ages of 45-74 years researchers found that,
in men, green tea consumption actually increased the risk of colon cancer development.[172] This suggests that in later stages of colorectal cancer progression, green tea may be having an adverse effect. A similar cohort study done in women consuming large amounts (≥ 5 g dry leaves/day) of green tea showed a reduced risk of colorectal cancer.[173] The beneficial effects were observed in two other prospective cohort studies although they were not statistically significant, further confusing the issue.[174] Using urinary levels of EGC and its metabolite, 4’-MeEGC, as a measure of microbiota function, researchers found a statistically significant inverse relationship between urinary tea catechins excreted and colon cancer risk.[175] In an intervention study, daily supplementation with green tea extract (1.5g) for 12 months reduced meachronous colorectal adenoma formation.[176] Taken together, this provides some evidence that colorectal carcinogenesis may be prevented through consumption of green tea.

1.3.2.3 Anti-Carcinogenic and Anti-Inflammatory Studies of Tea in Laboratory Models

Laboratory studies have generally supported the anti-carcinogenic and anti-inflammatory effects of tea. In a recent study done in azoxymethane (AOM)-induced diabetic obese mice, treatment with 0.01% or 0.1% EGCG in the drinking fluid for 7 weeks dose-dependently reduced aberrant crypt foci and β-catenin accumulated crypts, both precursor lesions for colon cancer.[177] Apc^{min/+} mice, a strain genetically susceptible to intestinal tumorigenesis, treated with EGCG as the sole source of drinking fluid (0.02 – 0.32%) reduced small intestinal tumorigenesis in a dose-dependent manner.[178] These researchers also controlled for caffeine and found that 0.044% caffeine had no effect. Similar results were observed in AOM-induced Apc^{min/+} mice treated with 0.6% green tea.[179] These authors found that methylation of the retinoic acid receptor alpha (RARα), a predictor for cancer, was decreased as a result of the green tea treatment suggesting a possible protective effect with carcinogenesis.[179, 180]
Studies of the effect of EGCG and green tea in DSS-induced mouse models have yielded conflicting results. In one study, the researchers induced low grade colitis by alternating DSS treatment with water. Oral gavage with 6.9 mg/kg EGCG and 2.9 mg/kg piperine reduced weight loss associated with colitis, improved histological scoring, and decreased lipid peroxidation products in the large intestine.[181] The piperine was used to slow gastric motility and increase the bioavailability of EGCG.[182]

By contrast, other studies with EGCG have reported an increase in tumorigenesis. In a recent study of mice treated with AOM and 1.5% DSS, EGCG treatment (0.03 – 0.3% in the diet) tended to decrease tumor formation, although the effects were not significant.[183] When the researchers examined the effects of EGCG in mice treated with a higher concentration (5%) of DSS, they found that 0.3% EGCG significantly increased tumor formation. Another study found that green tea induced nephrotoxicity and gene expression of antioxidant enzymes and heat-shock proteins in the kidney.[184] This study used 5% DSS which has the ability to cause severe acute colitis. The exact mechanism of this toxicity still remains unclear and should be considered when determining the appropriate upper safety limit of green tea in the context of IBD.

It has been consistently noted in studies of green tea and colitis that mice treated with green tea or EGCG and DSS typically lose weight to a similar degree as mice treated with DSS, despite the apparent improvements in markers of inflammation and tumorigenesis.[178, 183, 184] Recent studies involving high fat fed mice and EGCG treatment have demonstrated that weight gain decreased as a result of EGCG modulating fat absorption as measured by fecal lipids.[185-188] This increase in weight loss could also explain the weight loss seen in the DSS and EGCG treated mice.
1.3.3 Soybeans

1.3.3.1 Consumption and Components of Soy

Soybeans (Glycine max L., Fabaceae) are an important agricultural crop with over 288 million tons produced in 2013.[159] In the US, soy is second only to corn in terms of production, but consumption by Americans is low (less than 1 g per day per person).[189] By contrast, in Asian countries, consumption may be as high as 9.6 g per day per person.[190]

Soybeans are legumes containing approximately 36% protein, 20% fat, 30% carbohydrates, and 9% dietary fiber, and are rich in a number of different water and fat soluble vitamins and minerals (Table 1-1).[191] In addition, soybeans contain several classes of bioactive compounds that have been linked to cancer prevention including isoflavones, saponins, and bioactive peptides.[192]
Table 1-1. Nutrient breakdown of raw mature soybeans.[191]

<table>
<thead>
<tr>
<th>Macronutrient</th>
<th>(per 100g)</th>
<th>Amino Acids</th>
<th>(per 100g)</th>
<th>Vitamins</th>
<th>(per 100g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>8.54 g</td>
<td>Tryptophan</td>
<td>0.591 g</td>
<td>Vitamin C</td>
<td>6.0 mg</td>
</tr>
<tr>
<td>Energy</td>
<td>446 kcal</td>
<td>Threonine</td>
<td>1.766 g</td>
<td>Thiamin</td>
<td>0.874 mg</td>
</tr>
<tr>
<td>Protein</td>
<td>36.49 g</td>
<td>Isoleucine</td>
<td>1.971 g</td>
<td>Riboflavin</td>
<td>0.870 mg</td>
</tr>
<tr>
<td>Total lipid (fat)</td>
<td>19.94 g</td>
<td>Leucine</td>
<td>3.309 g</td>
<td>Niacin</td>
<td>1.623 mg</td>
</tr>
<tr>
<td>Carbohydrate, by difference</td>
<td>30.16 g</td>
<td>Lysine</td>
<td>2.706 g</td>
<td>Pantothenic</td>
<td>0.793 mg</td>
</tr>
<tr>
<td>Fiber, total dietary</td>
<td>9.3 g</td>
<td>Methionine</td>
<td>0.547 g</td>
<td>acid</td>
<td></td>
</tr>
<tr>
<td>Sugars, total</td>
<td>7.33 g</td>
<td>Cystine</td>
<td>0.655 g</td>
<td>Vitamin B-6</td>
<td>0.377 mg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phenylalanine</td>
<td>2.122 g</td>
<td>Folate, total</td>
<td>375 µg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tyrosine</td>
<td>1.539 g</td>
<td>Choline, total 1</td>
<td>115.9 mg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Valine</td>
<td>2.029 g</td>
<td>Betaine 1</td>
<td>2.1 mg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Arginine</td>
<td>3.153 g</td>
<td>Vitamin A, RAE</td>
<td>1 µg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Histidine</td>
<td>1.097 g</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Alanine</td>
<td>1.915 g</td>
<td>Carotene, beta</td>
<td>13 µg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Aspartic acid</td>
<td>5.112 g</td>
<td>Vitamin A, IU</td>
<td>22 IU</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glutamic acid</td>
<td>7.874 g</td>
<td>Vitamin E</td>
<td>0.85 mg</td>
</tr>
<tr>
<td>Glycine</td>
<td>1.880 g</td>
<td></td>
<td></td>
<td>(alpha-tocopherol)</td>
<td></td>
</tr>
<tr>
<td>Proline</td>
<td>2.379 g</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serine</td>
<td>2.357 g</td>
<td></td>
<td></td>
<td>Vitamin K (phyloquinone)</td>
<td>47.0 µg</td>
</tr>
</tbody>
</table>
Isoflavones are heterocyclic polyphenols that have been reported to have estrogenic/anti-estrogenic effects.\[193\] Levels of these bioactive isoflavones vary substantially between different cultivars of soybeans (1.1 – 3.3 mg/g) and are affected by processing.\[194-196\] For example, soy milk contains approximately 0.12 mg/g wet weight isoflavones whereas tofu contains close to 1.5 mg/g isoflavones; this is due mostly to a decreased water content in tofu.\[197\] Soy milk is produced by soaking dried soybeans and grinding them in water to produce a stable emulsion of oil, water and protein. A significant amount of soybean consumption is accounted for by consumption of fermented products including soy sauce, miso, and tempeh. Soy sauce has virtually no isoflavones, whereas miso may contain up to 0.2 mg/g and tempeh close to 0.5 mg/g.\[197\] As much as 90% of the total isoflavone content in soy-based foods is in the form of the glycosides genistin and daidzin.\[198, 199\] Although the aglycone forms, genistein and diadzein, are also present, they appear in much smaller amounts.

Another class of soy-derived bioactive components are saponins, which are amphiphilic glycosides that are thermally stable and found at concentrations of 0.5% w/w in dried soybeans.\[198\] Though more than 40 different saponins have been found in soy, oleanane-type triterpene aglycones are the most common and most studied.\[200\]

Soybeans contain up to 36% protein including a number of bioactive peptides that are either present naturally or that are formed via fermentative hydrolysis.\[201\] Of the storage proteins in soybeans, 50% is glycinin and 25% is β-conglycinin.\[202\] Glycinin is a 6-unit polypeptide with a size of about 320-380 kDa and β-conglycinin is a 150 kDa protein with 3 subunits. Both glycinin and β-conglycinin have been linked to both beneficial bioactivity as well as allergenicity.\[203, 204\]

Soybean protein is also composed of as much as 6% protease inhibitors by weight with the two major ones being Bowman Birk Inhibitor (BBI) and soybean trypsin (Kunitz) inhibitor.\[198\] Both BBI and Kunitz have been reported to decrease inflammation and
carcinogenesis.[205] The serine protease inhibitor, BBI, is the smaller of the two (8 kDa) and is held together by seven disulfide bonds.[205] The Kunitz inhibitor is larger (20 kDa) and has a unique β-Trefoil fold that is similar to IL-1β.[206] This is of particular interest as it may suggest a mechanism for soy blocking IL-1β signaling by interfering with binding of IL-1β to its receptor.

1.3.3.2 Anti-Carcinogenic Studies of Soy in Human Subjects

Numerous epidemiological and clinical intervention studies have looked at the mitigation of colorectal cancer risk by soy. Soy consumption was linked to a decreased risk (Overall Risk: 0.70, p<.001) of overall colon cancer risk in a meta-analysis of epidemiological studies.[207, 208] Another systematic review found no overall relationship between soy consumption and colorectal cancer risk, but did report that after stratification by gender, a decreased risk was observed in women who consumed soy (combined risk estimate: 0.79, p = 0.026).[192] As with many epidemiological studies, there are several issues that make comparison between studies difficult. Many case-controlled studies are not designed to measure only one dietary component. They typically rely on food frequency questionnaires which suffer from recall bias in the individual. Further confounding the issue, different studies will use different adjustments to account for cofounders making direct comparison between studies difficult.

An examination of the effects of BBI supplementation in a study of patients with ulcerative colitis showed a decrease in colitis severity as measured by the Sutherland Disease Activity Index.[209] A daily dose of 800 chymotrypsin inhibitors units given to the participants resulted in 36% of the patients going into remission. These suggest that it may actually be the protein component of soy, rather than the isoflavones, that is responsible for decreasing colorectal cancer risks.
In human intervention studies, markers for oxidative stress and inflammation have been used as potential markers for possible colorectal cancer progression. The majority of these experiments have shown anti-oxidative and anti-inflammatory effects with soy. One crossover study of 42 subjects with LDL cholesterol over 3.36 mmol/L found that soy protein and isoflavones diets reduced total antioxidant performance as compared to those that received animal protein.[210] In a similar experiment, antioxidant levels were higher in soy treated participants as compared to whey treatments.[211] What is particularly interesting about this study is that total antioxidant measurements did not differ between groups that received isoflavone-rich soy protein isolate and isoflavone-poor soy protein isolate. This suggests isoflavones may not be contributing to the antioxidant effect but, it is rather the protein within the isolate that is exerting this effect.

Changes in oxidative stress and inflammatory markers due to soy were not seen in a study done comparing dairy and soy protein isolate smoothies in obese men and women. Nor were they seen in another study done comparing dairy and soy milk intervention in females.[212] Confounding the issue more, diet supplementation with soy did show a decrease in total antioxidant content and lipid peroxidation in post-menopausal women with metabolic syndrome.[213] Taken together, these intervention studies do not provide a clear picture for soy consumption and its impact on oxidative stress or inflammation.

1.3.3.3 Anti-Carcinogenic and Anti-Inflammatory Studies of Soy Laboratory Models

The results of animal studies on the anti-carcinogenic and anti-inflammatory effects of soy have been mixed. An early study conducted in rats found an increase in AOM-induced colon tumors formation as a result of 250 mg/g genistein in the diet.[214] Similarly, intragastric administration of 75 mg/kg EGCG and 200 mg/kg genistein in APCmin/+ mice found an increase in colonic tumorigenesis.[215] Conversely, one study found that a 12% soy protein isolate
supplemented diet in AOM rats caused a reduction in tumor volume and multiplicity in AOM rats.[216] In another study, lifetime exposure to 20% soy protein isolate diet caused a significant decrease in aberrant crypt foci in AOM-treated rats.[216] This study also showed that rats exposed to soy protein, during gestation and weaning only, may carry an inherent carcinogenic protection with them later in life. Soy protein supplementation in another AOM study also decreased aberrant crypt foci; however, the effect was not as strong as a 0.015% genistein substituted diet suggesting that it may be the component of interest.[217]

In studies focused on oxidative stress, soy supplemented diets appear to decrease markers of inflammation. Soy protein reduced liver oxidation markers in rats treated with paraquat which is known to cause oxidative stress through superoxide radical generation.[218] This effect was not seen in rats that received soy isoflavones or saponins suggesting that elements of the protein are responsible for the antioxidant effect. Similarly, soy protein isolate and soy peptide diets caused reductions in serum thiobarbituric acid reactive substances (TBARS), a marker of oxidative stress, in rats that received paraquat.[219] Myocardial oxidative stress was also reduced in rats with myocardial infarctions that received 20% soy protein isolate diet.[220] Reduced oxidative stress markers have also been seen in healthy and exercising mice that received soy protein.[221, 222] Taken together, this further supports the idea that the soy protein may have a beneficial effect on oxidative stress and inflammation.

Looking at specific components of the soy protein, BBI has also been examined in colorectal cancer animal models. One study found that 0.5% BBI concentrate added to the diet reduced tumor multiplicity and incidence in dimethylhydrazine-treated rats.[223] In this study, the researchers found that when BBI was autoclaved the beneficial effects were lost. In DSS treated mice 0.5% BBI concentrate diet caused a decrease in histopathological inflammation scoring in the intestine.[224]
In DSS-induced colitis experiments, soy supplementation appears to decrease markers of inflammation and intestinal damage as measured by histological scoring. In stressed rats, 0.45 mg of isoflavone in its aglycone form administration to each rat showed a decrease in hyperpermeability and hypersensitivity within the colon.[225] A similar dose of isoflavones was administered to rats in a TNBS colitis model and yielded decreases in permeability and inflammation.[226] The issue with both of these studies is that the isoflavone doses also contained BBI, which has been shown to have significant impacts on inflammation regulation.

In a recent experiment, fermented soy sauce administration caused a decrease in inflammatory cytokine mRNA expression of TNF-α, interferon-γ (IFN-γ), IL-6, and IL-17a.[227] Interestingly, the researchers found the effect was lost when acid-hydrolyzed soy sauce was used. Soy protein given to DSS treated mice resulted in decreased inflammation scores, TNF-α mRNA, mucus glycoprotein (MUC), and trefoil factors (TFF); both MUC and TFF are elevated in DSS-induced colitis.[228] Conversely, an experiment using 20 mg/kg bodyweight of soy isoflavones daidzein or equol given to DSS treated mice caused an increase in weight loss and inflammation and reduction in anti-inflammatory cytokines.[229] This further suggests that the anti-inflammatory effects found from soy are actually due to the protein and peptide components.

Lunasin, a soy peptide, has shown anti-inflammatory activity in macrophage cells in culture.[230, 231] In a recent study in DSS treated piglets, soy derived di- and tri-peptides had an inhibitory effect on inflammation scores and gut permeability.[232] In this study, the researchers also found that this peptide treatment decreased pro-inflammatory pathways, Th1 and Th17, in the colon and ileum. In another study, a particular tripeptide, Val-Trp-Tyr, reduced inflammatory cytokine mRNA levels of TNF-α, IL-6, IL-1β, IFN-γ and IL-17 as compared to the DSS-only control.[233] Taken together, this makes a case for the anti-inflammatory ability found in soy coming from protein and peptides rather than isoflavones.
1.4 Hypothesis and Objectives

EGCG and soy protein have both been reported to have antioxidant and anti-inflammatory activities in a number of models. The underlying mechanisms of action for these components in the context of IBD, including their potential to mitigate intestinal permeability, have not been well-studied.

I hypothesize that dietary supplementation with dietary phytochemicals will protect mice from DSS-induced colonic inflammation as evidenced by reduction of markers of localized colonic inflammation and gastrointestinal permeability. Furthermore, I hypothesize that these effects are due to direct interactions with the epithelium.

I propose to test this hypothesis by determining:

a. The effect of EGCG on markers of inflammation, oxidative stress, gut barrier function, and nutrition in mice with DSS-induced ulcerative colitis and in DSS-treated Caco-2 cell monolayers in culture.

b. The effect of an isoflavone-free soy protein concentrate on markers of inflammation, oxidative stress, gut barrier function, and inflammasome formation in mice with DSS-induced ulcerative colitis and in DSS-treated Caco-2 cell monolayers in culture.

c. Extending the studies of the gut barrier improvements of other polyphenol rich foods such as tempeh, cocoa, cherry, and moringa in the Caco-2 transwell model.
Previous studies involving EGCG and its effects on ulcerative colitis in a mouse model have all yielded mixed results. Many of these studies involved either oral gavage, resulting in a large bolus dose of EGCG, or green tea supplementation in the diet. Many of these studies also focused on co-treatment with EGCG and colitis induction. These studies did not look at consumption of EGCG in the drinking fluid nor did they examine the effect of EGCG on the repair of epithelium and attenuation of inflammation after colonic insult. At the start of this study, there had been no reports of EGCG’s effects on gut barrier function. As a result, the following experiments were conducted to examine the effects of EGCG on markers of inflammation in acute DSS-induced ulcerative colitis in mice and gut barrier function in both mice and Caco-2 monolayer permeability studies.
2.1 Abstract

Ulcerative colitis (UC) is an inflammatory bowel disease that manifests as constant/cyclic episodes of colonic inflammation, oxidative stress, increased intestinal permeability, and ulceration followed by periods of quiescence. UC can lead to a significant increase in the risk of colon cancer. Green tea polyphenols, such as epigallocatechin-3-gallate (EGCG), have been shown to inhibit oxidative stress in animal and cell studies. In the present study, we examined the anti-inflammatory effects of EGCG in the dextran sulfate sodium (DSS)-induced CF-1 mouse model of UC. Following induction of colitis by treatment with 1.5% DSS for 7 days, mice were treated with EGCG (0.32%) as the sole source of drinking fluid for 3 days. In the colon homogenate, levels of inflammatory markers, IL-1β, IL-6, and TNF-α, were decreased as a result of the EGCG administration. In the plasma, inflammatory markers and endotoxins were also decreased. This suggests that EGCG is decreasing both the inflammatory response and the permeability of the intestine in the DSS-induced UC mouse model. Ongoing mechanistic studies are focused on determining the effect of EGCG on intestinal permeability and gut barrier function.


2.2 Introduction

Green tea (*Camellia sinensis*) is a commonly consumed beverage that is rich in polyphenolic compounds known as catechins.\(^{[165]}\) (\(\sim\)Epigallocatechin-3-gallate (EGCG) is the most abundant catechin in green tea (\(\sim\) 50% of total catechin content). EGCG and green tea have been reported to reduce the risk of a host of chronic diseases from diabetes and heart disease to lung and colon cancer.\(^{[161, 234-236]}\) This preventative effect is thought to be due do EGCG’s antioxidant activity.\(^{[144, 237, 238]}\)

Colorectal cancer is the second leading cause of cancer-related deaths. It is estimated that 136,830 people will be diagnosed with colorectal cancer and 50,310 people will die from it in the United States in 2014.\(^{[3]}\) Inflammation and oxidative stress have been linked to colon cancer development and progression.\(^{[47]}\)

Inflammatory bowel diseases (IBD), including ulcerative colitis and Crohn’s disease, are characterized by continuous or periodic inflammation of the colon. According to epidemiological studies, patients suffering from IBD are nearly 3 times more likely to develop colorectal cancer.\(^{[239]}\) IBD is characterized by increased colonic oxidative stress, production of inflammatory cytokines by both the colon epithelium as well as infiltrating inflammatory cells, and loss of gut barrier function.\(^{[114]}\)

Previous studies have reported that green tea polyphenols (GTP) can reduce markers of inflammation in mouse models of ulcerative colitis. One study found that APC\(^{\text{min/+}}\) mice treated with 0.08% and 0.16% EGCG in the drinking water had a dose response reduction in small intestinal tumors of 37% and 47% respectively.\(^{[178]}\) In another APC\(^{\text{min/+}}\) and AOM mouse model, researchers found that 0.6% green tea treatment reduced the number of newly formed tumors by 28%.\(^{[179]}\) In DSS mouse models of IBD, EGCG has yielded some confounding results. In a recent study, AOM and 1.5% DSS treated mice were given 0.03-0.3% EGCG in their
diet and showed a decrease in tumor formation for the high level EGCG dose, though it was not significant.[183] In the same study, the researchers examined the effects of EGCG after a high concentration (5%) of DSS and found that .3% EGCG in the diet actually caused an increase in the number of tumors formed. This dose mimics deleterious effects of high doses of green tea or EGCG on those with late stages colorectal cancer development.[172] Another study found that green tea induced nephrotoxicity and caused mRNA expression of antioxidant enzymes and heat-shock proteins to decrease significantly.[184] This study was also performed using 5% DSS, which has the ability to cause severe acute colitis, further suggesting that EGCG may contribute to the severity in highly inflamed areas. One study induced low-grade colitis by alternating DSS treatment with water and then gavaged the mice with 6.9 mg/kg EGCG and 2.9 mg/kg piperine.[181] The piperine was used to slow gastric motility and potentially increase the bioavailability of EGCG.[182] Oral gavage results in a large dose of EGCG once per day as opposed to ad lib access to EGCG in the drinking water which would provide a more constant level of EGCG throughout the experiment. The results of this study found that EGCG and piperine reduced weight loss associated with colitis, improved histological scoring, and decreased lipid peroxidation products in the large intestine. Other inflammation studies focused on the endothelium have found that EGCG improves barrier function however no studies have looked at its effect on epithelial cells.[240]

Some studies have indicated dose-limiting toxicities for GTP and EGCG. For example, at higher doses of GTP (10 mg/mL in drinking fluid), DSS-induced nephrotoxicity and hepatotoxicity are exacerbated compared to DSS-induced controls. Higher doses of EGCG (0.5 mg/g diet) exacerbated DSS-induced rectal bleeding and colonic inflammation.

In the present study, we selected a moderate dose of EGCG (3.2 mg/mL water) and examined the effect of EGCG on markers of acute inflammation, gastrointestinal permeability, and macronutrient availability in DSS-induced CF-1 mice. We observed that EGCG suppressed
DSS-induced markers of colon inflammation and increases in GI permeability compared to DSS-induced controls. EGCG treatment also induced transient weight loss and decreased feeding efficiency in DSS-induced mice. These effects appear to be due to decreased fat and protein digestion. Overall these results indicate that EGCG effectively mitigated DSS-induced colon inflammation, but that supportive nutritional therapy may be needed to prevent EGCG-induced body weight loss.
2.3 Materials and Methods

2.3.1 Reagents

EGCG (93% pure) was purchased from Taiyo Green Power (Jiangsu, China). DSS (mw; 36 – 50 kDa) was purchased from MP Biomedicals, LLC (Solon, OH). Bradford Reagent and fluorescein sodium salt (FITC-D, average mw 4,000) were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents were of the highest quality commercially-available.

2.3.2 Mice

All animal experiments were approved by the Institutional Animal Care and Use Committee at The Pennsylvania State University (IACUC #37550). Male CF-1 mice (5 wks old) were purchased from Jackson Laboratories (Bay Harbor, ME), allowed to acclimate for 1 wk prior to the start of the experiment. Mice were housed 5 per cage and had ad libitum access to AIN76A diet (Research Diets, New Brunswick, NJ) and water. All mice were maintained at 20 ± 2°C and on a 12 h light–dark cycle.

2.3.3 Dietary Treatments

All treatment periods lasted 10 d. 40 mice were randomized into 4 treatment groups (10 per group) based on body weight: DSS treated mice (D) were given DSS (1.5% w/v) as the sole source of drinking fluid for 7 d and then deionized water containing citric acid (5 mg/mL) for 3 days; DSS plus EGCG (DE) treated mice were treated for 7 d with 1.5% DSS and then 3 d with deionized water containing EGCG (3.2 mg/mL) and citric acid (5 mg/mL) for the final 3 d. The control mice (C) received deionized water for 7 d and then deionized water containing citric acid
(5mg/mL) for the last 3 d; the EGCG control mice (E) received deionized water for 7 d followed by EGCG (3.2 mg/mL) and citric acid (5 mg/mL) for the final 3 d. Food and water consumption, as well as body weight were monitored daily. Feeding efficiency was determined by dividing the change in body weight gained by the energy (in kJ) consumed.

2.3.4 Sample Collection

At the end of the experiment, blood was collected by cardiac puncture from anesthetized mice and plasma was prepared by centrifugation at 700xg for 15 min and stored at -80°C before analysis. Spleens were removed, rinsed with 0.9% NaCl, weighed, and stored at -80°C. The colons were harvested and fecal contents were collected and frozen at -80°C. The colons were flushed with 9 mg/mL NaCl and then snap frozen at -80°C.

2.3.5 Enzyme Immunoassay

Colon tissues were homogenized in ice-cold T-PER reagent (Thermo Scientific) containing nordihydroguaiaretic acid (10 μM), indomethacin (10μM), protease inhibitor (1:100), and phosphatase inhibitor cocktails (1:100) using a Bullet Blender (Next Advance) and 0.2 mm stainless steel beads. Samples were centrifuged at 15,000xg for 15 min and the protein concentration in the supernatant was determined using the Bradford Reagent. IL-1β, IL-6, TNF-α, and MCP-1 protein levels in colon homogenate and plasma were quantified by ELISA (R&D Biosystems). GLP-2 protein levels were also determined by ELISA (MyBioSource, Inc.,San Diego, CA).
2.3.6 Liver and Kidney Toxicity Assays

Plasma alanine aminotransferase (ALT) and blood urea nitrogen (BUN) levels were used as markers of hepatotoxicity and nephrotoxicity, respectively. ALT and BUN levels were determined using commercially-available assay kits (Catachem, Inc.) according to the manufacturer’s directions.

2.3.7 Fecal Macronutrient Analysis

Fecal samples obtained during dissection were pooled by cage, homogenized in water, freeze dried, and ground using mortar and pestle. Samples were analyzed for nitrogen using a CE Instruments EA 1110 CHNS-O elemental analyzer (Thermo-Fisher). The resulting dry weight nitrogen was converted to protein using 5.6 as a conversion factor.[241] For fecal lipids analysis, freeze dried feces were re-dissolving in an equal mass of water and extracted with methanol:chloroform (2:1, v:v). The organic phase was collected, filtered, and dried under vacuum. The resultant residue was weighed and normalized to freeze-dried fecal mass.

2.3.8 Plasma Endotoxin Analysis

Plasma endotoxin levels were analyzed using a Limulus Amebocyte Lysate (LAL) kit (Lonza) according to the manufacturer.

2.3.9 Intestinal Permeability Analysis

Gastrointestinal permeability was determined using the 4 sugar assay,[128, 242] In brief, mice from each treatment group (n = 5 per group) were fasted for 12 h prior to the end of the
experiment and given a single oral bolus dose (4mL/kg bw) of L-rhamnose (3.33 mg/mL), erythritol (6.66 mg/mL), lactulose (6.66 mg/mL), and sucralose (6.66 mg/mL) in water. Mice were housed in metabolic cages and the urine was collected for 3 h. During this time, mice had ad libitum access to their corresponding treatment waters, but not food.

Urine samples were extracted with a mixture of dichloromethane:methanol (2:1), filtered, and dried in a centrifugal evaporator. Samples were silylated as described previously and analyzed by gas chromatography-mass spectrometry. In brief, 1 µL samples were introduced into an HP 6890 gas chromatograph interface with a HP 6890 mass selective detector (GC-MS). A DB5-MS capillary column (30 m×0.25 mm I.D. and film thickness of 0.25µm, Agilent, Palo Alto, CA, USA) with helium (Airgas) as the carrier gas was used at a constant flow rate of 1.3 mL/min for chromatographic separation. The injector was maintained at 200°C and the MS source at 250°C. The temperature profile consisted of: injection at 180°C with a 2 min hold, a linear increase of 6°C/min to 300°C, and an isothermal hold at 300°C for 15 min. The MS was set to a scan of 50 to 650 Da at 1.27 scan/s. All samples were analyzed in splitless mode. All data was processed using HP-Chemstation software. Silylated sugars were quantified using total ion current (TIC) peak area.

2.3.10 Caco-2 Cell Culture and Monolayer Permeability Assay

Caco-2 human colon cancer cells (American Type Culture Collection, Manassas, VA) were maintained in sub-confluence in Dulbecco’s Modification of Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 IU/mL penicillin and 100 µg/mL streptomycin at 37°C under 5% CO₂ atmosphere. Cells were sub cultured by trypsinization. Paracellular permeability was observed by measuring the apical to basolateral flux of fluorescein isothiocyanate–dextran (FITC-D) as described previously. A trans-well system with was
used for these experiments (Corning, Tewksbury, MA). Caco-2 cells were seeded onto a polycarbonate insert (0.33 cm² area and 0.4 µm pore size) in a Transwell plate and allowed to differentiate for 21 d. Monolayer integrity was assessed using a Millicell-ERS2 Volt-Ohm Meter (Millipore, Co.Billerica, MA) and only monolayers with transepithelial electrical resistance of 500-600 Ω cm² were used for experiments.[243] EGCG (0, 5, and 25 µg/mL) was added to the apical chamber for 2 h prior to addition of 3% DSS to the media. After the addition of DSS, cells were co-incubated with EGCG for 48 h, washed with fresh media, and medium containing 1 mg/mL FITC-D was added to the apical compartment. Levels of FITC-D were determined in the basolateral compartment every 30 min for 6 h using a Fluoroskan Ascent FL fluorescent plate reader (Thermo Scientific, Waltham, MA).

2.3.11 Quantitative Reverse-Transcriptase PCR

Total RNA was isolated and DNA contamination was removed from homogenized colon samples using the RNeasy Mini Kit from Qiagen (Valencia, CA) and quantified with a Nanodrop 2000 spectrophotometer. The RNA was reverse-transcribed to cDNA using the RT² HT First Strand Kit (SA Biosciences, Valencia, CA) and real-time PCR was performed in 384-well plates using an Applied Biosystems 7900HT Fast Real-Time PCR System (San Francisco, CA). Reactions contained 5 µL PerfeCTa® qPCR SuperMix, ROX™ Quanta BioSciences (Gaithersburg, MD), 0.5µL TaqMan® hydrolysis probe (Table 2-1) Life Technologies (Grand Island, NY, Table 1), and 4.5 µL diluted cDNA. The PCR program included an initial incubation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. The data was analyzed with Sequence Detector Software (Applied Biosystems). Relative gene expression was determined using the $2^{-\Delta\Delta CT}$ method, where
\[ \Delta C_T = (C_{\text{target}} - C_{\text{reference}}), \text{with } Gapdh \text{ as the reference gene. } \Delta C_T \text{ values were used for statistical tests and } 2^{\Delta C_T} \text{ values were used for graphical representation.} \]

**Table 2-1.** Taqman® hydrolysis probes used in this study

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Gene name</th>
<th>Accession ID</th>
<th>Manufacturer’s no.</th>
<th>Amplicon Length (bp)</th>
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<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
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<td>Mm99999915_g1</td>
<td>107</td>
</tr>
<tr>
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<td>NM_016674.4</td>
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<tr>
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<td>Occludin</td>
<td>NM_008756.2</td>
<td>Mm0050912_m1</td>
<td>134</td>
</tr>
</tbody>
</table>

### 2.3.12 Statistical Analysis

Samples were analyzed for statistical significance using GraphPad Prism. One way ANOVA coupled with Tukey’s Multiple Comparison Test were used to compare samples. The mouse and cell experiments and all analyses were done in triplicate.
2.4 Results

2.4.1 Suppression of Colitis *in vivo*

DSS treatment lead to shortening of the colon and increased relative spleen weight indicating increased inflammation (*Figure 2-1a* and *Figure 2-1b*). EGCG significantly mitigated DSS-induced colon shortening and increases in relative spleen weight. Treatment with EGCG alone had no significant effect on either colon length or spleen weight. DSS induced body weight loss compared to control mice or mice treated only with EGCG. This loss began on d 6 and continued for the duration of the experiment (*Figure 2-1c*). DE mice showed a significantly greater body weight loss than DSS-treated controls for the first two days of treatment but their weight began to rebound on the third day of EGCG treatment. Plasma markers for liver toxicity (ALT) and kidney toxicity (BUN) were not significantly different among the controls or treatment groups (*Figure 2-1c* and *Figure 2-1d*). Due to lack of sample, group E was not measured for the kidney toxicity marker BUN.
Figure 2-1. The effects of dietary EGCG on gross measures of inflammation, weight gain, and toxicology markers in DSS-induced CF-1 mice.

(a) Colon length and (b) relative spleen weight were determined at the end of the experiment. (c) The feeding efficiency of the mice was determined based on the ratio of body weight gain to energy intake and averaged over the last 3 d of the experiment. (d) Liver toxicity marker, ALT, and (e) kidney toxicity marker, BUN, were determined from plasma samples. All values represent the means ± SEM and an n = 10 for each group. Different letters denote p < 0.05 using one-way ANOVA with a Tukey’s Multiple Comparison post-test.
2.4.2 Decreased Feeding Efficiency and Macronutrient Malabsorption

Treatment with DSS induced a 1.2-fold decrease in feeding efficiency (Figure 2-2a). Treatment of DSS-induced mice with EGCG exacerbated this decrease in feeding efficacy (3.5-fold decrease) compared to control mice. DSS treated groups had elevated fecal lipid levels of (Figure 2-2b). EGCG treatment significantly increased fecal lipids compared to DSS-only treated mice. DSS treatment also increased fecal protein levels of (Figure 2-2c). As with fecal lipids, fecal protein levels were also increased by treatment with EGCG.

Figure 2-2. The effects of dietary EGCG on feeding efficiency and macronutrient malabsorption in DSS-induced CF-1 mice.

(a) The feeding efficiency of the mice was determined based on the ratio of body weight gain to energy intake and averaged over the last 3 d of the experiment. Dry weight percentages of (b) fecal lipids and (c) fecal proteins were analyzed. All values represent the means ± SEM and an n = 10 for each group. Different letters denote p < 0.05 using one-way ANOVA with a Tukey’s Multiple Comparison post-test.
2.4.3 Biochemical Markers of Inflammation and Oxidative Stress in DSS-treated Mice

Protein levels of IL-1β, IL-6, TNF-α, and MCP-1 were significantly increased in colon homogenates from DSS-treated groups (Figure 2-3a – d). Due to lack of sample, TNF-α levels for group E were not measured.

![Figure 2-3](image)

**Figure 2-3.** Biochemical markers of colonic inflammation in DSS-induced CF-1 mice.

Protein levels of (a) IL-1β, (b) IL-6, and (c) TNF-α, and (d) MCP-1 were determined in the colon homogenate using commercially-available ELISAs and normalized to the total protein of each sample. (e) Lipid peroxide levels in colon homogenate were determined using the TBARS assay and normalized to total protein levels in the homogenate. (f) Plasma endotoxin content. All values represent the means ± SEM and an n = 10 for each group. Different letters denote p < 0.05 using one-way ANOVA with a Tukey’s Multiple Comparison post-test.
EGCG significantly decreased colonic levels of IL-1β (23%), IL-6 (54%), TNF-α (58%), and MCP-1 (21%) in DSS-induced mice. Colonic lipid peroxide levels were increased 193% in DSS-treated mice (Figure 2-3e). This increase was completely mitigated by treatment of DSS-induced mice with EGCG. Due to lack of sample, colonic lipid peroxide levels for group E were not measured. Plasma endotoxin levels tended to increase in DSS treated mice, but the effect was not statistically significant (Figure 2-3f).

2.4.4 Gut Barrier Function in DSS-Treated Mice

Treatment with EGCG reduced apical to basolateral flux of FITC-D across DSS-treated Caco-2 cell monolayers (Figure 2-4a). Pre-treatment with EGCG for 2 h followed by 48 h co-incubation reduced flux in a dose dependent manner. In vivo, EGCG blunted DSS-induced gastrointestinal permeability (Fig. 3b and c). The lactulose to rhamnose ratio in the plasma, a measure of small intestinal permeability, was increased by DSS-treatment, but the effect was blunted by treatment with EGCG (Figure 2-4b). Similarly, DSS treatment increased the plasma ratio of sucralose to erythritol, a marker of increased large intestinal permeability (Figure 2-4c). EGCG also mitigated increased large intestinal permeability in the mice treated with DSS (Figure 2-4c). GLP-2 protein levels in the colon homogenate of DSS treated mice was decreased by 47% compared to the water-treated control group however, EGCG supplementation normalized colonic GLP-2 levels (Figure 2-4d). DSS treatment tended to increase the mRNA expression of the tight junction protein, claudin-1, but decrease the expression of the tight junction protein, occludin compared to negative control mice (Figure 2-4e and Figure 2-4f). The result was a trend towards an increased ratio of claudin to occludin, although the effects were not statistically significant (Figure 2-4g). EGCG tended to mitigate these changes resulting in a claudin to occludin ratio that was more similar to the water-treated control mice.
Figure 2-4. Markers of intestinal permeability in Caco-2 cell transwell experiments and mouse colon homogenate.

(a) The effect of EGCG on DSS-induced increases in the permeability of Caco-2 monolayers was determined by measuring the apical to basolateral flux of FITC-D after 48 h incubation with 2% DSS and EGCG treatment. (b) Plasma sugar ratios lactulose:rhamnose and (c) sucralose:erythritol were determined by GC-MS. (d) GLP-2 protein levels in the colon homogenate as determined by ELISA and normalized to the total protein of each sample. Relative fold change of mRNA expression of (e) Claudin-1 and (f) Occludin in colon homogenate was determined by quantitative reverse transcriptase PCR and normalized to the expression of GAPDH expression. (g) The ratio of Claudin-1 to Occludin was used as a marker of gut barrier function, with a decreased ratio representing compromised barrier function. All values represent the means ± SEM and an n = 10 for each group. Different letters denote p < 0.05 using one-way ANOVA with a Tukey’s Multiple Comparison post-test.
2.5 Discussion

Epidemiological and animal model studies have generally shown that green tea polyphenols have colon cancer preventive effects. Green tea polyphenols have been shown to reduce markers of inflammation and colonic tumor incidence.[178, 179, 181, 240]

In this study, we examined the effect of oral EGCG on acute colonic inflammation in DSS-induced CF-1 mice. We found that EGCG supplementation modulated DSS-induced colon shortening and increased spleen weight, as well as colonic levels of inflammatory cytokines and colon malondialdehyde levels. These results confirm and extend previous studies on the anti-inflammatory effects of EGCG (alone or as part of a green tea polyphenol mixture).

We report for the first time, that EGCG can mitigate DSS-induced loss of gut barrier function in vitro and in DSS-treated mice. EGCG dose-dependently reduced the apical to basolateral flux of FITC-D across DSS-treated Caco-2 cells. In vivo EGCG supplementation reduced the ratio of lactulose to rhamnose and the ratio of sucralose to erythritol in the plasma of DSS-induced mice compared to DSS-only treated controls. The former ratio indicates permeability across the small intestine, whereas the latter indicates permeability across the large intestine.[128, 244, 245]

The peptide hormone, GLP-2, has been shown to improve gut barrier function and adaptation to barrier function injury.[246, 247] In our study, we found that DSS decreased colonic GLP-2 protein levels in colon, and treatment with EGCG ameliorated this decrease. Changes in tight junction proteins, such as increases in claudin-1 and decreases in occludin, have also been tied to colitis and other inflammatory bowel conditions [248]. We found mRNA expression claudin-1 were elevated in the DSS only treated group, and while the findings were not significant they do suggest a trend. Likewise, occludin was down-regulated in the DSS only treated group as compared to EGCG and DSS fed group. The trend is similar to the results of
clinical studies with IBD sufferers where an increased ratio of claudin-1 to occludin corresponded to increased severity of disease [248].

Loss of gut barrier integrity can allow gastrointestinal microorganisms and microbial components access to the basolateral side of the gastrointestinal epithelium and the systemic circulation resulting in systemic inflammation. Here we found that plasma endotoxin levels tended to increase in DSS-treated mice and that these results were mitigated by EGCG treatment. Taken together the results indicate that EGCG supplementation can enhance gut barrier function in the context of inflammatory bowel disease.

Although our studies demonstrate the anti-inflammatory effects of EGCG in the context of IBD, we also observed that EGCG-treated mice lost significantly more weight than DSS-only control mice. These results are similar to those published previously by Inoue et al. and suggest a dose-limiting toxicity for EGCG.[184]

Previously, we have reported that high dose, intragastric EGCG (750 – 1500 mg/kg, b.w.) can induce hepatotoxicity, and others have suggested that dietary doses of EGCG (525 mg/kg, b.w. as part of a green tea polyphenol preparation) can induce liver and kidney toxicity in DSS-induced mice.[16, 184] In the present study, however, we found that EGCG did not significantly alter either plasma ALT or BUN, markers of hepatotoxicity or nephrotoxicity, respectively. This difference could be due to differences in dose (our total daily dose was 480 mg/kg, b.w.) or dosage form (we used EGCG as a single component compared to the green tea polyphenol mixture used previously).

Alternatively, the difference could be due to differences in data interpretation. Although Inoue et al., have reported that dietary GTP can induce hepatotoxicity and nephrotoxicity in DSS-induced mice, a close examination of the results of that study show that plasma ALT and plasma aspartate transaminase levels were not increased relative to DSS-induced controls.[184]

Similarly, markers of oxidative stress in the kidney and liver tissue of GTP-treated, DSS-induced
mice were not significantly different than those in mice treated only with GTP. This suggests that the interactions between GTP and IBD are more complicated than previously reported.

We compared food intake to body weight gain and found that EGCG significantly decreased feeding efficiency compared to DSS-treated controls. Dietary polyphenols have historically been viewed as anti-nutritionals in animal husbandry, and previous work by our laboratory and others have demonstrated that dietary EGCG can decrease body weight gain and decreased dietary macronutrient (lipids and protein) digestion and absorption.[186, 188, 249] Here, we observed that EGCG treatment significantly increased fecal levels of both lipids and proteins indicating that EGCG interfered with macronutrient digestion and energy absorption. Within the context of over-nutrition and obesity, such effects may be viewed as beneficial; however in the context of compromised nutritional status (e.g. mice with active colitis) anti-nutritional effects may lead to undesirable effects including the weight loss observed in the present study.

In summary, the results of this study demonstrate that EGCG can mitigate colonic inflammation and oxidative stress in DSS-induced mice, confirming and extending previous studies. They indicate that EGCG can protect gut barrier function in the context of IBD. Our results, however, indicate that EGCG-induced macronutrient malabsorption represents a dose-limiting toxicity and that, if EGCG is to be translated to the clinic as a potential treatment for IBD, appropriate nutrient support is required to mitigate these deleterious effects. The present study may serve as a guide for the preparation of EGCG-containing formulations with increased efficacy and decreased side-effects for use in future human intervention studies.
Chapter 3

Dietary Soy Protein Concentrate Suppresses Colonic Inflammation and Loss of Gut Barrier Function In vitro and in Mice

Another dietary component that has been studied in addition to green tea as far as intestinal inflammation and colorectal cancer prevention is soy. Previous unpublished work done by our lab in an AOM/DSS mouse model found that long-term soy protein supplementation in the diet caused a decrease in inflammation scores and hyperplasia and dysplasia scores, however, these results were not significant. Due to the lack of significance, we set out to look at the biochemical markers of inflammation as a result of an acute colitis attack. Previous work had suggested that, like EGCG, soy protein had the ability to scavenge ROS in vitro and in vivo cell studies. As a result, we set out to examine the effects of soy protein concentrate supplementation on biomarkers of inflammation in acute DSS-induced colitis in mice and gut barrier modulation in Caco-2 monolayer permeability studies.

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2 To be published as:
Bitzer Z.T., Wopperer A.L., Tao L., Cooper T.K., Vanamala J., Elias R.J., Lambert, J.D. Dietary Soy (Glycine max) Protein Concentrate Suppresses Colonic Inflammation and Loss of Gut Barrier Function In vitro and in Mice
3.1 Abstract

Inflammatory bowel disease afflicts nearly 2.2 million people worldwide. Soy (*Glycine max* L., Fabaceae) is an important dietary constituent and may have beneficial effects against a number of chronic diseases. We examined the efficacy of soy protein concentrate (SPC) to suppress inflammation and oxidative stress, and to improve gut barrier function. SPC exhibited radical scavenging activity and protected Caco-2 human colon cells from H$_2$O$_2$-induced cytotoxicity. SPC also reduced dextran sulfate sodium (DSS)-induced increases in Caco-2 monolayer permeability. Dietary SPC reduced colonic protein levels of interleukin (IL)-1β, IL-6, and monocyte chemotactic protein-1 in DSS-induced CF-1 mice. A similar decrease in *Il1b* and EGF-like Module-containing Mucin-like Hormone Receptor-Like 1 and toll-like receptor 4 mRNA was observed. SPC increased colon mRNA levels of glucagon-like peptide 1 and tended to normalize the mRNA expression of claudin-1 and occludin. Our results suggest that SPC attenuates colonic inflammation in part by suppressing oxidative stress and intestinal permeability.
3.2 Introduction

Colon cancer is the second leading cause of cancer related deaths in the United States. The National Cancer Institute estimates that 136,830 men and women will be diagnosed with colorectal cancer in the United States in 2014.[3] Colon cancer progression has been linked to DNA damage caused by inflammation and reactive oxygen species (ROS).[47]

Inflammatory bowel disease (IBD) is a spectrum of diseases, including ulcerative colitis and Crohn’s Disease, characterized by either continuous or periodic inflammation of the colon. It has been estimated that nearly 4 million people worldwide suffer from IBD. Epidemiological studies have suggested that those suffering from IBD are nearly 3 times more likely to develop colorectal cancer.[239] In addition, IBD diminishes quality of life and represents a significant economic burden: in the United States, it is estimated that the total economic burden of ulcerative colitis and Crohn’s disease is US$19-30 billion annually.[27, 28]

IBD is characterized by increased colonic oxidative stress and inflammation as well as loss of gut barrier function.[114] ROS and inflammatory cytokines, such as interleukin-1 β (IL-1β) and interleukin-6 (IL-6), are released by inflamed epithelial cells and resident immune cells. Pro-IL-1β is converted to its mature form as a result of activated caspase-1. Caspase-1 is activated by inflammasome formations in the cell as a result of NFκB and ROS.[60] Inflammasome formation is dependent on NACHT, LRRm,and PYD domains-containing protein 3 (NLRP3) and its incorporation into the inflammasome as a result of ROS interactions with thioredoxin interacting protein (TXNIP).[250] Recent studies have also demonstrated that by blocking ROS generation, NLRP3 inflammasome response decreases and subsequently so does caspase-1 activation and maturation of IL-1β.[84]

Loss of gut barrier function and increased gut permeability occur when the tight junction proteins between intestinal epithelial cells are damaged or down-regulated.[30, 31] This decrease
in barrier function allows bulky or highly charged molecules, including gut bacteria-derived endotoxins, to pass into systemic circulation resulting in systemic inflammatory conditions. Increased intestinal permeability has also been linked to the development of colon cancer.[103]

Soybeans (*Glycine max* L., Fabaceae) have been widely studied for their cancer preventive effects.[199, 251-254] Many of these studies have focused on the soy isoflavones, which have yielded preventive effects in some model systems, but no effect or tumor promoting effects in others. To date, data suggests that colon cancer represents this latter case. For example, Rao et al., have reported that dietary supplementation of azoxymethane (AOM)-treated F344 rats with 0.025% genistein enhanced colon adenocarcinoma multiplicity and colonic mucosal levels of 8-isoprostane compared to AOM-treated controls.[255] We have previously reported that genistein (0.2%) in combination with the green tea polyphenol, (-)-epigallocatechin-3-gallate (0.01%), enhanced tumorigenesis in the *Apc^{Min/+}* mouse.[215] The underlying mechanisms for these increases in tumorigenesis are unclear, but seem to indicate that the isoflavone components of soy do not play a role in the associated reduction of colon cancer risk reported in previous epidemiological studies.[256]

In addition to their isoflavones, soybeans and soybean-derived foods are high in protein and peptides, either as endogenous components or resulting from fermentative or enzymatic hydrolysis of soy protein *in vivo.[257]* These proteins and peptides have been found to have antioxidant activity in cell-free models.[258-261] Soy-derived peptides, such as lunasin, have also been found to have anti-inflammatory activity in macrophage cells in culture.[231, 262] One study found that treatment of dextran sulfate sodium (DSS)-induced mice with 20% soy protein supplemented diet had significantly reduced colon shortening, colonic inflammation scores, and reduced colonic mRNA expression of tumor necrosis factor alpha.[228] More recently, studies in piglets and Balb/c mice with DSS-induced colitis found that soy derived di- and tripeptides, in particular the tripeptide, Val-Trp-Tyr, decreased inflammatory cytokine production as compared
to the DSS-only control.[232, 233] The relative contribution of this peptide to the overall activity of soy protein was not assessed.

The ability for a given protein to quench ROS is highly dependent on both the tertiary structure as well as its amino acid composition. Certain amino acids are more capable of chelating metals or scavenging free radicals than others, but if it is hidden within a hydrophobic pocket of a protein, these amino acids may be unable to react with hydrophilic pro-oxidant species.[263] In general, the hydrolysis of a protein increases its antioxidant activity, and this hydrolysis mimics the digestion of protein by gastric and pancreatic proteases (pepsin, trypsin and chymotrypsin) [264]. Disruption of the tertiary structure through heat treatment or enzymatic hydrolysis is a common approach for increasing the antioxidant activity of proteins in vitro. Several authors have reported that enzymatic hydrolysis of soy protein isolate to small peptides, but not free antioxidants, can enhance its antioxidant activity.[265, 266]

Although recent studies on the effect of soy protein are promising, many questions remain including the role of the antioxidant effects of soy protein in reduction of colonic inflammation and the impact of soy protein on gut barrier function. In the current study, we examined the radical scavenging activity of soy protein in cell-free and in vitro cytoprotection model; the anti-inflammatory effects of soy protein concentrate, specifically the development of IL-1β, in a mouse model of colonic inflammation; and ability of soy protein to modulate intestinal permeability in both cell and animal models.
3.3 Materials and Methods

3.3.1 Materials

Soy protein concentrate (SPC: 69% protein, 3% fat, 19% dietary fiber, 6% moisture, and 7% ash, Arcon SJ #066-408) was a gift from the Archer Daniels Midland Company (Decatur, IL). 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH), N-ethylmaleimide, fluorescein sodium salt (FITC-D, average mw 4,000), pancreatin, and pepsin, were purchased from Sigma-Aldrich (St. Louis, MO). 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate, di(acetoxymethyl ester) (DCDHFDA) was purchased from Invitrogen (Carlsbad, CA). DSS (mw = 30,000 – 50,000) was purchased from MP Biomedical, LLC (Solon, OH). Experimental diets for mouse studies were prepared by Research Diets, Inc. (New Brunswick, NJ) (Table 3-1). All other chemicals used were of the highest grade commercially available.

3.3.2 Solubilization and Fractionation of SPC

SPC was solubilized in water as follows for use in the ORAC and cell culture assays. SPC was suspended in deionized water (1:20 w/v) and the pH was adjusted to 11 using 1M NaOH under constant agitation to increase solubility. After 2 h, the pH was slowly adjusted back to 7.4 using 1M HCl. The solution was centrifuged at 3200 x g for 20 min at 20°C. The resulting supernatant was lyophilized and stored at -80°C until use. Solubilized SPC was dissolved in deionized water (1:20 w/v) and spun through a 10 kDa molecular weight cutoff centrifugal filter Millipore (Billerica, MA) according to the manufacturer’s instructions. The flow-through (SPC-L) was collected, lyophilized, and stored at -80°C until use. The slurry (SPC-H) that did not pass through the filter was also collected, lyophilized, and stored at -80°C until use. Protein content was determined in SPC, SPC-L, and SPC-H using the Bradford assay.
3.3.3 Soy Protein Concentrate Hydrolysis

The enzymatic hydrolysis of SPC was accomplished using a previously published method.[267] In brief, SPC was suspended in deionized water (1:20 w/v) and heated to 80°C for 5 min to reduce any potential bacteria population as well as to denature lipoxygenase. The pH was reduced to pH 2, pepsin was added (1:100 w/w, enzyme/SPC), and the solution was incubated at 37°C for 3 h. The pH was increased to 7.5, pancreatin was added (1:20 w/w, enzyme/SPC), and the mixture was then incubated at 37°C for 3 h. Hydrolysis was stopped by heating to 75°C for 20 min. The hydrolysate (HSPC) was centrifuged at 25,000 x g for 15 min, and the resulting supernatant was lyophilized and stored at -80°C.

3.3.4 Thiol analysis, Oxidation, and Thiol Blocking of SPC and HSPC.

Free thiol groups in SPC were blocked using N-ethylmaleimide (NEM) as previously described. [268] In brief, SPC and SPH were dissolved in phosphate buffer (0.1mM, pH 8) at a final concentration of 25 mg/mL and reacted with NEM (3.45 mmol/g of protein) for 15 min at 25 °C. SPC was oxidized in an analogous manner using H₂O₂ (10 mmol/g of protein). Excess NEM and H₂O₂ were removed by dialysis with 500 molecular weight cutoff dialysis tubing at 5 °C against 100 parts of 10 mM sodium acetate and imidazole buffer (pH 7). The buffer was changed at 6, 12, 18, and 24 h. [268] Sulfhydryl groups were measured afterwards using Ellman’s reagent (5,5'-dithio-bis-[2-nitrobenzoic acid). [269] Only samples where the levels of free thiols dropped below the limit of detection were used for studies. Samples were then lyophilized and stored at -80°C.
3.3.5 Antioxidant Capacity of SPC Preparations

The antioxidant capacity of the SPC preparations was determined using the oxygen radical absorption capacity (ORAC) assay. In brief, SPC samples were dissolved in phosphate buffer (10 mM, pH 7.4) to a final concentration of 100 µg/mL and combined with fluorescein (final concentration = 8.6 nM) in a 96-well black opaque plate. After incubation for 30 min at 37°C, fluorescence was measured (λ<sub>Ex</sub> 485 nm, λ<sub>Em</sub> 520 nm). AAPH (final concentration = 30 nM) or phosphate buffer was then added and fluorescence was measured every 90 seconds for 90 min. Fluorescence values were normalized to time 0 and the area under the curve determined for each treatment.

3.3.6 Cell Culture and Cytotoxicity

Caco-2 cells (American Type Culture Collection, Manassas, VA) were maintained in sub-confluence in Dulbecco’s Modification of Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 IU/mL penicillin and /100 µg/mL streptomycin at 37°C under 5% CO₂ atmosphere. Caco-2 cells were seeded to a 96-well plate (5 x 10<sup>3</sup> cells/well) and allowed to attach for 24 h before treatment. For the co-treatment experiment, cells were simultaneously exposed to 50 µM H₂O₂ and varying concentrations of SPC (0 – 0.5 mg/mL) in PBS for 60 min at 37°C. For the pre-treatment experiment, cells were incubated with varying concentrations of SPC for 30 min at 37°C. The cells were then washed with PBS, and treated with 50 µM H₂O₂ for 60 min at 37°C. After each experiment, cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.
3.3.7 Effect of SPC on Intracellular ROS

Caco-2 cells were seeded in Petri dishes (1.5 x 10^4 cells/cm²). After 48 h, cells were exposed to 0 or 50 µM H₂O₂ in PBS in the presence or absence of 1.0 mg/mL SPC for 60 min at 37°C. Cells were washed with DMEM and incubated with 10 µM DCDHFDA at 37°C for 30 min, washed twice with PBS, once with DMEM, and fluorescence was observed under an Olympus BX-51 Fluorescence Microscope (λ_{Ex.}= 490 nm, λ_{Em.} = 525 nm).

3.3.8 Caco-2 Monolayer Permeability

Caco-2 cells were seeded in polycarbonate transwell inserts (0.33cm² area and 0.4 µm pore size, Corning Life Sciences, Tewksbury, MA) and allowed to reach confluence and differentiate for 21 d. Based on previous studies, only monolayers with a transepithelial electrical resistance (TEER) of 500-600 Ω cm² were used.[243] The monolayers were treated with SPC samples (0 – 100 µg/mL) for 2 h prior to addition of 2% DSS to the media. After addition of DSS, cells were co-incubated for an additional 48 h. The paracellular permeability was observed by measuring the apical to basolateral flux of FITC-D as described previously.[136] Cells were then washed with fresh media, and 1 mg/mL FITC-D was added to the apical compartment and cells were incubated for 6 h. FITC-D levels were determined in the basolateral compartment every 30 min using a Fluoroskan Ascent FL fluorescent plate reader (Thermo Scientific, Waltham, MA).

3.3.9 Animals and Treatment

All experiments were approved by the Institutional Animal Care and Use Committee at The Pennsylvania State University (IACUC #29544). Male, CF-1 mice (Charles River
Laboratory, Wilmington, MA, 5 wks old) were randomized into 4 treatment groups (10 mice per treatment) and then housed 5 per cage in shoebox cages on corn cob bedding and maintained on a 12 h light/dark schedule with *ad libitum* access to food and water. Mice were allowed to acclimate for 5 – 7 d prior to the start of experiments.

3.3.10 DSS-Induced Colitis

Mice were randomized based on weight into control (water and AIN93G diet), DSS (1.5% DSS in drinking fluid and AIN93G), DS6 (1.5% DSS and 6% dietary SPC), and DS12 (1.5% DSS and 12% dietary SPC). Mice were treated with DSS and experimental diets for 7 d, and DSS was replaced with water for 3 additional days. Body weight, food, and fluid consumption were measured daily. Feeding efficiency was determined by dividing the body weight gained during the experiment by total energy intake (in kJ). Upon euthanasia, blood was collected by cardiac puncture. Spleens were removed, weighed, and fixed in formalin. Colons were resected and the length measured. They were then split longitudinally, washed with cold 0.9% NaCl solution, and frozen at -80°C.

3.3.11 Biochemical Analysis

Colon tissues were homogenized in T-PER reagent (Thermo Scientific) supplemented with nordihydroguaiaretic acid (10 μM), indomethacin (10 μM), protease inhibitor (1:100), and phosphatase inhibitors (1:100) using a Bullet Blender (Next Advance) with 0.2 mm stainless steel beads. The resulting homogenate was centrifuged at 16160 xg at 4°C for 15 min and supernatant collected for analysis. Protein levels in the supernatant was determined using the Bradford reagent (Sigma-Aldrich Chemical Co., St. Louis, MO) and the levels of IL-6, IL-1β, and MCP-1
were determined using enzyme-linked immunosorbant assays (ELISA)s (R&D Systems, Minneapolis, MN). Levels of GLP2 were determined using an ELISA from MyBioSource, Inc. (San Diego, CA). Lipid peroxidation was measured by thiobarbituric acid-reactive substances (TBARS) using previously described methods.[270] A standard curve was prepared using 1,1,3,3-tetraethoxypropane (TCI America). Caspase-1 activity was measured using a caspase-1 activity assay as per the manufacturer’s protocol (Abcam, Cambridge, MA).

3.3.12 Quantitative Reverse-Transcriptase PCR

Total RNA was isolated and DNA contamination was removed from homogenized colon samples using the RNeasy Mini Kit (Qiagen, Valencia, CA). Total RNA was assessed and quantified with a Nanodrop 2000 spectrophotometer. The RNA was reverse-transcribed to cDNA using the RT² HT First Strand Kit (SA Biosciences, Valencia, CA). Real-time PCR was performed using an Applied Biosystems 7900HT Fast Real-Time PCR System (San Francisco, CA) using primers (The Pennsylvania State University Genomics Core Facility, University Park, PA) or TaqMan® hydrolysis probes (Life Technologies, Inc, Grand Island, NY) (Table 3-2 and Table 3-3). The data was analyzed with Sequence Detector Software (Applied Biosystems). Relative gene expression was determined using the $2^{-\Delta CT}$ method, where $\Delta C_T = (C_{T,\text{target}} - C_{T,\text{reference}})$, with GAPDH as the reference gene. $\Delta C_T$ values were used for statistical tests and $2^{\Delta CT}$ values were used for graphical representation.
<table>
<thead>
<tr>
<th>Macronutrient Composition</th>
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<th>12% SPC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (% of energy)</td>
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<tr>
<td>Carbohydrate (% of energy)</td>
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<td>65</td>
<td>65</td>
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<tr>
<td>Fat (% of energy)</td>
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<td>17</td>
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<td>3.9</td>
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<td>3</td>
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<tr>
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<td><strong>Total</strong></td>
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<td>984.367</td>
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* Differences in cellulose and mineral mixes in the diets are as a result of the mineral composition and ash content in the SPC received from the supplier.

** Composed of calcium phosphate, magnesium oxide, potassium citrate, potassium sulfate, sodium chloride, chromium potassium sulfate, cupric carbonate, potassium iodate, ferric citrate, manganous carbonate, sodium selenite, zinc carbonate and sucrose.

*** Composed of Vitamin A, Vitamin D3, Vitamin E acetate, menadione sodium bisulfate, biotin, cyanocobalamin, folic acid, nicotinic acid, calcium pantothenate, pyridoxine-HCl, riboflavin, thiamin HCl and sucrose.
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<th>Manufacturer’s no.</th>
<th>Amplicon Length (bp)</th>
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Table 3-3. PCR primer sequences used in this study.*

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<td>Toll Like Receptor 4 (<em>Tlr4</em>)</td>
<td>21313256a1</td>
<td>AGCTCCTGACCTTGGTCTTG</td>
<td>CGCAGGGGAACTCAATGAGG</td>
<td>20</td>
</tr>
</tbody>
</table>

*All sequences were obtained from the Harvard’s PrimerBank [271-273]. Only validated sequences were used.
3.3.13 Data Analysis.

Statistical analysis was performed using GraphPad Prism (San Diego, CA). All data are presented as the mean ± standard error of the mean (SEM). One-way ANOVA with Tukey’s post-test was used to analyze cytoprotective effects, spleen-to-body weight ratios, markers of inflammation, mRNA gene levels, lipid peroxidation, and difference in monolayer permeability. Body weight gain and food and fluid consumption were analyzed by two-way ANOVA with Bonferroni’s post-test. Average DSS water consumption between groups was compared using Student’s t-test. Statistical significance was achieved at p < 0.05.
3.4 Results

3.4.1 Radical Scavenging Assay.

The peroxyl radical scavenging activity of SPC, SPC-H, SPC-L, SPC-NEM, SPC-H_2O_2, HSPC, HSPC-NEM, and HSPC-H_2O_2 was measured using the ORAC assay (Figure 3-1a).

Figure 3-1. Radical scavenging, cytoprotective, and permeability effects of SPC in vitro.
(a) The radical scavenging effects of SPC and its various modifications were determined chemically using the ORAC assay. (b) Thiol groups as measured by Elman’s reagent. (c) The protective effects of preincubation and coincubation with SPC on H_2O_2-induced loss of Caco-2 cell viability were assessed by MTT assay. (d) The effect of SPC on intracellular ROS was determined by measuring DCDHFDA fluorescence following treatment of Caco-2 cells with H_2O_2 alone or in combination with SPC. The effect of (e) SPC, HSPC, (f) SPC-H, SPC-L, (g) SPC-NEM, SPC-H_2O_2, (h) HSPC-NEM, and HSPC-H_2O_2 on DSS-induced increases in the permeability of Caco-2 monolayers was determined by measuring the apical to basolateral flux of FITC-D after 48 h incubation with 2% DSS and soy treatment. All data represent the mean and error bars indicate the SEM. All experiments were repeated at least twice. Different letters denote p < 0.05 using one-way ANOVA with a Tukey’s Multiple Comparison post-test.
Although all samples displayed radical scavenging activity, unmodified SPC had the greatest activity in terms of 100 μM Trolox per gram. Whereas the radical scavenging activity of SPC-H was 50% that of radical scavenging activity, the activity of SPC-L was negligible. Blocking the thiols of SPC using NEM and oxidation with H$_2$O$_2$ significantly decreased the radical scavenging activity of the SPC. Hydrolysis of SPC also decreased radical scavenging activity compared to SPC, and treatment with NEM of H$_2$O$_2$ caused a further reduction.

3.4.2 Thiol Measurements of SPC and HSPC

The levels of thiols in SPC, SPC-H, SPC-L, and HSPC were determined using Ellman’s reagent (Figure 3-1b). The concentrations of SPC-H and SPC-L analyzed were based on their relative amounts in unfractionated SPC. These amounts do not total 100% as there is some loss due to filter retention. SPC and SPC-H had equivalent levels of free thiols, whereas the levels of thiols in the SPC-L were significantly reduced. Hydrolysis reduced the thiol levels in SPC by approximately 50% compared to unfractionated SPC.

3.4.3 SPC-mediated Cytoprotection and Intracellular Antioxidant Activity

Both pre-treatment and co-treatment dose-dependently reduced H$_2$O$_2$-induced loss of Caco-2 cell viability with co-treatment being more efficacious (p=0.006) (Figure 3-1c). Co-treatment or pre-treatment with 0.5 mg/mL SPC reduced H$_2$O$_2$-induced loss of cell viability by 34% and 19%, respectively. Treatment with H$_2$O$_2$ significantly increased intracellular oxidative stress compared to vehicle-treated controls. Co-incubation with 0.5 mg/mL SPC reduced intracellular oxidative stress by 25% compared to cells treated with H$_2$O$_2$ alone (Figure 3-1d).
3.4.4 In vitro Permeability

Treatment with SPC and HSPC reduced DSS-induced increases in the apical to basolateral flux of the FITC-D across the differentiated Caco-2 cell monolayer by 54 – 65% (Figure 3-1e). Enzymatic hydrolysis did not significantly affect the ability of SPC to mitigate changes in monolayer permeability. By contrast, fractionation showed that both SPC-H and SPC-L contributed to the effects of SPC on the DSS-induced increase in monolayer permeability (Figure 3-1f). Thiol blocking by NEM significantly reduced the ability of SPC to mitigate DSS-induced permeability by 52% compared to unblocked SPC (Figure 3-1g). Although H2O2 treatment tended to decrease the efficacy of SPC, the effect was not significant. Treatment of HSPC, with either NEM or H2O2 had no effect on the mitigation of DSS-induced permeability by HSPC (Figure 3-1h).

3.4.5 Suppression of Colitis In Vivo

DSS treatment induced body weight loss beginning on day 6, which continued for the duration of the experiment (Figure 3-2a). This effect was related to a 414% decrease in feeding efficiency induced by DSS (Figure 3-2b). Dietary SPC mitigated both the body weight loss and decreases in feeding efficiency. Colon shortening and increased relative spleen weight were both observed in DSS-treated mice (Figure 3-2c and Figure 3-2d). SPC tended to normalize colon length (Figure 3-2c) and dose-dependently mitigated this increase in spleen weight (Figure 3-2d). DSS treatment increased lipid peroxidation in the colon by 46% compared to the negative control (Figure 3-2e). SPC supplementation normalized lipid peroxide levels.
Figure 3-2. The effects of dietary SPC on weight gain, feeding efficiency, and gross measures of colitis in DSS-induced CF-1 mice.

(a) Body weight change relative to starting weight was determined over the course of the experiment. (b) The feeding efficiency of the mice was determined based on the ratio of body weight gain to energy intake and averaged over the last 3 d of the experiment. (c) Colon length and (d) relative spleen weight were determined at the end of the experiment. (e) Lipid peroxide levels in colon homogenate were determined using the TBARS assay and normalized to total protein levels in the homogenate. All values represent the means ± SEM and an n = 10 for each group. Different letters denote p < 0.05 using one-way ANOVA with a Tukey’s Multiple Comparison post-test.
3.4.6 Biochemical Markers of Inflammation in DSS-treated Mice

Colonic protein expression of IL-1β, IL-6, and MCP-1 were significantly increased in DSS-treated mice (Figure 3-3a – c). These changes were dose-dependently mitigated by SPC supplementation. At the mRNA level, colonic Il1b and Emr1 were increased by DSS (Figure 3-3d and Figure 3-3e). These increases were mitigated by SPC supplementation (Figure 3-3d and Figure 3-3e).

![Figure 3-3. The effects of dietary SPC on colon inflammation in DSS-induced CF-1 mice.](image)

Protein levels of (a) IL-1β, (b) IL-6 and (c) MCP-1 were determined in the colon homogenate using commercially-available ELISAs and normalized to the total protein of each sample. Relative fold change of mRNA expression of (d) Il1b and (e) Emr1 in colon homogenate was determined by quantitative reverse transcriptase PCR and normalized to the expression of GAPDH expression. All values represent the means ± SEM and an n = 10 for each group. Different letters denote p < 0.05 using one-way ANOVA with a Tukey’s Multiple Comparison post-test.
3.4.7 Gut Barrier Function in DSS-Treated Mice

Colonic GLP-2 protein levels were reduced by 57% in DSS-treated compared to negative control mice (Figure 3-4a). Dietary SPC normalized the expression of GLP-2 in the colon.

Figure 3-4. Biochemical markers of intestinal permeability in DSS-induced CF-1 mice. (a) GLP-2 protein levels in the colon homogenate as determined by ELISA and normalized to the total protein of each sample. Relative fold change of mRNA expression of (b) Claudin-1 and (c) Occludin in colon homogenate was determined by quantitative reverse transcriptase PCR and normalized to the expression of GAPDH expression. (d) The ratio of Claudin-1 to Occludin was used as a marker of gut barrier function, with a decreased ratio representing compromised barrier function. All values represent the means ± SEM and an n = 10 for each group. Different letters denote p < 0.05 using one-way ANOVA with a Tukey’s Multiple Comparison post-test.

RT-PCR analysis showed that mRNA expression of claudin-1 tended to increase in DSS-treated mice compared to negative control mice (Figure 3-4b), whereas mRNA expression of occludin
tended to decrease (Figure 3-4c). SPC appeared to mitigate these effects somewhat, as seen in the ratio of claudin to occludin (Figure 3-4d), but the changes were not statistically different.

3.4.8 Inflammasome Formation and Caspase-1 in DSS-treated Mice.

DSS-treatment increased colonic expression of Nfκb1, Tlr4, and Nl rp3 compared to negative control mice (Figure 3-5a – c). SPC supplementation significantly decreased the expression of all of these genes.

Figure 3-5. Effects of dietary SPC on the inflammasome in DSS-induced CF-1 mice.
Relative fold change of mRNA expression of (a) NF-κB, (b) TLR4, (c) NLRP3, (d) TXNIP, and (e) Caspase-1 in colon homogenate was determined by quantitative reverse transcriptase PCR and normalized to the expression of GAPDH expression. (f) Caspase-1 enzyme activity was measured using a commercially available kit and normalized to the control. All values represent the means ± SEM and an n = 10 for each group. Different letters denote p < 0.05 using one-way ANOVA with a Tukey’s Multiple Comparison post-test.
The expression of Txnip and Casp1 were affected neither by DSS nor SPC supplementation (Figure 3-5d – e). By contrast, caspase-1 activity was significantly increased by treatment with DSS, and the increase was blocked by supplementation with SPC (Figure 3-5f).
3.5 Discussion

A number of epidemiological studies have reported an inverse relationship between the soy consumption and colon cancer.[192, 274] Although early work focused on soy isoflavones, more recent work has focused on other components in soy.[256] In the present study, we examined the efficacy of dietary SPC for mitigation of inflammation, oxidative stress, and gut permeability in vitro and in the DSS-induced mouse model of acute ulcerative colitis.

Caco-2 human colon cells are an accepted model of intestinal epithelial function: treatment with DSS can induce inflammation and increase permeability.[136] SPC decreased the DSS-induced permeability of Caco-2 cell monolayers, and reduced H2O2-induced oxidative stress and cytotoxicity in the same cells. We also found that digestive enzyme-mediated hydrolysis did not significantly reduce the ability of SPC to preserve Caco-2 cell monolayer integrity, but did reduce its radical scavenging effects indicating protective effects of SPC against epithelial barrier function, but the radical scavenging activity may be lost. These results also indicate that these two biological effects of SPC may result from different underlying mechanisms of action.

Soy protein is a complex food ingredient and the specific components in SPC that are responsible for the effects on inflammation and gut barrier function are unclear. Various studies have suggested that these effects are due to the presence of the Bowman-Birk protease inhibitor (MW = 8 kDa) [198, 209] or two small molecular weight, bioavailable tripeptides (MW < 1 kDa).[232, 233] These studies often examined purified components and did not assess the role of the particular component in the overall activity of SPC. Here, we fractionated SPC, into SPC-H (> 10 kDa) and SPC-L (< 10 kDa). SPC-H had similar amounts of reactive thiol groups as compared to SPC, whereas SPC-L had significantly lower thiol concentrations. The radical scavenging efficacy of SPC, SPC-H, and SPC-L mirrored differences in thiol concentration suggesting that these are the critical residues in the antioxidant activity of SPC. These findings
are supported by the observation that treatment with the thiol-blocking agent, NEM, or oxidation with H$_2$O$_2$ significantly reduced radical scavenging activity.

By contrast, the ability of SPC to mitigate DSS-induced monolayer permeability was only partially reduced by NEM or H$_2$O$_2$ treatment indicating other characteristics of SPC play a key role in this effect of SPC. Hydrolysis of SPC reduced its ability to scavenge radicals when oxidized or thiol blocked, however, these treatments did not affect its ability to prevent permeability \textit{in vitro}. This suggests that HSPC acts by a different mechanism than SPC. Further studies are needed to fully characterize the relative contribution of various protein components in SPC to the overall anti-inflammatory activity.

\textit{In vivo}, we found that dietary SPC dose-dependently ameliorated the DSS-mediated decreases in body weight and feeding efficiency as well as gross markers of inflammation including spleen weight and colon shortening. SPC also improved biochemical markers of inflammation (IL-6, IL-1β, MCP-1, and EMR-1) and oxidative stress. These results are similar to previous studies of purified di- and tripeptides derived from soy, but represent new findings for a whole soy protein preparation.\cite{232, 233}

Colonic inflammation leads to reorganization of the TJ proteins and loss of gut barrier function allowing intestinal microbiota and microbiota-derived components into the basolateral space of the intestine and into the systemic circulation. These effects have been linked to IL-1β-induced NFκB activation \cite{116}. We observed here that SPC improved gut barrier function as indicated by increased colonic GLP-2 levels, and decreased claudin to occludin ratio compared to DSS-treated controls.\cite{246-248}

IL-1β is synthesized as an inactive protein precursor that is activated by proteolytic cleavage: caspase 1 is the protease primarily responsible for this cleavage.\cite{275} We found that caspase-1 activity was elevated in DSS-treated mice, and that this effect was mitigated by SPC supplementation. By contrast, \textit{Casp}1 mRNA levels were not affected. Since caspase-1 is also
synthesized as a pro-enzyme that must be proteolytically activated, these results suggest that SPC is preventing this activation. Activation of caspase-1 occurs through the action of a multi-protein complex known as the inflammasome. This complex is formed by NLRP3 and ASC as a result of ROS mediated TXNIP interactions with NLRP3. [276] Although we found no significant effect of SPC on Tnip mRNA levels, we did observe a significant decrease in Nlrp3 expression in SPC mice compared to DSS-treated control mice. TLR4-induced NFκB activation regulates NLRP3 expression and thus IL-1β maturation. [93] This receptor plays a key role in innate immune response and is elevated in experimental models of IBD.[277, 278]

Figure 3-6. Proposed mechanism underlying the mitigation of colonic inflammation by SPC.
SPC reduces TLR4-NFκB-mediated activation of the NLRP3 inflammasome leading to decreased caspase 1 activation and IL-1β maturation. This leads to decreases inflammation as well as NFκB-mediated increases in colonic epithelium permeability. These effects may result from SPC-mediated decreases in oxidative stress, modulation of TLR4 ligand binding, or other unknown mechanism.
We found that mRNA levels of Tlr4 and Nfkb1 were elevated in DSS-induced mice and the effect was blunted in SPC-supplemented mice. Taken together, our results suggest, for the first time, that SPC interferes with caspase-1 mediated maturation of IL-1β, and these effects are mediated by SPC-induced changes in TLR4-NFκB-NLRP3 inflammasome-mediated signaling (Figure 3-6).

Overall, the findings of our study support the efficacy of dietary SPC as a means of reducing colonic inflammation and improving gut barrier function. These effects appear to result from multiple protein components, and involve modulation of TLR4-mediated inflammasome formation and innate immune signaling.
Chapter 4

A Survey of Dietary Procyanidins and Proteins and Their Modulation of Intestinal Permeability

The Caco-2 monolayer permeability assay data from the previous two studies correlates well with compound’s effectiveness in reducing inflammation and preserving gut barrier function. Using this model, we conducted a brief survey of extracts and fractions from a number of dietary components to look at their effectiveness at improving gut barrier function. These experiments were conducted in order to evaluate their potential in future in vivo animal studies.

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3 Cocoa sections to be published in part as:
4.1 Abstract

Chronic inflammation of the gastrointestinal tract as a result of inflammatory bowel disease (IBD) can result in disruptions of the normal gut barrier function. Investigations into the effects of different plant phytochemicals on modulating intestinal permeability have been scarce. As a result, we aim to determine the ability of dietary bioactive procyanidins and proteins to modulate intestinal permeability and oxidative stress. Using a Caco-2 human colorectal adenocarcinoma cell line transwell model, cherry extract, moringa leaf (*Moringa oleifera* Lam.) extract, tempeh, and different monomeric fractions of cocoa were all assessed for their ability to reduce inflammation induced permeability. All of the plant sources showed some ability to decrease permeability caused by dextran sulfate sodium (DSS)-induced inflammation. The cherry and moringa extracts exhibited a protective effect on the permeability. Tempeh and its high and low molecular weight fractions all exhibited similar reductions in inflammation induced permeability suggesting that they may be acting by different mechanisms. Of the different cocoa fractions, the polymeric fraction had the greatest protective effect on permeability. Collectively, this suggests that plant phytochemicals may reduce inflammation induced permeability and potentially improve gut barrier function during chronic inflammatory conditions.
4.2 Introduction

Gut barrier function is an important concern for those affected with inflammatory bowel disease (IBD). A chronic inflammation of the gastrointestinal tract, IBD, affects nearly 2.2 million people globally.\[7\] Of patients with IBD, 20% are estimated to develop colorectal cancer and half will die as a result.\[26\] IBD also dramatically diminishes the quality of life of those afflicted and presents a significant economic burden. The total economic burden of IBD in the United States is US$19-30 billion annually.\[27, 28\]

This chronic inflammation of the gastrointestinal tract is accompanied by the release of a number of different inflammatory cytokines and oxidative stress. These stressors have been linked to decreases in gut barrier function in both humans and animals.\[31, 127, 279-283\] This increase in permeability is a result of the modulation of tight junction proteins responsible for paracellular transport in the epithelium. Many studies have found that inflammatory cytokines cause a down-regulation in these tight junction proteins in patients with IBD.\[30, 31, 101, 113\] These inflammatory cytokines are generated in response to excessive oxidative stress which has also been shown to decrease gut barrier function in IBD.\[114\]

Procyanidins (PCs) are a subclass of flavonoids and are comprised of flavan-3-ol monomers such as (±)-catechin (C), (−)-epicatechin (EC), oligomers, and polymers. Flavanols are classified by the number of monomeric residues, or degree of polymerization (DP), and the average DP of all flavanols, or the mean DP (mDP).\[284\] Dietary PCs are present in many different types of food such as cocoa, grapes, and berries.\[285\] The biological activities of many PCs have been widely studied in relation to the prevention or treatment of diseases. The DP plays a significant role in determining the efficacy of PCs in disease models of inflammation.\[286-290\] DP also affects the efficacy of PCs in models of cancer.\[291-297\]
DP also influences the bioavailability of PC biological activity. For example, the typical oral bioavailability of monomers is <10%, lower for smaller PCs, and effectively none for larger PCs.[298-301]. Bioavailability is not necessarily the most important factor to consider when looking at the activities in the lumen or epithelium as direct contact with PCs is possible. As a result, in vitro screening assays may be more useful in predicting their activities than bioavailability alone. Direct interaction might be able to inhibit luminal and brush boarder digestive enzymes, inhibit intestinal inflammation, and modulate gut barrier function and endotoxin uptake. Modulation of these factors from PCs would be beneficial in obesity, diabetes, IBD, and possibly cancer prevention.[302, 303]

Cocoa is one of the most flavanol-rich food products and contains PCs of a wide range of DP [284, 304-308]. Recently, an oligomer-rich PC fraction of cocoa showed enhanced protection against diet-induced obesity and type-2 diabetes as compared to its monomeric and polymeric PC fractions.[309] Another study found that inflammation in mice fed high fat diets was reduced as a result of cocoa substitution in their diets.[310] PC-rich cherry extracts showed a dose-dependent decrease for NFκB transcriptional and binding activities as well as decreases in IL-1β and TNFα in RAW 264.7 murine macrophage cells stimulated with LPS.[311] Collectively, PCs have been shown to inhibit NFκB signaling and cytokine production, and thus could modulate intestinal permeability.[146-149]

Bioactive dietary proteins and peptides have also been shown to modulate inflammatory factors in vivo. The soybean peptide lunasin as well as di- and tripeptides have all been shown to exhibit anti-inflammatory effects in vivo and in vitro.[230-233] These proteins can be broken down during fermentation, yet fermented soy products still have been shown to decrease inflammation and colorectal cancer in animal models. [312, 313] Tempeh, a Rhizopus oligosporus fermented soy product, contains about 0.5 mg/g of isoflavones, high amounts of Vitamin B12, and enhanced antioxidant abilities as compared to unfermented soybeans.[314-316]
Recently, tempeh isolate was found to reduce neuroinflammation and scopolamine-induced amnesia significantly more than soy isolate in rats.\[317\] This is further evidence that fermentation does not necessarily remove the bioactive abilities of a particular food source and in some cases it may actually improve it.

The leaves from the moringa tree (\textit{Moringa oleifera} Lam.) are used as a protein-rich (27\% by dry weight) dietary and medicinal herb by African and Asian cultures.\[318\] In addition to having all of the essential amino acids and high levels of vitamins, the moringa leaves also have a number of bioactive components.\[319\] Recently, moringa leaf extract has been shown to be a powerful antioxidant and has inhibited expression of IL-1\(\beta\) and TNF\(\alpha\) in RAW 264.7 murine macrophage cells stimulated with LPS.\[318, 320\] Though the exact anti-inflammatory components are a source of debate, it does present an interesting option for the prevention of intestinal permeability and modulation of gut barrier function.

In the present study, we aim to determine the ability of dietary bioactive procyanidins and proteins to modulate intestinal permeability and oxidative stress.
4.3 Materials and Methods

4.3.1 Sample Preparation

4.3.1.1 Cherry Extract

Sulfite-free dried cherries (Travers Bay Dried Fruit Company, Travers City, MI) were blended in a Waring blender, diluted with 10 volumes of acetone:water:acetic acid (80:19.9:0.1, v:v:v), and stirred overnight at room temperature.[321] To remove solids, the extract was vacuum filtered and then rotary evaporated to remove the acetone. The remaining aqueous extract was passed through an Amberlite XAP-7HD column to remove sugars with water used as the eluting mobile phase. To remove the polyphenols from the column, methanol containing 0.1% acetic acid was used as the eluting mobile phase. The polyphenol extract was then rotary evaporated to remove the acetone, lyophilized, and stored at -80°C. The cherry extract powder was dissolved in DMSO before use.

4.3.1.2 Cocoa Fractions

Procyanidin-rich cocoa extract and fractions with distinct flavanol compositions (monomer-, oligomer-, and polymer-rich fractions) were produced from commercially available cocoa powder as described previously.[309] The concentrations of specific cocoa procyanidins in each fraction is presented in Table 4-1. Retention time correlates roughly with DP as larger species tend to elute later.[309] Eluting peaks from the cocoa extract were categorized into early and late-eluting PC peaks. These peaks corresponded to monomer-, oligomer-, and polymer-rich fractions. The polymer fraction was enriched with late-eluting large PCs as compared to cocoa, monomer- and oligomer-rich fractions, although these were not quantifiable by UPLC-MS/MS.
(Table 4-1). Table 4-1 reflects quantities of PCs for which authentic standards were available, and most larger PCs are therefore excluded from these data.[309] Data regarding the characterization of these fractions (Folin, mDP, etc.) have been published previously.[309] A cocoa polymer fraction with even greater enrichment of high MW PCs (92% by weight of DP7+) was a generous gift from The Hershey Co. (Hershey, PA the fraction was originally prepared for Hershey by Planta Analytica, Danbury, CT). The composition of this fraction is shown in Table 4-2.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Composition (mg compound/g dried fraction)</th>
<th>Enrichment Factor</th>
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<tr>
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<td>CE</td>
<td>M</td>
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<td>(±)-catechin</td>
<td>1.67±0.183 b</td>
<td>0.119±0.0183 a</td>
</tr>
<tr>
<td>(−)-epicatechin</td>
<td>5.90±0.0635 b</td>
<td>0.667±0.0152 a</td>
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<tr>
<td>(−)-epigallocatechin</td>
<td>0.015±0.00498 b</td>
<td>0.0199±0.00102 b</td>
</tr>
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<td>PC dimer B1</td>
<td>0.356±0.0126 b</td>
<td>1.12±0.0765 d</td>
</tr>
<tr>
<td>PC dimer B2</td>
<td>4.88±0.149 b</td>
<td>15.4±0.201 d</td>
</tr>
<tr>
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<td>3.10±0.0298 b</td>
<td>7.52±0.246 c</td>
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<td>0.114±0.00897 c</td>
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<td>0.43±0.0222 c</td>
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<td>0 a</td>
<td>0.311±0.156 b</td>
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<td>26.3±0.427 d</td>
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<td>10.6±0.209 d</td>
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<td>3.69±0.261 b</td>
<td>9.77±0.438 c</td>
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<td>PC hexamers</td>
<td>2.70±0.164 b</td>
<td>3.65±0.193 c</td>
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<tr>
<td>PC heptamers</td>
<td>2.14±0.116 b</td>
<td>1.93±0.187 b</td>
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<tr>
<td>PC octamers</td>
<td>0.736±0.0757 c</td>
<td>1.88±0.0598 d</td>
</tr>
<tr>
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<td>3.03±0.186 c</td>
</tr>
<tr>
<td>PC decamers</td>
<td>0 a</td>
<td>0 a</td>
</tr>
</tbody>
</table>

| Total monomers                 | 7.59±0.0806 b | 0.806±0.0129 a | 0.0899±0.0146 a | 1 | 5.30 | 0.106 | 0.011 |
| Total dimers                   | 8.48±0.155 b | 24.7±0.267 d | 0.34±0.0408 a | 1 | 1.77 | 2.91 | 0.040 |
| Total PCs DP 3:6               | 18.0±0.763 c | 50±1.08 d | 5.04±0.231 a | 1 | 0.72 | 2.80 | 0.280 |
| Total PCs DP 7:10              | 4.65±0.208 b | 6.83±1.141 b | 5.82±1.45 b | 1 | 0.072 | 1.47 | 1.25 |

Note: Adapted with permission from: Domenkott, et al. Oligomeric cocoa procyanidins possess enhanced bioactivity compared to monomeric and polymeric cocoa procyanidins for preventing the development of obesity, insulin resistance, and impaired glucose tolerance during high-fat feeding. J. Agric. Food Chem. 2014, 62, 2216-2227. Copyright 2014 American Chemical Society.

Data are mean ± SEM (n=3). Treatments with different letters for the same compound (or sum of compounds) are significantly different.

Enrichment factor = the ratio of mean levels of the compound in each fraction compared to mean levels of the same compound in CE.

CE = cocoa extract
M = monomer-rich fraction
O = oligomer-rich fraction
P = polymer-rich fraction

1. Likely procyanidin dimers B3, B4, and either B6, B7, or B8
2. N/A = this compound was not detected in CE
3. Enrichment factor cannot be calculated for this compound relative to CE, as this compound was not detected in CE.
Table 4-2. Composition of a cocoa extract fraction enriched for high molecular weight polymeric procyanidins (DP 7+).

<table>
<thead>
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<th>% (w/w)</th>
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</tr>
<tr>
<td>8</td>
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<td>11</td>
<td>9</td>
</tr>
<tr>
<td>12</td>
<td>7</td>
</tr>
<tr>
<td>other</td>
<td>8</td>
</tr>
</tbody>
</table>

4.3.1.3 Moringa Extract

The moringa extract was prepared from dried, powdered *Moringa oleifera* leaves (Ariti Herbal, Addis Ababa, Ethiopia). 5 g of the leaf powder was extracted overnight in 10 volumes of hexane. The hexane was decanted and the remaining leaf powder was extracted twice overnight with 10 vol of acetone:water:acetic acid (80:20:0.1, v:v:v). The acetone:water: acetic acid fractions were combined, dried under vacuum, and then freeze-dried. The moringa extract powder was stored at -80°C and dissolved in DMSO before use.

4.3.1.4 Tempeh Fermentation, Extract Preparation, and Fractionation

Soybeans were sourced locally (International Marker, State College, PA) and split into two groups: unfermented soybeans (S) and tempeh (T). Both groups were soaked in water overnight (1:4 w/v). The beans were then boiled for 15 minutes, peeled, and group S was frozen at -20°C for later processing. Group T after being peeled was allowed to soak in the water again overnight. The beans were then boiled again for 15 minutes and drained. Once drained, 1 g Tempeh Starter Culture from Cultures for Health (Sioux Falls, SD) was added to 300 g of beans.
and stirred. Tempeh was then placed in a zip-top bag containing holes (2 cm apart) and allowed to incubate for 36 hours at 37°C in a 95% humidified chamber.

Unfermented cooked soybeans (S) or mature tempeh (T) were blended in a Waring blender with deionized water (1:20 w/v). This mixture was defatted by mixing with hexane and then centrifuged at 3200 x g for 20 min at 20°C. The aqueous fraction was collected for further processing. Under constant agitation, the pH of the aqueous phase was adjusted to 11 using 1M NaOH to increase the solubility. After 2 h, the pH was adjusted back to 7.4 using 1M HCl. The mixture was then centrifuged at 3200 x g for 20 min at 20°C. The supernatant was lyophilized and stored at -80°C until use. Solubilized soybeans or tempeh was dissolved in deionized water (1:20 w/v) and spun through a 10 kDa molecular weight cutoff centrifugal filter according to the manufacturer’s instructions. The low-molecular weight flow-through of the different samples (Tempeh – T-L and Soybeans – S-L) were collected, lyophilized, and stored at -80°C until use. The high molecular weight slurry of the different samples (Tempeh – T-H and Soybeans – S-H) that did not pass through the filter was also collected, lyophilized, and stored at -80°C until use. Protein content of each of the samples was determined using the Bradford assay. To check fermentation, T and S samples were run through a 4-20% gradient protein gel and stained with Coomassie blue.
4.3.2 Methods

4.3.2.1 Antioxidant Capacity

Samples or 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were dissolved in phosphate buffer (10 mM, pH 7.4). 100 µg/mL of each sample or Trolox standards were combined with fluorescein (final concentration = 8.6 nM) in a 96-well black opaque plate. After an incubation period of 30 min at 37°C, fluorescence was measured (ʎ<sub>Ex</sub>, 485 nm, ʎ<sub>Em</sub>, 520 nm). 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) (final concentration = 30 nM) or phosphate buffer was then added and fluorescence was measured every 90 seconds for 90 min. Fluorescence values were normalized to time 0 and the area under the curve was determined for each treatment. Areas were then converted to their Trolox equivalents using the standard curve.

4.3.2.2 Cell Culture and Permeability

Caco-2 cells (American Type Culture Collection, Manassas, VA) were maintained in sub-confluence in Dulbecco’s Modification of Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 IU/mL penicillin and 100 µg/mL streptomycin at 37°C under 5% CO2 atmosphere. The paracellular permeability was observed by measuring the apical to basolateral flux of fluorescein isothiocyanate–dextran (FITC-D) as described previously.[136] Caco-2 cells were seeded in polycarbonate transwell inserts (0.33cm<sup>2</sup> area and 0.4 µm pore size, Corning Life Sciences, Tewksbury, MA) and allowed to reach confluence and differentiate for 21 d. Based on previous studies, only monolayers with a transepithelial electrical resistance (TEER) of 500-600 Ω cm<sup>2</sup> were used.[243] The monolayers were treated with the different extracts (0 – 100 µg/mL) for 2 h and then co-treated with 2% DSS for an additional 48 h. Cells were then washed with fresh media, and 1 mg/mL FITC-D was added to the apical compartment and cells were incubated
for 6 h. FITC-D levels were determined in the basolateral compartment every 30 min using a Fluoroskan Ascent FL fluorescent plate reader (Thermo Scientific, Waltham, MA).
4.4 Results

4.4.1 Cocoa Fraction Anti-Oxidant and Permeability Effects

Peroxyl radical scavenging activity of the different fractions were measured using the ORAC assay (Error! Reference source not found.a). The oligomers-rich and monomer-rich fractions had a statistically greater scavenging ability than the cocoa fraction and the oligomers-rich fraction had a greater ability than the polymer-rich fraction. This may suggest that DP affects a PC’s ability to quench free radicals. Typically radical scavenging ability increases with DP, however, that was not observed in this experiment.[322, 323]

![Graph showing antioxidant potential and gut permeability effects of cocoa procyanidins.](image)

**Figure 4-1.** Antioxidant potential and gut permeability effects of cocoa procyanidins. (a) Trolox equivalence of 1g dry weight of each sample as measured by the ORAC assay. (b) The effects of cocoa procyanidin fractions and (c) high molecular weight polymeric cocoa procyanidin extract (DP 7+, 10 or 25 μg/mL) on DSS-induced increases in the permeability of Caco-2 monolayers was determined by measuring the apical to basolateral flux of FITC-D after 48 h incubation with 2% DSS and soy treatment. All data represent the mean and error bars indicate the SEM. All experiments were repeated at least twice. Different letters denote p < 0.05 using one-way ANOVA with a Tukey’s Multiple Comparison post-test.
The ability of cocoa PCs to protect intestinal epithelial cells from DSS-induced loss of membrane integrity is shown in Error! Reference source not found. DSS significantly increased apical-basolateral flux of FITC-dextran compared to vehicle (DMSO) treated cells. Cocoa extract and all cocoa PC fractions significantly inhibited DSS-induced loss of barrier function. The polymer-rich fraction possessed the greatest protective activity, followed by the monomer-rich fraction. Cocoa extract and the oligomer-rich fraction were least protective. The results for cocoa extract and the monomer-rich fraction correlate with a previous study, in which it was found that these were the least and most effective at reducing serum levels of endotoxin, respectively, in high fat-fed mice [309]. By contrast, the data from the oligomer- and polymer-rich fractions do not correlate well with this same study. In order to further confirm the impacts of high MW cocoa PCs in these models, a more concentrated extract (92% by weight PCs with DP 7+) was assayed for activity using the same models. Due to the greater enrichment for DP 7-12 compared to the polymer-rich fraction previously assayed, lower concentrations (10-25 μg/mL) were employed for the DP 7+ fraction. The ability of this fraction to protect intestinal epithelial cells from DSS-induced loss of membrane integrity is shown in Error! Reference source not found. Similar to the previous experiment, the DP 7+ enriched fraction inhibited the effects of DSS on membrane integrity in a dose dependent-fashion (10 μg/mL partly inhibited the effects of DSS, while 25 μg/mL completely blocked the effects of DSS).

4.4.2 Cherry and Moringa Extract Anti-Oxidant and Permeability Effects

Peroxy radical scavenging activity of the cherry and moringa leaf extract were measured using the ORAC assay (Figure 4-2a). Though not directly comparable, the cherry extract did have a statistically greater scavenging ability than the moringa extract. The ability of cherry and moringa extracts to protect intestinal epithelial cells from DSS-induced loss of membrane
integrity is shown in Figure 4-2b. The cherry extract was able to significantly reduce permeability as compared to the DSS only treated group, however it still allowed more apical-basolateral FITC flux than the control. The moringa extract was also able to statistically lower the permeability as compared to the DSS only treated group and was not statistically different from the control group or the cherry group. This suggests that the moringa extract may have a more positive effect in protecting gut barrier function. Also, these results do not correspond to the radical scavenging results, suggesting that regulation of oxidative stress by cherry and moringa extracts may not be responsible.

![Figure 4-2](image)

**Figure 4-2.** Antioxidant potential and gut permeability effects of cherry and moringa extracts.  
(a) Trolox equivalence of 1g dry weight of each sample as measured by the ORAC assay.  
(b) The effects of 100μg/mL cherry extract and moringa extract on DSS-induced increases in the permeability of Caco-2 monolayers was determined by measuring the apical to basolateral flux of FITC-D after 48 h incubation with 2% DSS and soy treatment. All data represent the mean and error bars indicate the SEM. All experiments were repeated at least twice. Different letters denote p < 0.05 using one-way ANOVA with a Tukey’s Multiple Comparison post-test.
4.4.3 Tempeh Extract Anti-Oxidant and Permeability Effects

The protein gel shows that the starting soybeans had a number of well-defined protein bands above 35 kD whereas the fermented tempeh had many of those proteins cleaved resulting in a much higher concentration of smaller molecular weight proteins (Figure 4-3a). Peroxyl radical scavenging activity of the different tempeh and soy treatments were measured using the ORAC assay (Figure 4-3b).

![Figure 4-3. Antioxidant potential and gut permeability effects of tempeh.](image)

(a) Protein gel showing the transformation of high molecular weight proteins into low molecular weight proteins as a result of the fermentation process. (b) Trolox equivalence of 1 g dry weight of each sample as measured by the ORAC assay. (c) The effects of 100 μg/mL tempeh, high molecular weight tempeh, and small molecular weight tempeh on DSS-induced increases in the permeability of Caco-2 monolayers was determined by measuring the apical to basolateral flux of FITC-D after 48 h incubation with 2% DSS and soy treatment. All data represent the mean and error bars indicate the SEM. All experiments were repeated at least twice. Different letters denote p < 0.05 using one-way ANOVA with a Tukey’s Multiple Comparison post-test.

It showed that tempeh (T) had a greater radical scavenging ability than soybeans (S) than the high molecular weight soybean fraction (S-H). Both low molecular weight fractions of tempeh (T-L)
and soy (S-L) demonstrated a stronger radical scavenging ability over their unfractionated counterpart. The low molecular weight tempeh fraction (T-L) had the greatest radical scavenging ability of all of the groups. Since these extracts were not solely protein, a number of different small molecular weight isoflavones and other antioxidants may be present in each of the samples. This would explain why the low molecular weight fractions for tempeh and soybeans had enhanced scavenging ability.

Figure 4-3c shows the protective effects of tempeh on membrane integrity in DSS-treated intestinal epithelial cells. The tempeh (T) was able to significantly reduce FITC-D apical-basolateral flux as compared to the DSS only treated groups. The high molecular weight (T-H) and low molecular weight (T-L) fractions also significantly reduced the permeability between cells. All three treatments reduced the permeability back to levels of the control. The fractionation of the tempeh had no apparent effect suggesting that two different mechanisms of action are involved in tempeh’s overall gut barrier protection.
4.5 Discussion

The results of the present study suggest that cocoa, cherry, moringa, and tempeh all may be effective in preventing the loss of gut barrier function, which is a critical step in the pathogenesis of endotoxemia, inflammatory bowel disease, and colon cancer.

Gut barrier function is thought to be modulated in part by inflammation and, as previous studies have shown, cocoa, cherries, moringa, and fermented soy products possess anti-cancer and anti-inflammatory activities.[311-313, 324-328] The present data shows that this anti-inflammatory ability may be translatable to preservation of gut barrier function. In the case of the cherry, moringa, and tempeh groups, these extracts contained both large molecules that may not be bioavailable and smaller molecules that would be bioavailable. These extracts represent more of a whole food extract whereas the cocoa experiments focused more on the effects of different DPs. In the case of cocoa, our findings indicate that the polymeric PC components of cocoa are the most effective as compared to the smaller oligomer-rich and monomer-rich fractions.

The Caco-2 transwell model of permeability may be particularly translatable to *in vivo* situations as epithelial cells do not require a bioactive component to be systemically available and instead can have direct contact with the epithelium. Therefore, larger, less bioavailable molecules such as polymeric PC or indigestible proteins may modulate key mechanistic targets directly in the epithelium resulting in improved prevention or disease outcomes. Processing can also affect the activity of certain protease inhibitors and other bioactive peptides and proteins in foods by possibly making them less bioavailable but still active.[329]

In many of the extracts that were examined in this study, the *in vitro* antioxidant potential did not correlate well with the *in vivo* data. The reasoning behind this may be two-fold. Many *in vitro* antioxidant studies do not correlate with results from *in vivo* oxidation models.[330] While the ORAC assay provides a cheap chemical test to look at antioxidant capacity, cell culture
experiments to study antioxidants are more biologically relevant as a compound may induce a
cell’s natural response to oxidative stress rather than directly quenching the radical on its own.
Another reason for our in vitro and in vivo data not correlating may be simply due to antioxidant
activity not playing a large role in the permeability of the cell monolayer and another anti-
inflammatory mechanism occurring.

Additional research is required in animal models in order to fully understand the potential
activities of these extracts. Such in vivo studies possess additional difficulties as they are often
time and cost intensive for both extracting and isolating compounds as well as the time and cost
associated with running and analyzing the animal samples. Likewise, efficient extraction and
fractionation are needed to support these studies and eventually translate them into clinical
studies in humans. This cocoa fractionations done in this study and others show that larger PCs,
often ignored due to complexities associated with their qualitative and quantitative analysis, have
strong potential bioactivities.[331, 332] Nevertheless, this work highlights the need for novel
approaches to investigate the activities of these complex dietary polyphenols, proteins, and
peptides mixtures.
Chapter 5

Conclusions and Future Work

5.1 Introduction

The incorporation of different putative food-derived bioactive components into the diet in order to inhibit inflammation and improve gut barrier function in those at risk of IBD and colorectal cancer appears to be a viable option. Unlike drugs, where a specific molecule targets a particular pathway, food systems are much more complex and may contain multiple beneficial bioactive components. Isolation of a single bioactive compound from the whole food may lead to a decrease in bioactivity compared to the whole food. Conversely, while one particular compound from a food may have an effect, it may not be present in high enough quantities in the whole food to exhibit an effect. Climate, harvest time, cultivar, and a number of different environmental effects can all affect the quantity of a particular compound in a food. Nevertheless, it is important to understand how these active compounds function as they can be beneficial for understanding disease progression and the effect of low-level consumption of the bioactive component over time.

I investigated EGCG from green tea and soy protein concentrate along with other botanical products for their effects on colon inflammation and gut barrier function. In these studies, I found that EGCG and soy protein concentrate both decreased colonic inflammation in DSS-induced mice. I also found that both components reduced intestinal permeability in this mouse model as well as in DSS-treated Caco-2 cell monolayers in culture. Furthermore, extracts prepared from cherry, cocoa, moringa, and tempeh also mitigated DSS-induced permeability in Caco-2 cell monolayers. The antioxidant capacity of each component did not appear to correlate well with many of our results. Further studies are needed to identify the mechanisms and specific
components involved. Despite this, it does provide further evidence that reduction of inflammation and improvement of gut barrier function can be accomplished through dietary treatments. While dietary treatment may be beneficial, it should not usurp the role of routine checkups and screenings.

5.2 Impact of EGCG on Colonic Inflammation in vitro and in vivo

The findings from the EGCG study demonstrate not only the anti-inflammatory effects of EGCG, but a putative dose-limiting toxicity in the context of IBD. While EGCG did indeed improve gut barrier function in both the mouse and cell culture models, it also increased the body weight loss and appeared to induce significant anti-nutritional effects. When the mice were treated with EGCG via intraperitoneal injections, these beneficial effects were lost suggesting that EGCG does need direct contact with the epithelial cells and/or gastrointestinal microbiota. Because the Caco-2 monolayer model lacks the microbiota component, it appears that EGCG can exert a protective effect by directly interacting with the epithelial cells, however further studies are needed to better understand the underlying mechanism. The microbiota should not, however, be ignored entirely as it may be that microbial metabolites of EGCG that play a role in the protective effects of EGCG in vivo.

To investigate this effect further, the major metabolites of EGCG produced by the colonic microflora, including 5-(3’, 5’-dihydroxyphenyl)-γ-valerolactone (M6’), should be isolated and tested directly. These could be prepared by incubating EGCG in a slurry of fresh excreted human feces and isolated using preparative scale HPLC. The identified metabolites could then be tested for their ability to mitigate inflammation and intestinal permeability in cell culture models. This would allow for the comparison of EGCG to its microbiota specific metabolites. Alternatively, a germ-free mouse model could be used to determine the effect of EGCG in the absence of EGCG
metabolites from microbiota or the effects of EGCG on microbiota. These mice are, however, expensive and the germ-free condition complicates efforts to induce colitis.

The anti-nutritional effects of EGCG may represent a dose-limiting toxicity in the context of IBD. The inhibition of pancreatic lipase and fat digestion, as well as protein digestion, by EGCG has been studied with the aim of understanding the role of these activities in the obesity-preventive effects of green tea.[186] These beneficial effects of green tea in the context of obesity, appear to become liabilities in the context of IBD.

Very little research has been done looking at the anti-nutritional effects of dietary components in disease models. Much of the research has been focused on prevention or elimination of symptoms of a disease and often ignore malabsorption of nutrients as a symptom. This is typically because malabsorption is assumed to be a result of inflammation-induced dysfunction of the intestinal tract. Our findings with EGCG suggest that this is not necessarily the case as inflammation markers were decreased yet malabsorption of fat and protein increased.

This poses a potential problem for those with IBD who turn to green tea for its beneficial effects as it may increase the malnutritional state that they are already in as a result of the disease. So the question becomes, how do we get the beneficial effects of EGCG without making people sicker? As we found in our studies, direct contact with the epithelium and EGCG studies exhibited beneficial effects. It would be interesting to somehow wrap the EGCG molecules in a casing of slowly digestible or resistant starch. This would allow EGCG to pass through the stomach and small intestine unabsorbed before gut microbiota digest the starch and release the EGCG into the colon. This technology is already being studied as a drug delivery system and as a delayed nicin packaging enhancement for some food products.[333-335] Upon successful encapsulation, a similar DSS mouse experiment could be run with the encapsulated EGCG given in the diet.
5.3 Impact of Soy Protein Concentrate on Colonic Inflammation in vitro and in vivo

In our soy experiments, we found that a low isoflavone soy protein preparation decreases markers of inflammation and intestinal permeability in DSS-induced mice. This effect was related to an inhibition of inflammasome-related signaling. While the exact mechanism for the formation of the inflammasome is still a source of debate, it is clear that soy protein concentrate inhibits part of that process. One thing that was not examined in our study was the comparative inhibitory activity of the protease inhibitors BBI and soybean trypsin (Kunitz) inhibitor against activation of the inflammasome. These two inhibitors have both been shown to decrease anti-inflammatory activity in cell and animal models. It would be interesting to determine if these inhibitors are present in our mouse diets, and the extent to which they play a role in our results. The larger, Kunitz inhibitor is of particular interest as it has a unique β-Trefoil fold that is similar to IL-1β.[206] There has been little to no research done looking at the potential ability for this β-Trefoil component of the inhibitor to bind to IL-1β receptors. This is an exciting prospect as it may be yet another component of soy that accounts for its ability to prevent inflammation and may be a mechanism for inhibition of the caspase-1 activation. Molecular modeling could be done to look at how this particular structure of the inhibitor fits into the receptors that sense IL-1β. If the results of molecular modeling yielded a positive result, a receptor binding assay study could be done in vitro to see if there is actual blocking of the receptors.

In our studies, both the high and low molecular weight fractions yielded a decrease in permeability. The effects of the high molecular weight fraction may be due to the Kunitz inhibitor while the low molecular weight fractions may be inhibiting permeability through BBI, di- and tripeptides, or very low levels of isoflavones. To investigate this further, obtaining pure standards of each inhibitor and conducting another monolayer permeability study could be done. Likewise, further fractionation of the low and high molecular weight fractions via prep-HPLC
could be done. This would help to focus in on a particular peptide, protein, or inhibitor that is exhibiting the greatest effect. The same approach could be done in mice fed different fractions that exhibited activity in cell culture. As mentioned earlier, the beneficial effects observed may also be due to metabolites created by the microbiota from the soy protein. Similar to the suggested experiment above involving EGCG, using a germ-free mouse model that would ignore the effects of the microbiota could also be used to determine the role the microbiota play in soy protein’s attenuation of inflammation.

As much of the research done on soy has been focused on isoflavones, it would be interesting to look at the combined effect of isoflavones and proteins as it would better emulate a whole food. This could be done in an experiment where treatment groups would receive isoflavones, isoflavone free soy protein, or both. While isoflavones and protein components may both act by different mechanisms as a whole or more complete system, their beneficial effects may be additive.

Similarly, the effects of tempeh observed in the final study also showed that fermented soy products may induce similar beneficial effects. Different processing techniques of soy could yield a soy-derived product with enhanced anti-inflammatory effects. Soy can be transformed into a number of different products such as miso, tempeh, and soy sauce. Soybeans have also been used widely as a meat analogue and the effects of processing on these products should also be looked at as extreme heat treatment or pH changes may affect the conformation of potential bioactive proteins or peptides and thus their effectiveness.

5.4 Impact of Dietary Phytochemicals on Intestinal Permeability in Caco-2 Cells

In the final study looking at the effects of cherry, cocoa, moringa, and tempeh we found that all of them did inhibit intestinal permeability to some degree. While the exact mechanisms
of these extracts are unknown, it does show that direct interaction with epithelial cells improves gut barrier function with these extracts. It would be useful to look at these in both cell and IBD animal studies to observe their anti-inflammatory properties and effectiveness at preventing carcinogenesis. Further fractionation of all of these extracts would be extremely useful in investigating the particular components that are exerting this beneficial effect. This could be done by fractionating them using a prep-HPLC, testing the effectiveness of each fraction in cell models, and then characterizing the compounds in the most effective fractions.

5.5 Conclusions

Using permeability as an endpoint of IBD is useful however it does not provide an exact mechanism. While inhibition of inflammation is clearly an important factor of intestinal permeability, it would be interesting to look at how long-term treatment with these components affects protein and mRNA expression of tight junctions. It may be that longer treatment with these dietary components causes an up-regulation in tight junctions or other cellular mechanisms that protect them from acute inflammatory or oxidative stresses.

In our animal studies, we induced acute colitis by using DSS and observed the short-term effects on inflammation. It would be interesting to look at long-term treatment with these dietary components on carcinogenesis mouse models such as the AOM/DSS model. Similarly, looking at pre-treatment as a measure of prevention of colitis would be interesting as it would attempt to emulate results of epidemiological studies finding that soy or green tea consumption leads to lower risks for developing IBD and colorectal cancer.

Collectively, we have demonstrated that different dietary treatments in acute DSS-induced ulcerative colitis in mice reduce inflammation and improve gut barrier function. While the exact mechanism of each of the treatments we used still needs to be investigated further, it
does suggest that dietary treatment may be a viable alternative to medication in some situations.

The results from the studies presented here show that by adding a simple dietary component, like EGCG, soy protein, cherry, cocoa, moringa, or tempeh to their diets, IBD patients may reduce inflammation and improve gut barrier function, thereby decreasing their risk of developing colorectal cancer.
Appendix A

Effects of Intraperitoneal EGCG on DSS-induced Ulcerative Colitis

As was found in our previous study involving, EGCG exhibits anti-inflammatory and gut barrier improvements effects in the DSS-induced ulcerative colitis mouse model. Despite the decrease in inflammatory markers, EGCG also decreases feeding efficiency and macronutrient absorption. In order to investigate this duel nature of EGCG, we examined the anti-inflammatory and anti-nutritional effects of systemic EGCG through the use of intraperitoneal injections of EGCG. This was done to identify if systemic or direct epithelial contact with EGCG was needed for the anti-inflammatory or the anti-nutritional effect.

Mice were treated as colitis was induced as described in 3.3.10 DSS-Induced Colitis. After the 7 days of DSS treatment, all groups were switched to normal drinking water. The mice were injected intraperitoneally with either 40mg/kg EGCG (DEIP) or .9% saline solution (DIP). Two control groups received no DSS and either saline injection (CIP) or 40mg/kg EGCG (EIP) were also analyzed (CIP). Feeding efficiency was determined by dividing the weight gained by the kJ consumed. Spleen weights, colon length measurement, and sample prep was done as described in 3.3.10 DSS-Induced Colitis. Measurements of colonic IL-1β protein were measured as described in 2.3.5 Enzyme Immunoassay. Fecal macronutrient analysis was done as described in 2.3.7 Fecal Macronutrient Analysis.

Weight loss induced by DSS began on day 6 and continued for the duration of the experiment (Figure A-1a). Group DEIP lost significantly more weight than group D during the treatment period. The DEIP group continued to lose weight and did not rebound on the third day of EGCG as they had in the dietary EGCG experiment. The DEIP group had a significantly lower feeding efficiency as compared to the other groups over the three day treatment period.
(Figure A-1b). The feeding efficiency of the other groups were not significantly different than the control. Groups treated with DSS had significantly reduced mean colon length, however group DEIP had statistically significantly longer colons than group DIP (Figure A-1c).

![Figure A-1](image)

**Figure A-1.** The effects of intraperitoneal EGCG injections on weight gain, feeding efficiency, gross measures of inflammation, and macronutrient malabsorption in DSS-induced CF-1 mice. (a) Body weight change relative to starting weight was determined over the course of the experiment. (b) The feeding efficiency of the mice was determined based on the ratio of body weight gain to energy intake and averaged over the last 3 d of the experiment. (c) Colon length and (d) relative spleen weight were determined at the end of the experiment. (e) IL-1β was determined in the colon homogenate using commercially-available ELISAs and normalized to the total protein of each sample. (f) Fecal lipids and (g) fecal proteins were analyzed. All values represent the means ± SEM and an n = 10 for each group. Different letters denote p < 0.05 using one-way ANOVA with a Tukey’s Multiple Comparison post-test.
Colon lengths of groups CIP, DEIP, and EIP were not significantly different. Increases in relative spleen weight were seen in the groups that received DSS (Figure A-1d). While both DSS groups showed significantly higher relative spleen weights, there were no statistical differences between groups DIP, DEIP, or EIP. IL-1β levels between groups DIP and DEIP were not statistically different and were both higher than the controls (Figure A-1e).

DSS treated groups had statistically higher levels of malabsorbed lipids in their feces as compared to the control group (Figure A-1f). Groups DIP and DEIP did not differ significantly. DSS treated groups also had higher levels of malabsorbed proteins in their feces (Figure A-1g). Levels of fecal proteins were statistically higher in group DIP as compared to DEIP. There was no statistical difference between DEIP and the control group.

To investigate if beneficial effects of EGCG were a result of direct interaction with mucosal cells and microbiota or if it was due to EGCG metabolites formed systemically, we administered EGCG via intraperitoneal injections. The similar changes in weight were observed however the mice treated with EGCG did not rebound after the three days of intraperitoneal treatments as they had in the dietary EGCG experiment. The anti-inflammatory effects of dietary EGCG in both crude measurements, colon length and spleen weight, or inflammatory cytokine levels, IL-1β, were not observed in the EGCG intraperitoneal treated mice. Levels of fecal lipids in DEIP mice were not significantly different from DIP mice and the protein found in the feces was significantly less than DIP mice. Together this data suggests that anti-nutritional and anti-inflammatory effects of EGCG seen in mice with experimentally induced colitis are influenced more by the direct interaction with intestinal mucosa and biota than systemic circulation. Unfortunately, this increased weight loss and decreased feeding efficiency suggest that EGCG may be inducing some other toxicity. Future experiments could be done to expand this study by using lower, potentially non-toxic, IP injections.
Appendix B

Abbreviations Used

The following is a list of the abbreviations used throughout this dissertation.

8-OHdG  8-hydroxy-2’-deoxyguanosine
AAPH  2,2’-azobis(2-methylpropionamidine) dihydrochloride
ALT  Alanine aminotransferase
ANOVA  Analysis of variance
AOM  Azoxymethane
APC  Adenomatous polyposis coli
ASC  Apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain
BBI  Bowman Birk Inhibitor
BRAF  B-Raf proto-oncogene, serine/threonine kinase
BUN  Blood urea nitrogen
cDNA  Complementary deoxyribonucleic acid
DAMP  Damage associated molecular patterns
DCDFDA  6-carboxy-2’,7’-dichlorodihydrofluorescein diacetate, di(acetoxymethyl ester)
DHA  Docosahexaenoic acid
DMEM  Dulbecco’s modification of Eagle’s medium
DMSO  Dimethyl sulfoxide
DNA  Deoxyribonucleic acid
DP  Degree of polymerization
DSS  Dextran sulfate sodium
EGCG  Epigallocatechin-3-gallate (EGCG)
EGCG  Epigallocatechin
ELISA  Enzyme-linked immunosorbent assay
EMR-1  EGF-like Module-containing Mucin-like Hormone Receptor-Like 1
EPA  Eicosapentaenoic acid
FAP  Familial adenomatous polyposis
FITC-D  Fluorescein isothiocyanate–conjugated dextran
GCMS  Gas chromatography–mass spectrometry
GI  Gastrointestinal
GLP-2  Glucagon-like peptide 2
GTP  Green tea polyphenols
HPLC  High-performance liquid chromatography
IBD  Inflammatory bowel disease
IFN-γ  Interferon-γ
IKK-β  Inhibitor of nuclear factor kappa-B kinase subunit beta
IL  Interleukin
IP  Intraperitoneal injection
IRAK  Interleukin-1 receptor-associated kinases
IU  Inhibitor units
\( \text{IkB}\alpha \) | Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
---|---
JAM | Junctional adhesion molecule
KO | Knock-out
KRAS | Kirsten rat sarcoma viral oncogene homolog
LAL | Limulus amebocyte lysate
LDL | Low-density lipoprotein
LPS | Lipopolysaccharide
MAPK | Mitogen activated protein kinase
MCP-1 | Monocyte chemotactic protein-1
mDP | Mean degree of polymerization
MLCK | Myosin light-chain kinase
mRNA | Messenger ribonucleic acid
MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MUC | Mucus glycoprotein
MW | Molecular weight
NADPH | Nicotinamide adenine dinucleotide phosphate
NEM | N-ethylmaleimide
NF\( \kappa \)B | Nuclear factor kappa-light-chain-enhancer of activated B cells
NLR | Nucleotide-binding domain and leucine-rich repeat-containing
NLRP3 | NACHT, LRRm, and PYD domains-containing protein 3
ORAC | Oxygen radical absorbance capacity
PAC | Proanthocyanidin
PAMP | Pathogen-associated molecular patterns
PBS | Phosphate buffered solution
PCR | Polymerized chain reaction
PCR | Procyanidins
PRR | Pattern recognition receptors
RAE | Retinol activity equivalents
RAR\( \alpha \) | Retinoic acid receptor alpha
RNA | Ribonucleic acid
RNS | Reactive nitrogen species
ROS | Reactive oxygen species
SEM | Standard error of the mean
SNP | Single nucleotide polymorphism
SOD | Superoxide dismutase
SPC | Soy protein concentrate
TBARS | Thiobarbituric acid reactive substances
TEER | Transepithelial electrical resistance
TFF | Trefoil factors
TGF\( \beta \) | Transforming growth factor beta
TIC | Total ion current
TJ | Tight junction
TLR | Toll-like receptor
TNBS | Trinitrobenzene sulfonic acid
TNF | Tumor necrosis factor
TP53 | Tumor protein p53
T-PER | Tissue Protein Extraction Reagent
TXNIP | Thioredoxin interacting protein
<table>
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<tr>
<th>Acronym</th>
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<td>TYK2</td>
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VITA

Zachary T Bitzer
ZBitzer@gmail.com

Education

**Ph.D. Food Science**  
The Pennsylvania State University, University Park, PA  
August 2010 - May 2015

**Bachelor of Science in Chemistry**  
Minor in Fine Art/Photography  
Ursinus College, Collegeville, PA  
August 2002 - May 2006

Publications


Poster Presentations


Certifications

Certified ESL/EFL (English as a Second/Foreign Language) Instructor. Certified June 2009.