QUERCETIN AND CHLOROGENIC ACID MITIGATE DSS-INDUCED CHANGES IN EXPRESSION OF SELECT PRO-INFLAMMATORY CYTOKINES AND SHORT CHAIN FATTY ACID TRANSPORTER GENES

A Thesis

by

LEIGH ANN PIEFER

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2012

Major Subject: Nutrition

Quercetin and Chlorogenic Acid Mitigate DSS-Induced Changes in Expression of Select Pro-Inflammatory Cytokines and Short Chain Fatty Acid Transporter Genes

QUERCETIN AND CHLOROGENIC ACID MITIGATE DSS-INDUCED CHANGES IN EXPRESSION OF SELECT PRO-INFLAMMATORY CYTOKINES AND SHORT CHAIN FATTY ACID TRANSPORTER GENES

A Thesis

by

LEIGH ANN PIEFER

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Approved by:

Chair of Committee,	Nancy D. Turner
Committee Members,	Clinton D. Allred
	David H. Byrne
Interdisciplinary Faculty Chair,	Rosemary Walzem

August 2012

Major Subject: Nutrition

ABSTRACT

Quercetin and Chlorogenic Acid Mitigate DSS-Induced Changes in Expression of Select Pro-Inflammatory Cytokines and Short Chain Fatty Acid Transporter Genes.

(August 2012)

Leigh Ann Piefer, B.S., Texas A&M University

Chair of Advisory Committee: Dr. Nancy D. Turner

Quercetin (Q) and chlorogenic acid (CA), two bioactive compounds found in stonefruits, may protect against inflammation and cancer because of anti-cancer, anti-oxidant, and anti-inflammatory properties. Since these compounds reach the colon undigested, they affect the luminal environment before they are metabolized by the microbiota and transported into epithelial cells. We hypothesized that Q and CA may suppress expression of pro-inflammatory molecules, alter the luminal environment, and alter the cell cycle, thereby protecting against injury/colitis. To test this hypothesis, 63 male weanling rats were given one of three diets (basal, 0.45% Q, 0.05% CA). After 3 wk of acclimation, colitis was induced in 11 rats/diet [3% dextran sodium sulfate (DSS), 48 h, 3 treatments, 2 wk separation] and 10 rats/diet served as control (0% DSS). All rats were terminated at wk 9. Measurements included: fecal moisture content, fecal short chain fatty acid (SCFA) concentrations (gas chromatography), epithelial injury and inflammation in the distal colon, proliferation (PCNA), and NF-κB activity (ELISA method) and gene expression (real time RT-PCR) in mucosal scrapings. Fecal moisture

content was significantly increased by DSS exposure ($p<0.05$), and never returned to control levels. Fecal SCFA concentrations also increased with DSS (acetate, $p<0.05$; butyrate, $p<0.05$). Increased SCFA concentrations could indicate decreased SCFA uptake. Experimental diets were able to mitigate DSS-induced decreases in SLC5A8 (SCFA transporter) expression. DSS significantly increased injury ($p<0.0001$) and inflammation ($p<0.01$) scores. Compared to the basal diet, CA decreased NF-κB activity in DSS-treated rats ($p<0.05$). Q and CA may maintain healthy regulation of NF-κB through maintaining expression levels of IκBα and Tollip, molecules that inhibit NF-κB activation. Q and CA mitigated DSS-induced increases in pro-inflammatory cytokine expression, specifically IL-1. Q enhanced expression of injury-repair molecule FGF-2 ($p<0.01$), but neither diet nor DSS treatment altered proliferation. Although Q and CA did not protect against DSS-induced increases in injury and inflammation scores or fecal SCFA concentrations, their influence on expression of injury repair molecules, pro-inflammatory cytokines, SCFA transport proteins, and NF-κB inhibitory molecules suggests beneficial influences on major pathways involved in DSS-induced injury/inflammation. The combined benefit of these compounds could have additive/synergistic effects and, therefore, deserve further examination.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my committee. Their support and guidance with my research project is greatly appreciated. I would like to thank my committee chair, Dr. Nancy Turner for her never-ending support. With Dr. Turner's faith in my intelligence and abilities as a scientist, I developed a confidence in myself that cannot be taught. I would also like to thank my committee members, Dr. Clint Allred and Dr. Dave Byrne, for their support and advice. My appreciation is extended to Lauren Ritchie and Stella Taddeo. The obstacles I encountered in the lab could not have been overcome without their patience, advice, and direction. Beyond that, their support inside and outside the lab helped me to succeed.

I am so thankful to have so many loving and supportive people in my life. With my family's unconditional love and guidance, I was able to pursue my graduate degree in a subject area that I truly love. I am appreciative to Amanda, for her constant enthusiasm and optimism. To all of my friends, I thank for their support and willingness to listen. I am also grateful to Lauren, for her friendship and mentorship. Lastly, I could not have reached this goal without Keith's endless encouragement, love, patience, and understanding. I am truly lucky to have so many supportive people involved in my life.

This research has been funded by the grant support from the United States Department of Agriculture/National Institute of Food and Agriculture (2008-34402-19195 and 2009-34402-19195).

NOMENCLATURE

CD	crohn's disease
COX	cyclooxygenase
CRC	colorectal cancer
ddH_2O	distilled deionized water
DSS	dextran sodium sulfate
ELISA	enzyme-linked immunosorbent assay
EtOH	ethanol
FGF	fibroblast growth factor
GI	gastrointestinal
IBD	inflammatory bowel disease
IKK	NF-κB kinase
IL	interleukin
iNOS	inducible nitric oxide synthase
LPS	lipopolysaccharide
LS	least squares
MCT-1	monocarboxylate transporter-1
MyD88	myeloid differentiation factor-88
NF-κB	nuclear transcription factor kappa B
PBS	phosphate buffered solution
PFA	paraformaldehyde
RT-PCR	reverse transcriptase polymerase chain reaction

RIP	receptor interacting protein
SEM	standard error of the mean
SLC5A8	sodium-coupled monocarboxylate transporter-1
SCFA	short chain fatty acids
TGF	transforming growth factor
TFF-3	trefoil factor-3
Th	T-helper
TLR	toll-like receptor
TNBS	2,4,6-trinitrobenzene sulfonic acid
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
Tollip	toll interacting protein
UC	ulcerative colitis

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION

Patients with chronic ulcerative colitis (UC) have a 10-fold greater risk than healthy individuals to develop colorectal cancer (CRC), one of the most commonly diagnosed cancers (1, 2). Regular screenings, early detection, and a diet rich in fruits and vegetables are a few ways to reduce risk of colon cancer (2, 3). These food groups are nutrient-dense and a good source of fiber, phytochemicals, and antioxidants (3). Peaches, for example, contain high levels of phenolic compounds like quercetin and chlorogenic acid (4). In peaches, as well as nectarines and plums, it has been shown that phenolic content correlates with antioxidant activity (5). Antioxidants, or free radical scavengers, can inhibit the development of colon cancer. Quercetin and chlorogenic acid may also have anti-inflammatory properties, by reducing expression of some pro-inflammatory cytokines (6, 7). Due to their poor absorption, phenolic compounds reach the colon undigested, where they are metabolized by the microbiota (8, 9). Exposure of the microbiota to phenolic compounds can alter the composition of bacterial populations, in addition to altering their function and fermentation rates and products. Quercetin and chlorogenic acid alter the luminal environment and inflammatory pathways in the colon, suggesting that quercetin and chlorogenic acid may have potential for mitigation of inflammation and injury repair in chronic UC (5, 6, 8, 10-15).

This thesis follows the style and form of The Journal of Nutrition.

Overview of Inflammatory Bowel Diseases

Inflammatory bowel diseases

The incidence and prevalence of inflammatory bowel diseases (IBD), such as ulcerative colitis (UC) and Crohn's disease (CD), has increased in the United States, Canada, and Western Europe since the 1980's (16, 17). In fact, IBD is one of the top five digestive diseases in the United States, with UC being more prevalent (up to 238/100,000) than CD (17, 18). Unlike the gastrointestinal (GI) inflammation observed in CD, the inflammation characteristic of UC is localized to the distal colon and, because colon cancer is more prevalent in the distal colon and rectum, patients with UC are at a particularly high risk of developing colon cancer (1, 17). Therefore, identifying dietary modifications that suppress inflammation, such as that occurring in UC, will not only reduce the morbidity associated with UC, but it may also serve to reduce the incidence of colon cancer.

Ulcerative colitis

In a healthy colon, the mucosa creates a protective barrier, separating the outside environment (luminal contents) and inside the body. A healthy mucosal barrier is characterized by functioning goblet cells, adequate mucin production, and strong tight junctions between epithelial cells (**Fig. 1**). UC is characterized by a reduction of goblet cells (mucin production) and thinning of the protective mucus, resulting in increased exposure of intestinal cells to luminal contents and bacteria (**Fig. 2**) (1, 19-21).

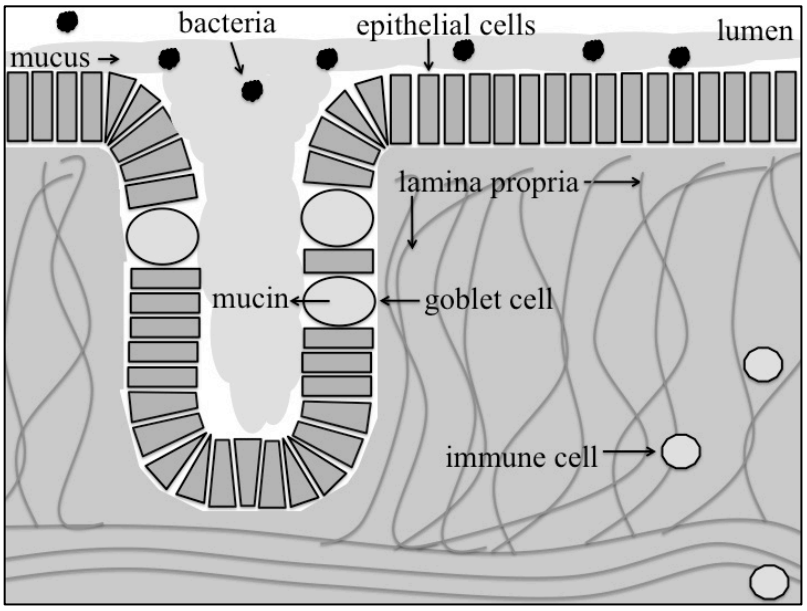


FIGURE 1 Epithelial cells are the colon's primary defense against the outside environment (luminal contents). A healthy and well-maintained epithelium, in addition to adequate production of protective mucin is necessary for healthy colonic tissue.

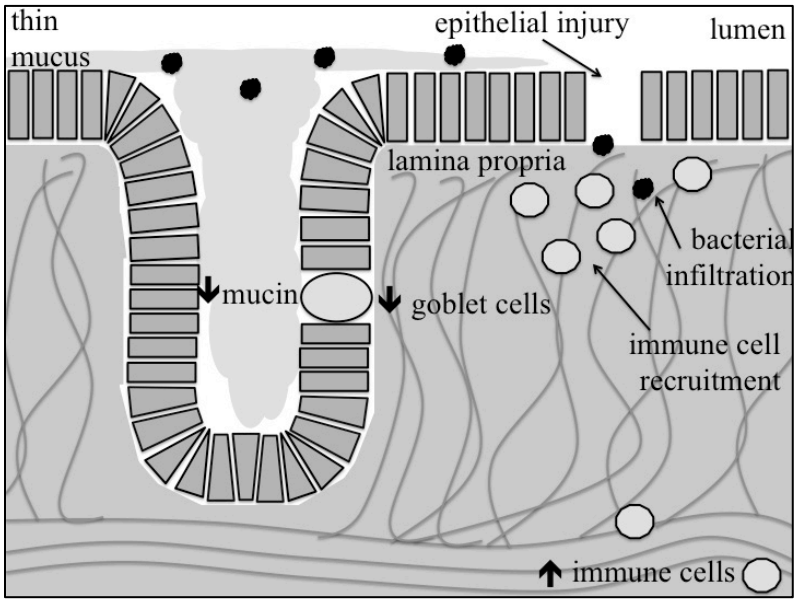


FIGURE 2 In active UC, impaired mucin production and decreased integrity of the epithelium leads to an increased exposure of the lamina propria to the luminal contents. This causes severe immune and inflammatory responses.

The increased exposure of colonocytes to luminal contents can cause epithelial injury, allowing infiltration of fecal material and bacteria into the lamina propria, triggering inflammation in the both the epithelium and lamina propria of the distal colon.

Diet and Colon Health

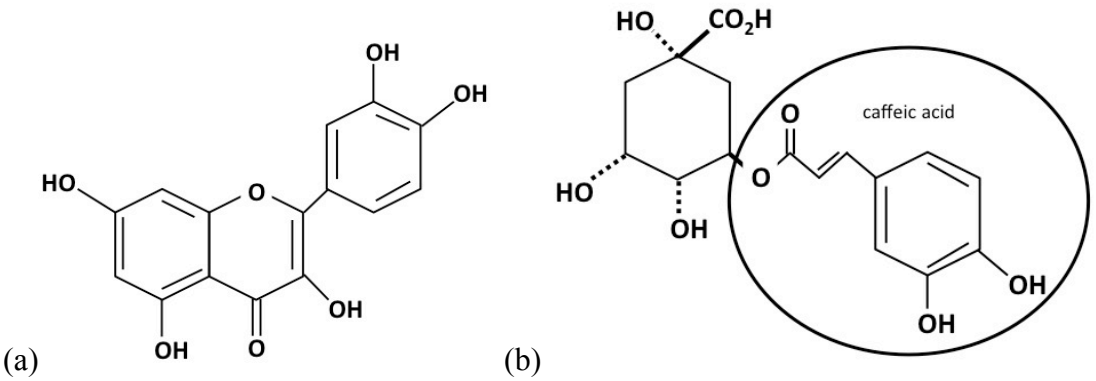
Phytochemicals in the diet

Diets rich in fruits and vegetables are associated with a reduced risk of many diseases, including colon cancer and inflammatory bowel disease (IBD) (2, 3, 22). Fruits and vegetables are good sources of fiber, vitamins, and minerals, as well as many different types of phytochemicals. Phytochemicals are secondary metabolites synthesized by plants, and include chemical classes such phenols, anthocyanins, and flavonols. The composition of phytochemicals varies between not only the types of fruits and vegetables, but also between the genotypes of a single fruit.

Stonefruits, such as peaches and plums, are of particular interest since they are high in phenolic compounds (4, 5, 11). Peaches and plums contain high levels of quercetin and chlorogenic acid, two bioactive compounds that could be used as preventative or therapeutic agents in disease (4, 5, 8, 11, 15). Stonefruit contain a variety of bioactive compounds, but chlorogenic acid and quercetin are found at very high concentrations compared to other bioactive compounds (4).

Quercetin and chlorogenic acid in the diet

Like all phytochemicals, quercetin and chlorogenic acid are synthesized by plants, and therefore are only found in plant foods. Quercetin, the main flavonol in the diet, is found in varying concentrations in a variety of fruits and vegetables (**Fig. 3a**). Chlorogenic acid, an ester of caffeic and quinic acids, is the most abundant hydroxycinnamic acid in the diet, found in high concentrations in coffee and the skins of fruits, such as apples and peaches (**Fig. 3b**) (4, 9, 11). In addition to the widespread presence of these parent compounds in the diet, there are several derivatives of each compound that can be found in each food item. For example, neochlorogenic acid, an isomer of chlorogenic acid, is also found in high levels in peaches and plums (4, 5). Quercetin is most often consumed as a glycoside, where quercetin aglycone is bound to a carbohydrate molecule. Quercetin aglycone and caffeic acid are found as several esters and isomers in varying concentrations in plant foods.



(a) (b)

FIGURE 3 Quercetin is shown as quercetin aglycone (a), not bound to a carbohydrate molecule. Chlorogenic acid (b) is an ester of caffeic acid and quinic acid.

Quercetin and chlorogenic acid in colon health and colonic inflammation

It has been demonstrated that phenolic extracts containing quercetin and chlorogenic acid have anti-cancer and anti-inflammation effects *in vitro* and *in vivo*. (A detailed description of the pathways involved in the regulation of inflammation is provided in the section following this overview.) In lipopolysaccharide (LPS)-stimulated murine macrophages, phenolic extracts of Kakadu plums suppressed inflammation by reducing levels of cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) (23). Kakadu plum extracts also decreased phosphorylation and degradation of IKBα, the inhibitory subunit of nuclear transcription factor- κB (NF-κB) (23). Although not a stonefruit, *Ginkgo biloba* extract inhibits tumor necrosis factor (TNF)-α, NF-κB, and interleukin (IL)-6 expression in 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced murine colitis, possibly due to its high content of quercetin aglycone (24). Phenolic extracts can contain a variety of bioactive compounds, but there is sufficient evidence that quercetin and chlorogenic acid play a major role in contributing to the anti-inflammatory properties observed.

Several studies have evaluated quercetin and chlorogenic acid in inflammation models. Observing each compound individually, rather than as a part of their respective phenolic extracts, provides more information on how each compound contributes to anti-inflammatory properties. Quercetin has been studied extensively *in vivo,* inhibiting inflammation in acute dextran sodium sulfate (DSS)-induced colitis (6). Suppressing activation of NF-κB and expression of COX-1 and COX-2, quercetin may play a strong anti-inflammatory role by decreasing expression of pro-inflammatory cytokines

downstream of NF-κB (6, 15). Quercetin has also shown to enhance epithelial barrier function *in vitro* by increasing expression of claudin-4, a tight junction protein (25). This flavonol may be important for colon health and inhibiting inflammation by altering gene transcription of pro-inflammatory cytokines and tight junction proteins.

Chlorogenic acid may also be beneficial due to its antioxidant properties, although there is limited *in vivo* data defining the effects of chlorogenic acid in colitis (9). *In vitro,* chlorogenic acid inhibits DNA damage and the formation of mutagenic and carcinogenic compounds (9). Other esters of caffeic acid have been shown to have protective effects *in vitro*, through inhibition of lipoxygenase and cyclooxygenase enzymes (26). Metabolites of lipoxygenase (leukotrienes) and cyclooxygenase (prostaglandins) pathways play a role in inflammatory diseases and in tumorigenesis (26). This *in vitro* data suggests that chlorogenic acid could not only protect against inflammation, but also reduce cancer risk by acting as an antioxidant and reducing production of prostaglandins. Few *in vivo* studies have been reported on the anti-inflammatory effects of chlorogenic acid. However, hydrocaffeic acid, a derivative of caffeic acid, has shown to alleviate DSS-induced increases in IL-1β, TNF-α, and IL-8 (7). Upon reviewing previous *in vitro* and *in vivo* studies, chlorogenic acid may be protective through a variety of pathways, including those that regulate inflammation in the colon.

Inflammation in the Colon

Nuclear transcription factor-κB pathway

Inflammatory mediators, along with pro-inflammatory cytokines, can have varied effects based on the type of cell. For example, cyclooxygenase-2 (COX-2) limits bacterial translocation into the lamina propria when expressed in epithelial cells, but can lead to neoplasia and tumorigenesis when expressed in the lamina propria (2, 27). Like COX-2, the downstream effects of nuclear transcription factor kappa-B (NF-κB) vary depending on the type of cell in which it is being expressed (**Fig. 4**) (27).

In order to have downstream effects, NF-κB must be activated. NF-κB is present in the cytoplasm of cells in its inactive form, bound to the inhibitory molecule IκBα. NF-κB is activated by the presence of pro-inflammatory molecules, like interleukin (IL)-1 and tumor necrosis factor-α (TNF-α) (20, 21, 27, 28). IL-1 is one of the primary cytokines responsible for triggering the NF-κB-inflammation cascade (21, 29). NF-κB can also be activated through toll-like receptor (TLR) pathways, which are activated by interactions with bacterial cell components (21, 30).

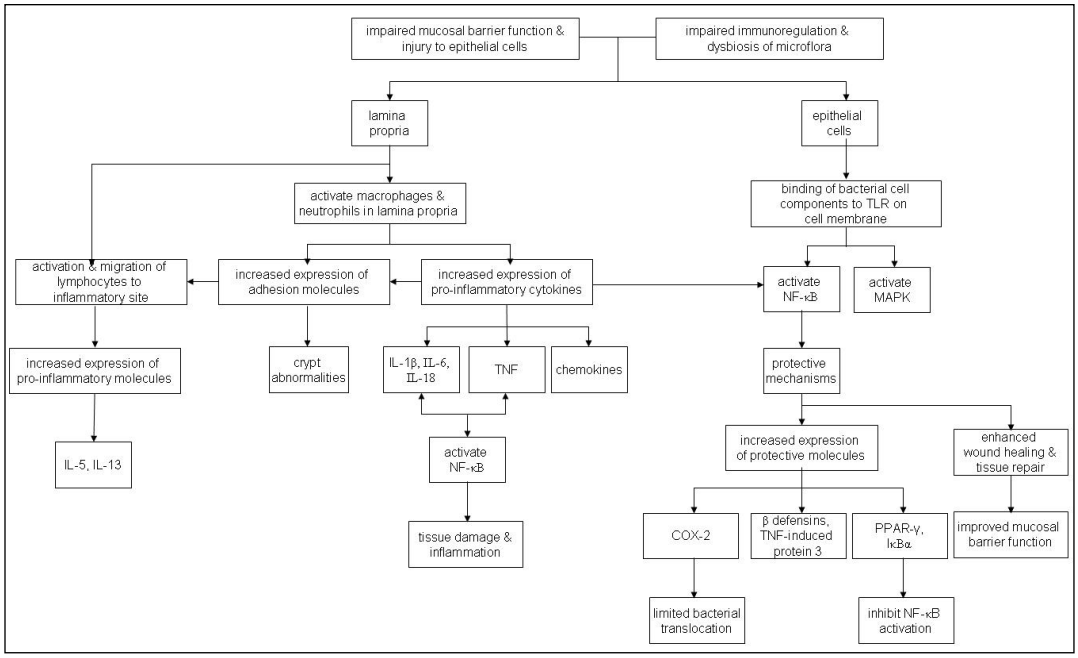


FIGURE 4 Effects of NF-κB activation vary depending on the type of cell in which it is expressed. A review of the literature reveals that UC-associated injury triggers different responses in lamina propria and epithelial cells (1, 20, 21, 27).

When IκBα is phosphorylated and removed from NF-κB, activated NF-κB translocates to the nucleus where it induces transcription of cytokines (20, 21, 27, 30). There are multiple downstream effects of activated NF-κB including the stimulation of COX-2 expression, a key link between inflammation and cancer (2, 27). NF-κB can increase expression of TNF-α, IL-1, and IL-6, and therefore propagate activation of NF-κB in neighboring cells through positive feedback (20, 21, 28-31). This positive feedback is further promoted by IL-6, which increases TNF-α and IL-1 expression (32). IL-1 and TNF-α also injure the colonic tissue by damaging the extracellular matrix and compromising the integrity of the protective mucosa (20). The propagation of NF-κB activation by pro-inflammatory cytokines through positive feedback causes an uncontrolled inflammatory response.

NF-κB activation causes increased expression of other pro-inflammatory molecules including interferon (IFN)-γ, IL-6, IL-12, and COX-2 (20, 21, 28-33). These cytokines are directly involved in mucosal damage and inflammation that is characteristic of active inflammatory bowel disease (IBD) (20, 32). Just as immune signals can increase expression of pro-inflammatory cytokines, pro-inflammatory cytokines can alter the function, differentiation, and recruitment of immune cells. For example, IFN-γ expression is stimulated by increased expression of IL-12, but IFN-γ also induces the differentiation of T-helper (Th)-1 cells (20, 32). Th-1 immune response, while predominantly found in Crohn's disease, is found in most experimental colitis models (32). In chronic UC, both Th-1 and Th-2 immune responses are active (33). IL-6 expression in colonocytes, for example, is elevated in UC and plays roles in both Th-1 and Th-2 immune responses (31). In the lamina propria, dysregulation of NF-κB signaling and Th-1 and -2 immune responses can lead to severe inflammation (pro-inflammatory cytokines) and injury (degradation of the extracellular matrix).

While NF-κB activation can exacerbate injury and inflammation in the lamina propria, NF-κB activation can be protective in epithelial cells. For example, downstream effects of activated NF-κB in the epithelium include enhanced integrity of the epithelial cell barrier (anti-apoptosis) and limited bacterial translocation into the lamina propria (COX-2 expression) (2, 27, 32). Through suppressing apoptosis and enhancing proliferation, NF-κB can facilitate restoration of epithelial barrier integrity and wound healing (1, 21). Further evidence suggests NF-κB may play an anti-inflammatory role in colonic epithelial cells by suppressing further activation of NF-κB in neighboring cells

by stimulating expression of the inhibitory molecule IκBα (1, 20, 21, 28, 32). While NF-κB could exacerbate inflammation through increased activity in macrophages, activated NF-κB could play a protective role by promoting cell repair and integrity of the epithelial barrier (1, 20, 21).

Tumor necrosis factor-α effects on cell cycle

Expression of tumor necrosis factor (TNF)-α is increased in colitis, but can have numerous downstream effects (33). Not only does TNF-α stimulate inflammation and activate immune cells, it also influences the cell cycle, altering proliferation and cell death in the epithelium (**Fig. 5**) (31). Tumor necrosis factor receptor (TNFR)-1 mediates cell death, cytokine production, and can affect targeted gene expression (31). Through receptor interacting protein (RIP) and caspase-8, TNFR-1 is able to induce apoptosis and necrotic cell death (34). Apoptosis, or programmed cell death, differs from necrotic cell death. In necrotic cell death, the cell lyses, releasing contents of the cell, including pro-inflammatory cytokines. Activation of TNFR-1 could be the reason why both necrosis and apoptosis are observed in inflammation-associated cell death.

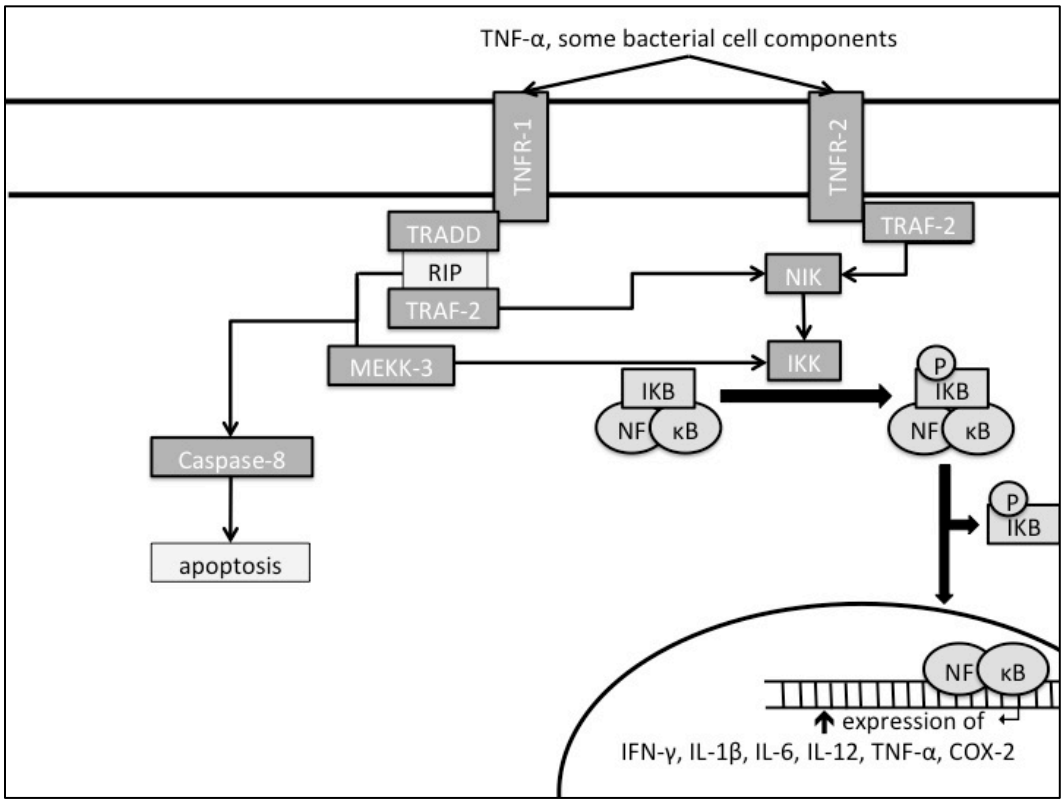


FIGURE 5 TNFR-1 and -2 stimulation causes biological responses involving inflammation and the cell cycle. TNFR-1 stimulates inflammation by activating NF-κB and cell death through apoptosis and necrosis. TNFR-2 promotes cell survival through the anti-apoptotic signals of NF-κB, in addition to the increased expression of pro-inflammatory cytokines associated with activated NF-κB.

Tumor necrosis factor receptor (TNFR)-2 also plays a role in the cell cycle during inflammation, although it is responsible for cell proliferation and survival, rather than cell death (31). Some downstream effects of TNFR-2 overlap with effects TNFR-1, while some downstream effects are independent of TNFR-1 (31). Like TNFR-1, TNFR-2 activates NF-κB, resulting in anti-apoptotic signals, promoting cell survival (31). In UC, TNFR-2 expression is elevated, possibly due to the increased concentrations of pro-inflammatory cytokines like TNF-α and interleukin-6 (IL-6) (31).

While an elevation in TNFR-2 has the potential to be beneficial for cell survival, the elevations of NF-κB activity via TNFR-2 can increase inflammation. This provides further support that proper expression of TNFR-1 and -2 and tight regulation of NF-κB activation is vital for healthy colonic tissue. Despite the protective mechanisms of TNFR-2, its increased expression could increase the inflammatory response observed in UC.

Microbiota Homeostasis and Metabolism

Intestinal microbial interactions through Toll-like receptors

Toll-like receptors (TLRs) are a family of receptors found in a variety of cells, including epithelial cells in the gastrointestinal (GI) tract. Normally, TLR expression is low in epithelial cells, so the large concentrations of commensal bacteria do not cause an immune response (32). TLR stimulation by pathogenic or misrecognized commensal bacteria in the GI tract causes increased recruitment and activation of neutrophils, enhancing the innate immune response (35). In addition to an acute inflammatory response, TLR are thought to play a role in chronic inflammatory processes, like that found in ulcerative colitis (UC).

TLRs trigger downstream inflammatory responses via the TLR signal transduction molecule myeloid differentiation protein-88 (MyD88) (**Fig. 6**) (35, 36). MyD88 activates kinases that activate NF-κB kinase (IKK), and therefore NF-κB (35). Although MyD88-independent signal transduction does exist in TLR pathways, similar

transduction molecules will also activate NF-κB (35). In chronic inflammatory diseases like UC, the impaired regulation of TLR can be detrimental to intestinal inflammation.

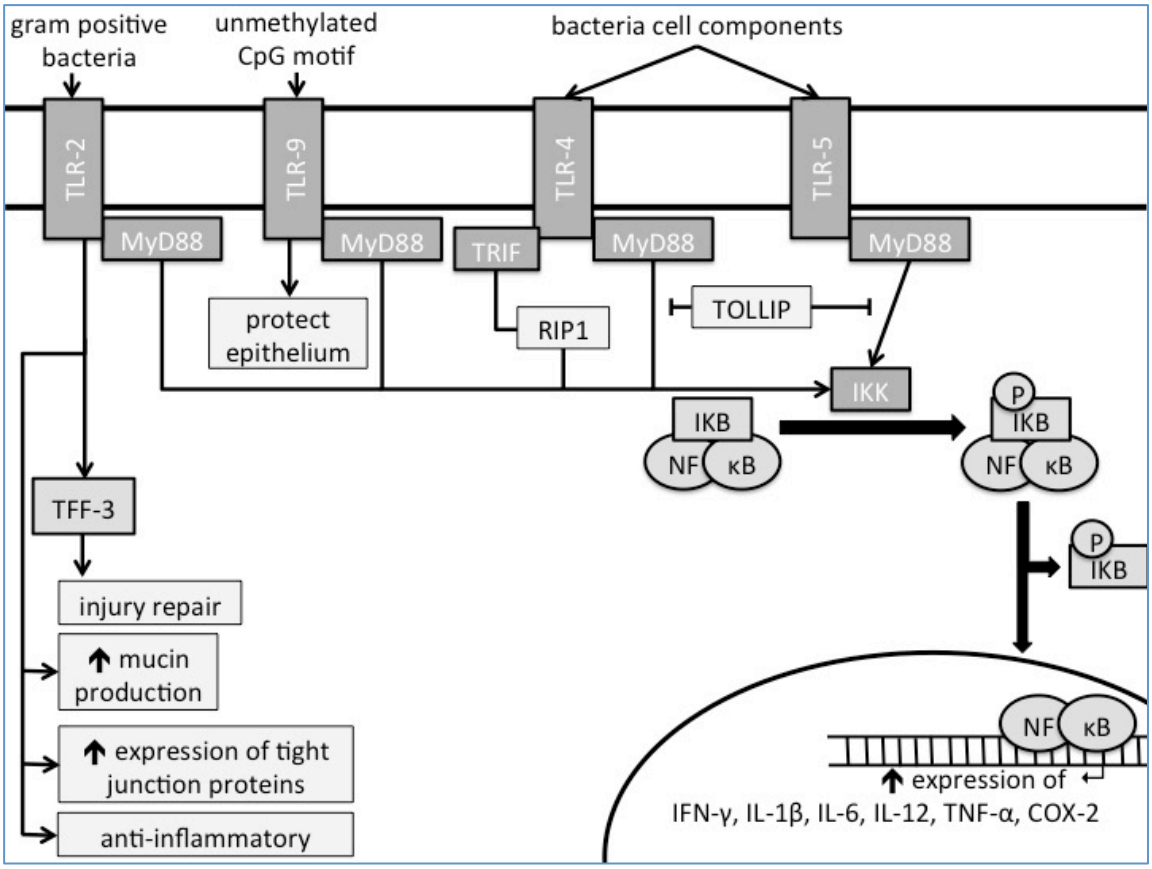


FIGURE 6 TLR mediates activation of NF-κB via MyD88 and other transduction molecules. My-D88-stimulated activation of NF-κB is inhibited by TOLLIP.

Tight regulation of TLR limits cell damage and prevents unnecessary immune and inflammatory responses. Specific endogenous and exogenous TLR ligands can activate TLR and downstream inflammatory processes (35). Exogenous TLR ligands vary by the type of TLR, but all TLR are activated in bacterial or viral infection (35). TLR-2 is stimulated by gram positive bacterial cell wall components and can play a

protective role through the maintenance of the epithelial barrier (35, 37). Maintaining epithelial barrier integrity, TLR-2 increases tight junction protein expression and enhances the function of mucin-producing goblet cells (37, 38). When TLR-2 is activated, trefoil factor-3 (TFF-3) expression is increased, a peptide important for epithelial barrier integrity (38, 39). TFF-3 is thought to enhance injury repair through enhanced cell survival and reduced recruitment of inflammatory cells (38, 39). In TNBS-induced colitis, a reduced expression of TFF-3 has been observed, which is reflected by the high levels of injury and inflammation seen in colitis (39). TLR-2 also has downstream anti-inflammatory effects, suggesting possible protection in UC (38). In addition to TLR-2, TLR-9 also functions to protect the epithelium. TLR-9 is stimulated by unmethylated CpG motifs, motifs most often found in prokaryotic and viral DNA (32, 37, 40, 41). CpG motifs are cytosine and guanidine nucleotides found next to each other, which are tightly regulated (suppressed and/or methylated) in mammalian DNA (40). TLR-9, a pattern-recognition receptor, is unique in that it can differentiate between self and non-self CpG DNA motifs (40). If TLR-9 is dysfunctional, self CpG DNA can be identified as non-self, causing an auto-immune response (37). In colitis, TLR-9 stimulation can be protective via increased expression of IFN-α/β (32, 40). These type I IFNs can suppress dextran sodium sulfate (DSS)-induced colitis and prevent epithelial barrier dysfunction (41). Although TLR-2 and TLR-9 play an active role in maintaining epithelial barrier integrity, some TLR modulate microflora-mediated protection against pathogenic bacteria.

Some TLR, like TLR-4 and -5, identify pathogenic bacteria and trigger responses to protect the body from the pathogens. TLR-4 plays an important role in commensal microflora-mediated protection (37). TLR-4 will identify pathogenic bacteria causing an inflammatory response (NF-κB activation), neutralizing the pathogenic threat to the epithelial cells (35, 37). Normally, NF-κB is activated via the TLR-MyD88 pathway. However, TLR-4 is able to activate NF-κB using different signal transduction molecules (TRIF, RIP-1) (37). In addition, the presence of inflammatory cytokines, like TNF-α and IFN-γ, have shown to increase expression of TLR-4 *in vitro*, which may exacerbate TLR-4-induced inflammatory response (37). In UC, pathogenic bacteria populations may be higher than normal (32, 42). With an increased population of pathogenic bacteria and an increased number of TLR-4 receptors on epithelial cells, this can lead to severe inflammation. Another inflammation-associated TLR is TLR-5, which is stimulated by flagellin, a bacterial component found in both commensal and pathogenic bacteria (35, 37). If pathogenic bacteria are present, or commensal bacteria are misrecognized to be pathogenic, TLR-5 stimulates cytokine and chemokine secretion in intestinal epithelial cells, via NF-κB (32, 37). In UC, an intolerance of commensal bacteria develops, leading to inflammatory and exaggerated immune responses.

Since colonic epithelial cells are constantly in contact with bacteria, it is important that non-pathogenic, commensal bacteria do not trigger an immune response. This is achieved by an increased expression of toll like receptor inhibiting protein (Tollip) in response to non-pathogenic bacteria and transforming growth factor (TGF)-β-regulation of immune response (43, 44). Tollip allows colonic tissue to tolerate the

presence of the microflora, inhibiting TLR-induced pro-inflammatory responses (43). Tollip expression is seen to be similar in patients with and without UC, while a decreased sensitivity of TGF-β is observed in colitis (43, 44). Decreased sensitivity of TGF-β causes an increase in T cell-mediated autoimmune and auto-inflammatory responses (44). Although Tollip activity is normal in UC, the decreased sensitivity of TGF-β and the over-stimulation of TLR by bacteria are responsible for the increased TLR-induced inflammatory and immune responses.

In the GI tract, TLR play an important role in controlling homeostasis, injury repair, and prevention of injury. Whether through maintaining the epithelial barrier or preventing pathogenic or viral infection, TLR are important for GI health. The increased expression of TLR, dysregulation of TLR signaling, and misrecognition of commensal bacteria as pathogens, however, can be detrimental to health status.

Role of short chain fatty acids in colon health

Fermentation of substrates, including dietary fiber and other carbohydrate structures, by colonic microflora plays a part in colonic health and homeostasis of colonocytes. Dietary fiber is not digested in the small intestine, so it reaches the colon where it is fermented by bacteria, yielding short chain fatty acids (SCFA) (45). It is also a prebiotic, promoting a healthy, non-pathogenic microbial population (42, 46). Dietary fiber not only affects the populations of bacteria found in the colon, but also their fermentation rates and patterns.

Release of SCFA causes beneficial changes to the luminal environment: decreased colonic pH, enhanced water and mineral absorption, and impaired growth of pathogenic bacteria (42). While the rate of fermentation is higher in the proximal colon, fermentation is still active in the distal colon, and therefore its benefits are applicable to the distal colon (42, 47). Fermentation products vary, but the most important SCFA are butyric, acetic and propionic acids (45, 47, 48). Butyric acid is particularly important, as it serves as a primary energy source for colonocytes and decreases production of pro-inflammatory cytokines (via inhibition of NF-κB activation) (42, 49, 50). In addition, butyrate decreases the activity of co-carcinogenic enzymes (42, 46). In normal cells, SCFA stimulate epithelial cell proliferation and increase mucosal blood flow, improving epithelial barrier integrity (45, 46, 49). SCFA are important for the homeostasis of both the microflora (inhibit growth of pathogenic bacteria), and the colonocytes (energy source and anti-inflammatory).

An imbalance between healthy and pathogenic bacteria in the colon, or dysbiosis, is thought to trigger the onset of ulcerative colitis (UC) (32, 42). Changes in the microbial population in the colon will not only alter SCFA concentrations in the lumen, but can also exacerbate immune and inflammatory responses. Caffeic acid, a component of chlorogenic acid, has shown to inhibit growth of pathogenic bacteria, like *Clostridium perfringens* (51). In addition, 3-(Quercetin-8-yl)-2,3-epoxyflavanone, a product of quercetin oxidation, has shown to have antibacterial activity against multidrug-resistant *S. aureus* and *H. pylori* (52). By influencing the populations of microbes present in the colon, chlorogenic acid and quercetin may contribute to changes in the amount and

relative distributions of SCFA produced. Monitoring fecal SCFA levels and relative concentrations will therefore serve as a biomarker of changes in either microbial populations or their fermentation patterns.

Metabolism of phytochemicals by the microbiota

In addition to fermentation of fiber, the bacteria in the colon also metabolize phytochemicals like flavonols and phenols. Chlorogenic acid is degraded by bacteria-produced esterases into caffeic and quinic acids (53). Quercetin, found as a glucoside, is extensively metabolized by the microbiota (53). When metabolized, quercetin-3-O-glucoside yields quercetin aglycone, formate, lactate, ethanol, and 3,4-dihydroxyphenlyacetic acid, acetate, and butyrate (53). Quercetin aglycone is thought to be more beneficial than its glucoside counterpart, as the aglycone is more effective at preventing experimental murine colon carcinogenesis than the glucoside (54). Therefore, breakdown of quercetin-3-O-glucoside is essential for release of the bioactive quercetin aglycone. Since the microbiota is a vital component of quercetin and chlorogenic acid metabolism and absorption, it is safe to assume that changing levels of phytochemicals in the diet could impact the health, homeostasis, and proper function of the microbiota.

Short chain fatty acid absorption

While the microflora is needed for short chain fatty acid (SCFA) synthesis from fiber, transport proteins are necessary for proper absorption of SCFA into the epithelial

cells. Absorption of SCFA can enhance water and ion transport across the epithelial cell membrane. Proper water and electrolyte balance is important for gastrointestinal health, as water imbalance can lead to constipation or diarrhea. A transport protein necessary for ion balance and SCFA absorption is sodium-coupled monocarboxylate transporter-1 (SLC5A8). SLC5A8 transports both SCFA and sodium into colonocytes (39). The SCFA uptake is important for maintenance of luminal pH and transport of energy-yielding substrate into the cells; sodium uptake is important for water balance. Water and electrolyte imbalances exacerbate the symptoms of UC, so sufficient expression and localization of SLC5A8 in epithelial membranes is desired in the colon.

Butyrate is an important energy source for colonocytes and is vital for the integrity of the epithelial barrier (39, 55). Monocarboxylate transporter-1 (MCT-1) is an important transporter of butyrate into colonic epithelial cells, playing a key role in the maintenance of the epithelial barrier (39, 55). In UC, a strong epithelial barrier can limit bacterial infiltration into the colonic tissue. However, in dextran sodium sulfate (DSS)-induced murine colitis, MCT-1 expression (both protein and mRNA levels), butyrate uptake, and butyrate utilization are reduced (55). In fact, an indirect relationship between MCT-1 expression and colonic mucosal inflammation (IFN-γ and TNF-α) is observed in DSS-induced colitis (39, 55). In UC, impaired production, uptake, and β–oxidation of butyrate are observed (42, 45, 46, 50, 55, 56). The decreased absorption of butyrate observed in UC could impair butyrate's ability to act as an energy source and an anti-inflammatory agent.

Summary

A review of the literature suggests that quercetin and chlorogenic acid may be beneficial in colitis. Examination of both *in vivo* and *in vitro* studies provides sufficient evidence that both compounds have anti-inflammatory properties in colon cells. Expression of pro-inflammatory molecules is decreased by quercetin and chlorogenic acid through a variety of pathways. NF-κB activation is primarily responsible for the increased expression of pro-inflammatory molecules, increasing inflammation in some cells, while activating protective mechanisms in others. Quercetin down regulates NF-κB, and therefore its downstream pro-inflammatory effectors. Chlorogenic acid has also shown to reduce expression of pro-inflammatory molecules. NF-κB activation and increased expression of pro-inflammatory cytokines can be triggered through multiple pathways including toll-like receptor and tumor necrosis factor receptor signaling. Since there is little evidence suggesting which pathways are influenced by quercetin and chlorogenic acid, each pathway will be investigated.

In addition, quercetin and chlorogenic acid may play a role in altering microbial populations, their fermentation rates, and ratios of short chain fatty acids (SCFA) in the colon. Since these compounds are metabolized by the microbiota, they may function to promote survival of beneficial bacteria and/or inhibit growth of pathogenic bacteria. In addition, quercetin and chlorogenic acid may alter fermentation of carbohydrates, altering the rates and/or ratios of SCFA released into the lumen. These SCFA alter the luminal environment and can enhance water and mineral absorption. One SCFA,

butyrate is the primary energy source for colonocytes, making its production and absorption a necessity for properly functioning colonocytes. Butyrate has also shown to have anti-inflammatory properties, which could be beneficial in colitis. As a result of this review, quercetin and chlorogenic acid will be examined individually to determine their effects on various aspects of inflammation in an *in vivo* murine model of chronic colitis.

Objective of Study

Based on current literature, we hypothesize that the stonefruit bioactive compounds, quercetin and chlorogenic acid, may protect against inflammation in a colitis/injury state via suppression of pro-inflammatory molecules, and regulation of the cell cycle and the luminal environment. To assess the effects of quercetin and chlorogenic acid on inflammation and injury in the colon, this study aims to:

- evaluate structural changes in colon crypts (injury and inflammation)
- evaluate proliferation in colon crypts
- quantify and characterize short chain fatty acid concentrations in fecal material
- evaluate NF-κB activity in mucosal scrapings
- quantify expression of genes involved in regulation of inflammation, injury repair, and SCFA transport

CHAPTER II

CHLOROGENIC ACID AND QUERCETIN MITIGATED DSS-INDUCED INCREASES IN IL-1 EXPRESSION AND DECREASES IN SLC5A8 EXPRESSION

Introduction

Inflammatory bowel disease (IBD) is one of the top five digestive diseases in the United States, with ulcerative colitis (UC) being more prevalent than Crohn's disease (17, 18). Inflammation in UC is localized to the distal colon, and, because colon cancer is more prevalent in the distal colon and rectum, patients with UC are at a particularly high risk of developing colon cancer (1, 17). The ultimate causes of ulcerative colitis are not known, however, the disease is associated with exaggerated immune responses to normal commensal microbiota, a probable dysbiosis in which the distribution of microflora is altered, and altered epithelial barrier function (32). Treatment regimens relying upon pharmaceuticals and biological therapies are currently inefficient in that they are often ineffective in approximately half of subjects (57). Therefore, identifying alternative interventions, such as dietary modifications that suppress inflammation in the colon, by mediating the contributors to colitis or their downstream effects, will not only reduce the morbidity associated with UC, but it may also serve to reduce the incidence of colon cancer.

Diets rich in fruits and vegetables are associated with a reduced risk of many diseases, including colon cancer and IBD (2, 3, 22). Fruits and vegetables are good sources of fiber, vitamins, and minerals, as well as many different types of phytochemicals. Stonefruits, such as peaches and plums, contain many phytochemicals with biological activity, including quercetin and chlorogenic acid (4, 5, 11). Quercetin is the main flavonol in the diet, and chlorogenic acid, an ester of caffeic acid, is the most abundant hydroxycinnamic acid in the diet (4, 9, 11). There are a limited number of studies that have attempted to determine the effect of stonefruits on colon health, with most of the work being conducted *in vitro.* However, Yang and Gallaher (2005) found that consumption of dried plums alters activities of bacterial enzymes leading to a reduction in fecal levels of secondary bile acids, which are known irritants that can promote colon carcinogenesis (58). Phenolic extracts of Kakadu plums have been reported to decrease activation of NF-κB and levels of cyclooxygenase (COX)-2 in LPS-stimulated murine macrophages (23). Since stonefruit extracts contain many different compounds, it is desirable to identify the protective mechanisms of each compound.

In an attempt to begin to understand how the individual compounds in these extracts influence the colon, our lab demonstrated that quercetin reduces colon carcinogenesis *in vivo*, and that the suppression may have occurred in part due to a reduction in expression of pro-inflammatory mediators like COX-2 (15). It has also been shown that quercetin suppresses activation of NF-κB in an acute model of colitis and enhances epithelial barrier function *in vitro* by increasing expression of claudin-4, a tight junction protein (6, 25). In UC, epithelial barrier integrity is reduced, resulting in

increased paracellular transport of luminal contents, triggering immune and inflammatory responses (32). The effects of quercetin on pro-inflammatory cytokines and epithelial integrity have not been studied in chronic colitis, however, and must be further investigated.

Chlorogenic acid also may protect against inflammation, as well as inflammation-associated carcinogenesis. Chlorogenic acid has demonstrated the ability to protect against carcinogenesis *in vitro* via inhibition of DNA damage and of formation of mutagenic and carcinogenic compounds (9). Through inhibition of pro-tumorigenic enzymes, lipoxygenase and cyclooxygenase, esters of caffeic acid have shown to have protective effects *in vitro* (26). Few *in vivo* studies have been reported on the anti-inflammatory effects of chlorogenic acid. However, hydrocaffeic acid, a derivative of caffeic acid, alleviates DSS-induced increases in IL-1, TNF-α, and IL-8 in acute colitis (7). The anti-cancer and anti-inflammatory properties demonstrated *in vitro* and *in vivo* warrant further investigation of chlorogenic acid in chronic colitis.

Diet can not only alter the production of pro-inflammatory cytokines, but can also influence the microbial populations in the colon (59). Chlorogenic acid and quercetin are both metabolized by bacterial-produced enzymes and, therefore, may influence fermentation patterns (53). *In vitro*, quercetin and chlorogenic acid have shown to have anti-microbial properties (51, 52). Through changes in the microbial populations and their fermentation patterns, these bioactive compounds could impact the epithelium and production of pro-inflammatory cytokines.

The purpose of this study was to evaluate the effects of quercetin and chlorogenic acid on inflammation, injury, and SCFA metabolism in the colon using a chronic model of colitis. To determine the mechanisms involved, the influence on NF-κB, toll-like receptor (TLR), and tumor necrosis factor receptor (TNFR) pathways were examined. Based on the literature, we hypothesized that stonefruit bioactive compounds, quercetin and chlorogenic acid, may protect against inflammation in a chronic colitis/injury state via suppression of pro-inflammatory molecules, and alteration of the cell cycle and of the luminal environment.

Materials and Methods

Animals and study design

Animals in this project were treated in accordance with National Institutes of Health guidelines using a protocol approved by the Texas A&M University Animal Care Committee. Sixty-three male weanling Sprague-Dawley rats (Harlan Sprague-Dawley, Houston, TX) were acclimated for 6-9 d and then assigned to one of three diet groups (basal, 0.45% quercetin, 0.05% chlorogenic acid), which were further divided into dextran sodium sulfate (DSS, 3%) and control (0% DSS) groups (**Fig. 7**). Previously, our lab has used 0.45% quercetin in the diet, which inhibited expression of COX-1 and COX-2 in an AOM model (15). Therefore, this level of quercetin was used as a positive control. Assuming the rats consumed approximately 20 g/d of diet, this would result in an intake of 0.09 g quercetin/d and 0.01 g chlorogenic acid/d. Adjusting for body

weight, this is equivalent to a human consuming 1.8 g quercetin/d and 0.2 g chlorogenic acid/d. In order to get 1.8 g quercetin/d, humans would need to supplement a diet rich in fruits and vegetables with quercetin supplements. On the other hand, drinking ~60 mL of coffee (strong brew, pale roast robusta) is sufficient to obtain this level of chlorogenic acid (60). This amount of chlorogenic acid can also be obtained in the diet by 150-300 g of peaches, plums, or nectarines (4).

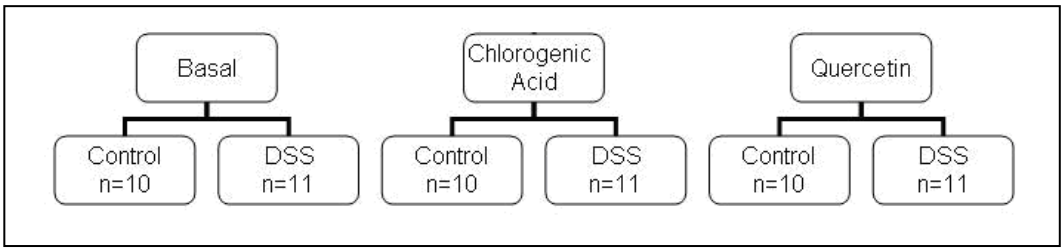


FIGURE 7 Twenty-one rats were assigned to each diet (basal, 0.05% chlorogenic acid, 0.45% quercetin), with 10 rats assigned to the control (distilled H_2O), and 11 rats assigned to the 3% DSS treatment group in each diet.

After 3 wk of consuming the experimental diets, DSS animals began DSS treatment (**Fig. 8**). DSS animals were given a total of three 48-h DSS treatments (3% DSS in H_2O), separated by 2 wk, during which they received distilled H_2O. Control animals received only distilled H_2O. Since DSS is one of the most widely used reagents for inducing colitis, the clinical and histopathological characteristics, as well as associated gene expression, are already known to reflect aspects of human UC (1, 61, 62). Clinical symptoms exhibited by animals on DSS treatment include weight loss, diarrhea, and blood in the stool (61). Although DSS is often used as an acute injury-inducing agent, the histopathology, inflammatory cytokines, microflora effects, and

clinical symptoms of DSS reflect UC. To mimic human chronic UC, animals were given multiple low-dose DSS-treatments with periods of remission (1).

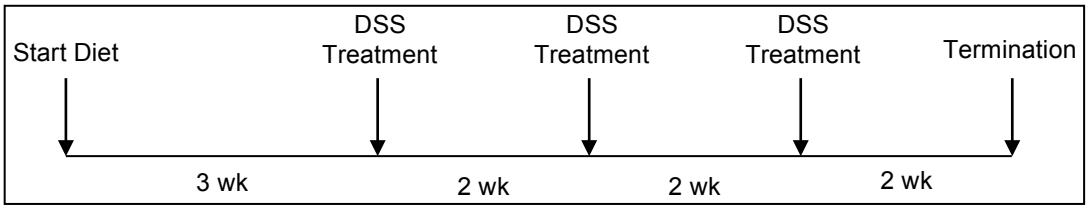


FIGURE 8 Animals were acclimated before starting experimental diets. After 3 wk of experimental diets, DSS exposures began (3% DSS for 48 h, 2 wk separation, 3 treatments total).

Body weight and dietary intake

Body weights were recorded throughout the experiment: wk 0, 3, 4, 6, 8, 9. Body weights were used to assess growth rates and weight loss that may be attributed to onset or progression of disease. Intake was measured at wk 3, 6, and 9. Body weights and intake were measured twice over 48 h and averaged. Measuring dietary intake ensured that the animals received adequate nutrition and a known amount of bioactive compounds, in addition to monitoring disease state, since intake is usually decreased in UC. Experimental diets were extracted with methanol and analyzed using mass spectroscopy to verify levels of quercetin (0.466%) and chlorogenic acid (0.047%).

Fecal output and moisture content

Feces was collected at seven time points corresponding to before and after each DSS treatment, and prior to termination. After being observed for consistency and

presence of blood, fecal samples were placed in vials. Fresh samples were collected within 15 min of defecation over a 48 h period and snap-frozen in liquid nitrogen, and stored at -80ºC until further analysis. Daily fecal production was determined using samples collected over 24 h, which were weighed, snap-frozen, and stored at -80ºC until further analysis. Fecal production was calculated by averaging the daily fecal weights if two consecutive 24-h collections occurred. Fecal moisture content was determined to track disease state in fresh fecal samples. Feces were dried at 60-80°C for 24-48 h, then placed in a dessicator until cooled (2-4 h). This process was repeated until a constant weight was obtained for each sample (49). In fresh fecal samples, fecal moisture content was used to calculate SCFA concentrations on a dry weight basis, which was combined with the 24 hour fecal production data to estimate daily short chain fatty acid concentrations (See Appendix A for calculations).

Short-chain fatty acid analysis

SCFA concentrations were quantified in fecal samples collected before and after the first and third DSS treatments and prior to termination. Samples (~0.30 g) were powdered, prepared, and analyzed as previously described using liquid-gas chromatography, with few modifications (49). To each diluted supernatant (100 µL supernatant, 100 µL 70% ethanol), 200 µL internal standard solution (heptanoic acid in 70% ethanol) and 20 µL H_3PO_4 was added. A Varian CP3841 Autoinjector injected 1 µL into a Varian 3900 GC fitted with an HP-FFAP 30 m, 0.53 mm i.d. capillary column (19095F-123, Agilent, Santa Clara, CA). A 1 m deactivated glass capillary precolumn

(25703, Supelco, Bellmonte, PA) was in place as a guard column. Data were integrated and plotted using the Varian Star Workstation Program (Varian, Walnut Creek, CA). Standards and a blank were run before and after the sample runs each day to calculate SCFA (acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid) concentrations in the feces. Fecal production and moisture content data were used to calculate SCFA concentrations on a dry weight basis and 24-h SCFA excretion (See Appendix A for calculations).

Injury and inflammation scores

From the distal colon, 1 cm was removed and fixed in 4% paraformaldehyde (PFA) (400 mL 1x PBS, 100 mL 20% PFA) (**Fig. 9**). PFA sections were stained with hematoxylin and eosin (H&E), a stain commonly used for histopathological analyses (63). Histopathological signs induced by DSS include crypt distortion and loss, infiltration of immune and inflammatory cells, as well as edema and ulceration (1, 61, 62). Stained sections were scored by a board-certified pathologist for injury (0-3) and inflammation (0-3) (See Appendix A for scoring details) (63).

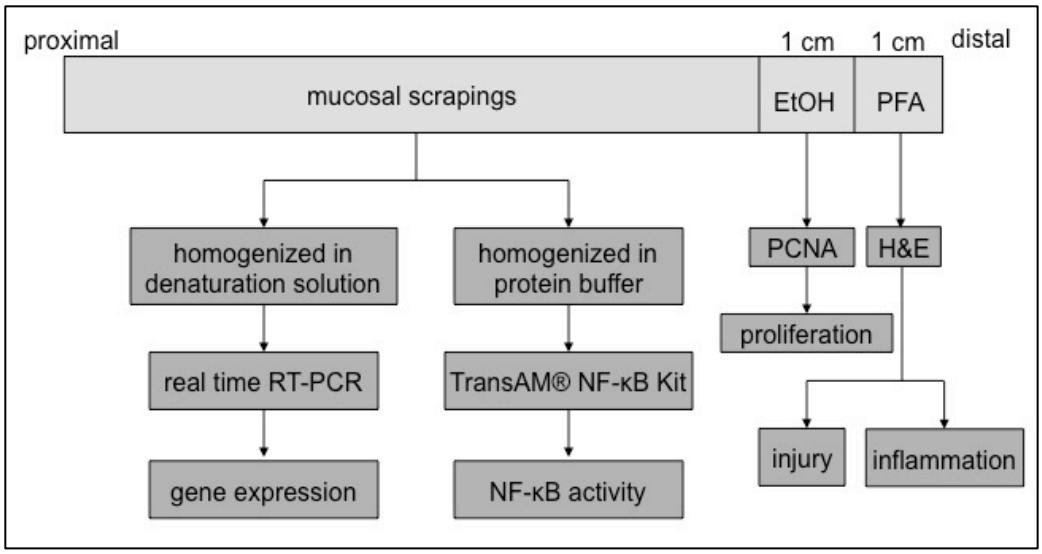


FIGURE 9 Once the colon was rinsed with PBS, two 1 cm sections were removed from the distal end of the colon. The sections were fixed in either 70% EtOH or 4% PFA, as described. From the remaining tissue, aliquots of mucosal scrapings were homogenized, and stored in denaturation solution or protein buffer.

Proliferative index, zone, and crypt height

From the distal colon, an additional 1 cm was removed and fixed in 70% ethanol (EtOH). The staining procedure (concentration of antibodies and incubation times) was optimized using manufacturer suggestions (Vector Labs and Covance) to the protocol described by Vanamala et al. (14). Tissues were pre-treated with Reveal Decloaker (Biocare Medical, Concord, CA), then stained with Vectastain® Elite® ABC Kit (Vector Lab, Burlingame, CA), and purified proliferating cell nuclear antigen (PCNA) monoclonal antibody (SIG-3523, Covance, Emeryville, CA). To visualize positively stained cells, slides were then stained with 3,3'-diaminobenzadine (DAB) Substrate Kit for Peroxidase (SK-4100, Vector Lab, Burlingame, CA) until optimal staining intensity was reached (30-60 s) and counterstained with hematoxylin solution (HHS16, Sigma

Aldrich, St. Louis, MO). Twenty-five crypt columns per animal were observed to calculate crypt column height, proliferative index, and proliferative zone as previously described (14).

NF-κB activity

Post mortem, the colon was removed and rinsed twice with RNAse free PBS. Mucosal scrapings were collected using a RNase free slide and RNase free glass surface on ice. An aliquot of mucosal scrapings was homogenized in protein buffer ([1 mL 500 mM Tris-HCl (pH 7.2, Sigma), 2.5 mL of 1.0 M sucrose (Sigma), 100 µL 200 mM EDTA (pH 7.6, Sigma), 100 µL 100 mM EGTA (pH 7.5, Aldrich), 1.25 µL 0.4 M NaF (Sigma), 4.554 mL H2O, 1 mL 10% Triton X-100 (Sigma), 100 µL 10 mM orthovanadate (Sigma), 400 µL Protease Inhibitor (Sigma)] as previously described by Leonardi et al. (64). Supernatant was aliquoted and stored at -80ºC until analysis. One protein aliquot was used for the NF-κB activity analysis, using an ELISA based method (6). The TransAM® NF-κB Chemi p65 kit (40097, Active Motif, Carlsbad, CA) was used as described in the manufacturer's instructions. NF-κB activity is reported in relative luminescence units, which is the level of NF-κB activation in the samples compared to the positive control (1 µL Jurkat nuclear extract, 19 µL Complete lysis buffer/well) provided in the TransAM® NF-κB Chemi p65 kit. Activity was normalized to protein concentrations, which were quantified using the Coomassie Plus (Bradford) Assay Kit (23236, Thermo Scientific, Rockford, IL).

Real time RT-PCR

A second aliquot of the mucosal scrapings was homogenized in denaturation solution (ToTALLY RNA™ Kit, Ambion, Austin, TX) and the mRNA samples were stored at -80ºC until further analysis (15). As described by Warren et al., total mRNA was isolated and treated with DNase, with the additional use of Phase Lock Gel™ Tubes (5 Prime, Gaithersburg, MD) for centrifugation (15). The Phase Lock Gel™ Tubes aid in the separation of phases, limiting the concentrations of contaminants in the mRNA samples. Before PCR analysis, mRNA quality was measured using an Agilent Bioanalyzer, while mRNA concentrations were measured using spectrophotometry. Using Superscript™ III Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA), Taqman® Array Microfluidic Cards (Applied Biosystems, Foster City, CA), ABI 7900 HT thermocycler (Applied Biosystems, Foster City, CA), and SDS 2.4 software (Applied Biosystems, Carlsbad, CA) real time RT-PCR was performed on select gene targets (TFF-3, TGF-B, TLR-2, TLR-4, TLR-5, TLR-9, TNF-α, TNFR-1, TNFR-2, Tollip, FGF-2, IκBα, IL-1, IL-6, IL-12, MyD88, COX-2, RelA/p65, RIP kinase, SLC5A8, and MCT-1). The use of Taqman® Array Microfluidic Cards reduces the potential for loading differences for each of the targets. These targets allowed for analysis of several pathways that contribute to regulation of processes involved in maintaining colon health. To complement NF-κB activity data, RelA/p65 and IκBα were measured, in addition to the pro-inflammatory cytokines downstream of NF-κB (IL-1, IL-6, IL-12, TNF-α, COX-2, TGF-B). Upstream of NF-κB are the TLR (TLR-2, TLR-4, TLR-5, TLR-9, MyD88, Tollip) and TNFR (TNFR-1, TNFR-2, RIP kinase, TNF- α) pathways, both of

which can function to activate NF-κB. In addition, injury repair molecules TFF-3 and FGF-2 were measured since DSS is an injury model, in addition to an inflammation model. Lastly, SCFA transport proteins MCT-1 and SLC5A8 were measured to complement SCFA concentrations measured in the feces. Data was analyzed using the comparative C_T ($\Delta\Delta$ C_T) method in RQ Manager 1.2.2 (Applied Biosystems, Carlsbad, CA), with 18S as the calibrator gene.

Statistics

Data were analyzed using a two-way analysis of variance (ANOVA) (diet, treatment, diet*treatment) using SAS 9.3 software. All data are reported as least squares (LS) means ± standard error of the mean (SEM), using a significance of $p<0.05$.

Results

Body weight, intake, and fecal moisture content

There were no differences in body weight at baseline, or wk 3, 4, 6, 8, and 9 with diet or treatment (See Appendix B). There were no significant differences in intake at wk 3 (prior to first DSS treatment) or 9 (prior to termination) with experimental diets and DSS treatment (**Table 1**). At wk 6 (~d 63), non-DSS treated animals on the quercetin diet had significantly lower intake than non-DSS treated animals consuming the basal diet ($p=0.01$) and DSS-treated animals consuming the quercetin diet ($p=0.02$).

TABLE 1 Intake (g) measured prior to DSS treatment, immediately following the second DSS treatment, and prior to termination[1]

	Basal Diet		Chlorogenic Acid Diet		Quercetin Diet	
wk	Control	DSS	Control	DSS	Control	DSS
3	16.6 ± 0.6^{a}	17.5 ± 0.5^{a}	16.2 ± 0.5^{a}	16.2 ± 0.5^{a}	16.3 ± 0.5^{a}	16.5 ± 0.5^{a}
6	20.4 ± 0.8^{c}	19.4 ± 0.7^{abc}	19.5 ± 0.9^{abc}	18.1 ± 0.8^{ab}	17.6 ± 0.8^{a}	20.0 ± 0.7^{bc}
9	20.0 ± 0.9^{a}	19.4 ± 0.8^{a}	19.4 ± 0.9^{a}	20.7 ± 0.8^{a}	19.3 ± 0.9^{a}	20.1 ± 0.8^{a}

[1] Values are LS means ± SEM. Means with superscripts without a common letter differ ($p<0.05$). n=7-11 rats/group.

Severity of disease was not reflected in body weight or intake, but fecal moisture content increased with DSS treatment ($p<0.05$) and remained elevated compared to non-DSS treated animals ($p<0.05$). Even after 2 wk of recovery, animals were unable to recover to control levels of fecal moisture content (**Fig. 10**). Prior to termination, DSS animals persisted to show symptoms of disease, including increased fecal moisture content ($p<0.05$). In addition to causing diarrhea, there was an increased incidence of blood in the stool and diarrhea with DSS treatment (data not shown).

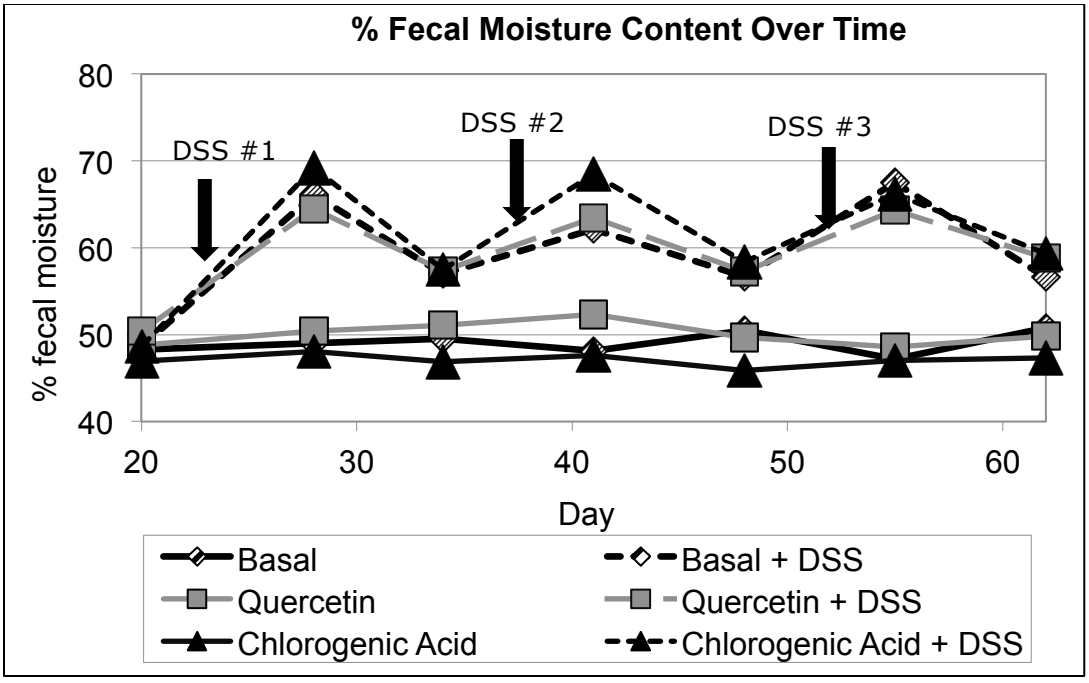


FIGURE 10 Baseline measurements revealed no diet differences in fecal moisture content (n=8-11 rats/diet). DSS increased fecal moisture content ($p<0.05$) and remained significantly higher than control animals for the remainder of the experiment. See Appendix B for actual values.

SCFA concentrations

Due to the severity of diarrhea observed in DSS-treated animals, SCFA concentrations were corrected for percent water in the feces and reported on a dry weight basis (For calculations, See Appendix B). After the induction of DSS treatment (post DSS 1), acetic acid ($p<0.05$), butyric acid ($p<0.05$), and total SCFA ($p<0.05$) concentrations increased for all diets compared to their non-DSS counterparts (See Appendix A). This pattern was also observed after the final DSS treatment for acetic acid ($p<0.0001$), butyric acid ($p<0.05$), and total SCFA ($p<0.0001$) (**Fig. 11**). The final measurement of SCFA concentrations is most reflective of the possible luminal

environment close to termination (**Fig. 12**). After the final 2 wk recovery, all DSS treated animals showed significantly higher levels of SCFA (acetic acid, $p<0.05$; butyric acid, $p<0.05$; total SCFA, $p<0.05$) compared to their non-DSS counterparts.

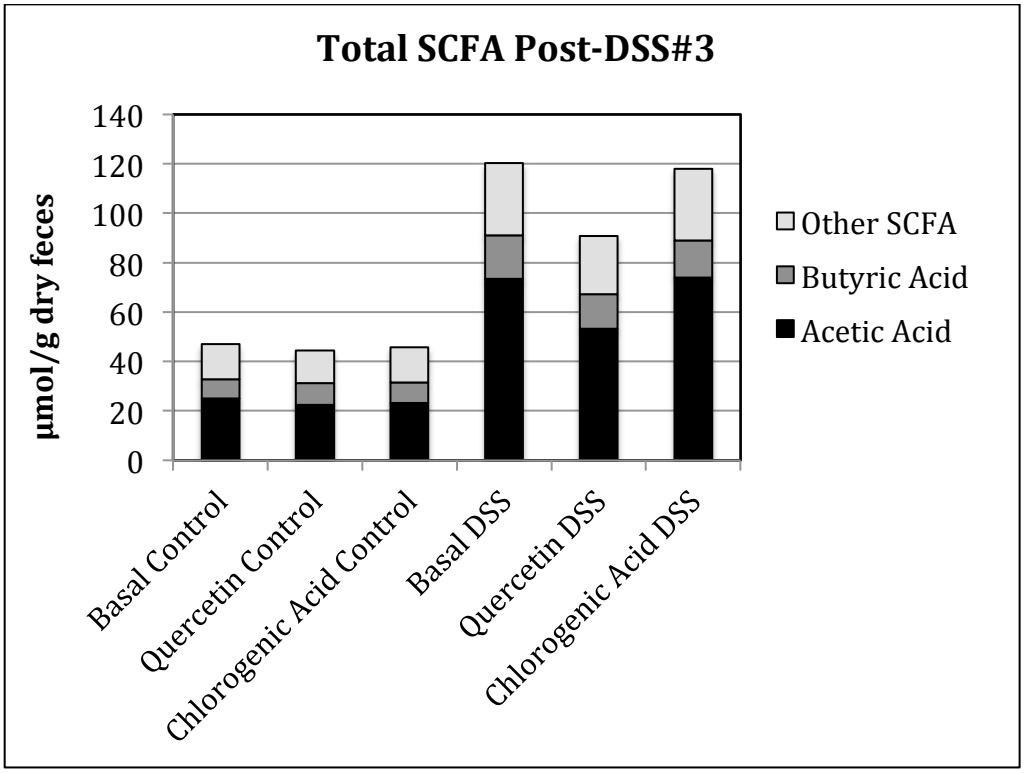


FIGURE 11 SCFA concentrations following the final DSS treatment revealed significant increases in acetic acid ($p<0.0001$), butyric acid ($p<0.05$), and total SCFA ($p<0.0001$; n=8-11 rats/diet). Relative concentrations of acetic acid were increased ($p<0.05$), while relative concentrations of butyric acid were decreased ($p<0.05$) by DSS treatment in all diet groups. See Appendix B for actual values.

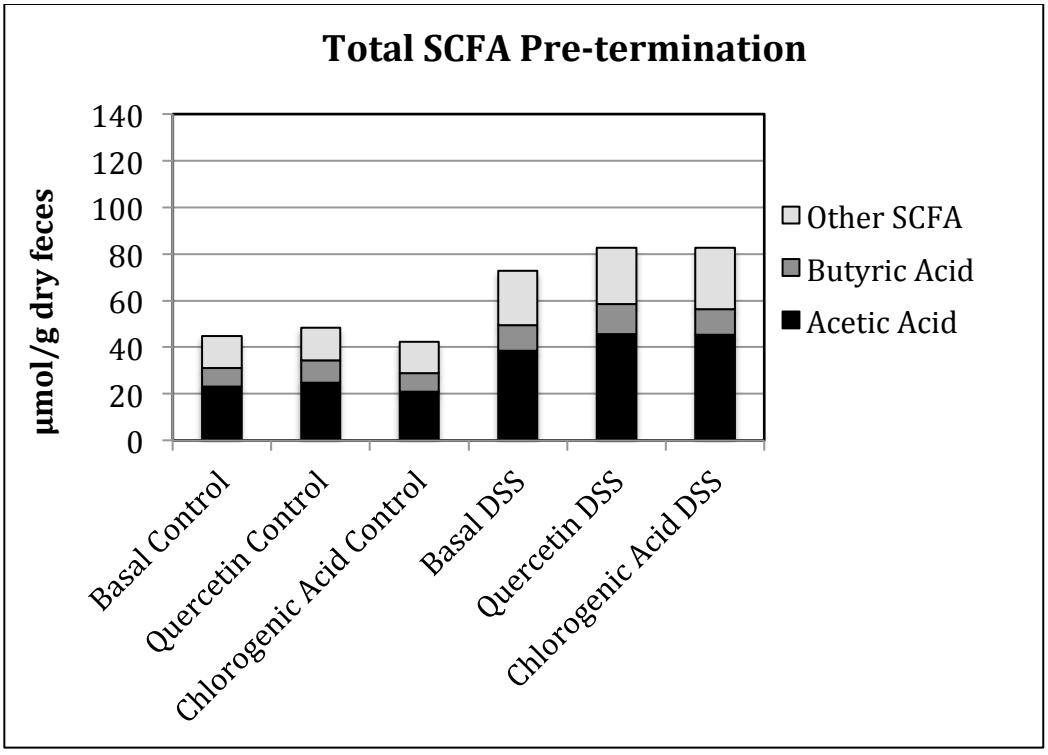


FIGURE 12 Prior to termination, SCFA concentrations remained significantly higher in DSS-treated animals compared to control animals (acetic acid, $p<0.05$; butyric acid, $p<0.05$; total SCFA, $p<0.05$; n=8-11 rats/diet). Animals fed a basal diet had comparable relative concentrations of acetic acid between DSS and non-DSS treated rats. Relative concentration of acetic acid ($p<0.05$) and butyric ($p<0.05$) acids, however, still showed prominent differences between DSS and non-DSS treated rats on experimental diets. See Appendix B for actual values.

Relative concentrations were calculated for acetic and butyric acids as a relative percentage of total SCFA. Baseline measurements reflect no diet effect on relative concentrations of acetic and butyric acids. Following the final DSS exposure, differences were observed between DSS-treated rats and their non-DSS counterparts. DSS increased relative concentrations of acetic acid ($p<0.05$) and decreased relative concentrations of butyric acid ($p<0.05$) (**Fig. 11**). After 2 wk of recovery following the final DSS treatment, animals fed a basal diet had comparable relative concentrations of

acetic acid between DSS and non-DSS treated rats. Relative concentration of acetic acid ($p<0.05$) and butyric ($p<0.05$) acids, however, still showed prominent differences between DSS and non-DSS treated rats on experimental diets (**Fig. 12**).

Increased concentrations of SCFA in the feces could be caused by an increased production or a decreased uptake, so expression of SCFA transport proteins SLC5A8 and MCT-1 were measured. In animals fed the basal diet, DSS significantly reduced expression of both SCFA transport proteins ($p<0.05$). Chlorogenic acid was able to partially mitigate the decrease in MCT-1 expression, while both quercetin and chlorogenic acid mitigated the DSS-induced decrease in SLC5A8 expression (**Fig. 13**).

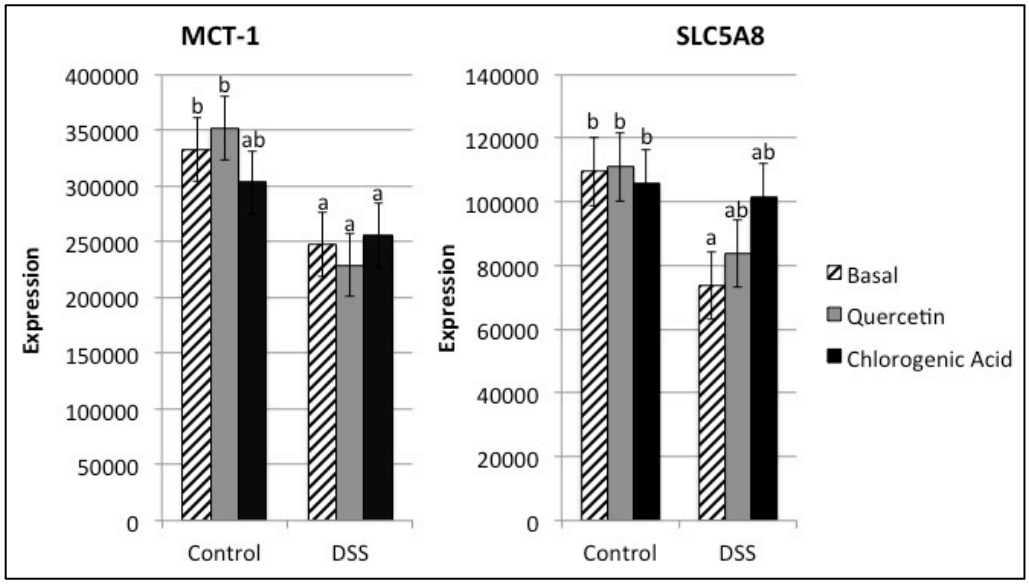


FIGURE 13 MCT-1 and SLC5A8 mRNA levels show that DSS significantly reduced expression of both genes in the basal diet ($p<0.05$). Animals fed chlorogenic acid were able to mitigate DSS-induced decreases in MCT-1 expression. Both experimental diets were able to mitigate DSS induced decreases in SLC5A8 expression. Means not sharing a common superscript are significantly different ($p<0.05$). Data are LS means ±SEM, n=7-8 rats/diet. See Appendix B for actual values.

Injury and injury repair

Treatment with DSS significantly increased injury ($p<0.0001$) scores (**Fig. 14**). Non-DSS treated animals fed the chlorogenic acid diet had lower injury scores than non-DSS treated animals fed the basal diet ($p=0.0272$). Although diet did not have an effect on distal colon injury scores, bioactive compounds did alter expression of some injury repair molecules. DSS-treated animals fed a quercetin diet had significantly higher expression of fibroblast growth factor-2 (FGF-2) than any other diet/treatment group ($p<0.01$) (**Fig. 15**). FGF-2 is an injury repair molecule found on the basolateral membrane of epithelial cells. In animals fed the quercetin diet, trefoil factor-3 (TFF-3) expression was lower in DSS-treated animals compared to non-DSS treated animals ($p<0.05$). TFF-3 is a wound-healing molecule found on the apical membrane of colonic epithelial cells and is expressed predominantly by goblet cells (38). A decrease in TFF-3 expression could reflect impaired wound healing on the apical membrane of epithelial cells. Bioactive compounds did not mitigate DSS induced injury, however quercetin did beneficially alter expression of FGF-2.

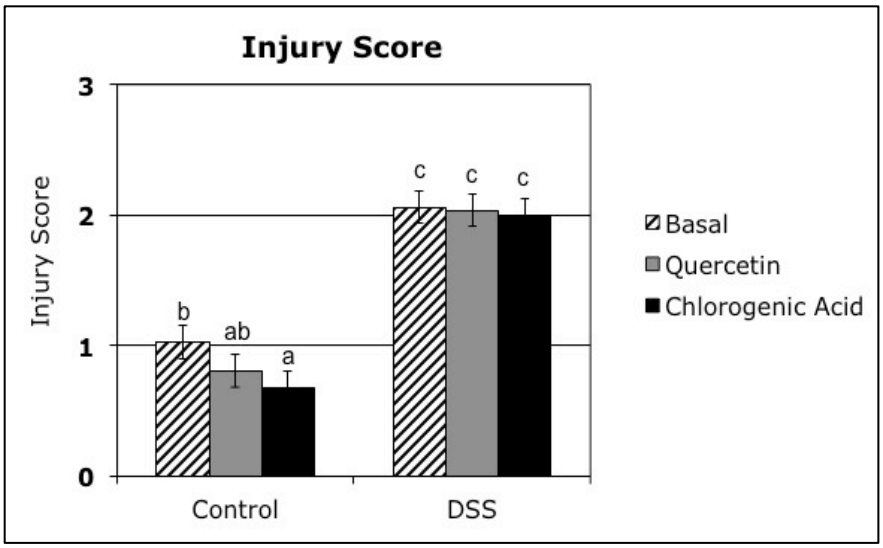


FIGURE 14 DSS significantly increased injury score in all diet groups ($p<0.0001$). In non-DSS treated animals, chlorogenic acid reduced injury score compared to the basal diet ($p=0.0272$). Means not sharing a common superscript are significantly different ($p<0.05$). Data are LS means ±SEM, n=7-8 rats/diet. See Appendix B for actual values.

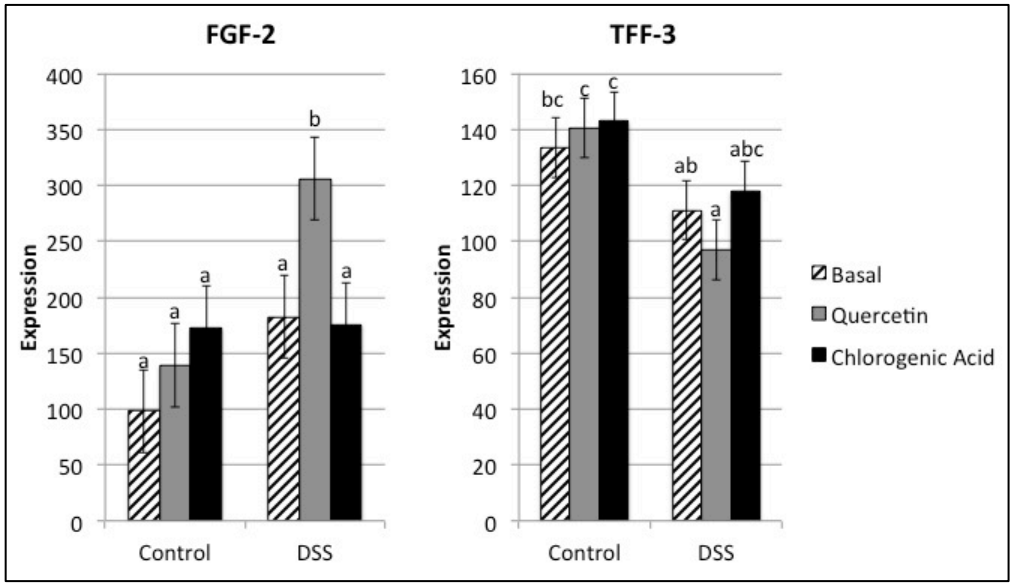


FIGURE 15 Expression of injury repair molecules suggests that quercetin may be protective by increasing expression of basolateral repair molecule FGF-2 ($p=0.0005$). DSS-treated animals fed quercetin had lower levels of TFF-3 expression, an apical wound healing molecule ($p=0.0035$). Means not sharing a common superscript are significantly different ($p<0.05$). Data are LS means ±SEM, n=7-8 rats/diet. See Appendix B for actual values

NF-κB activity and expression of downstream effectors

DSS increased inflammation scores ($p<0.01$) in all diet groups (see Appendix B). However, NF-κB activity, a pro-inflammatory transcription factor, was not significantly increased by DSS (**Fig. 16**). DSS-treated animals fed a chlorogenic acid diet had lower levels of NF-κB activity compared to those fed a basal diet ($p<0.05$). Expression of NF-κB-associated molecules was also measured in mucosal scrapings (**Fig. 17**). In all diet groups, treatment with DSS resulted in significantly lower levels of RelA/p65 expression ($p<0.05$), the regulatory subunit of NF-κB. In animals fed the basal diet, DSS-treatment also reduced expression of IκBα expression, the molecule that prevents NF-κB translocation into the nucleus ($p<0.05$). However, both experimental diets were able to mitigate the decrease in IκBα expression.

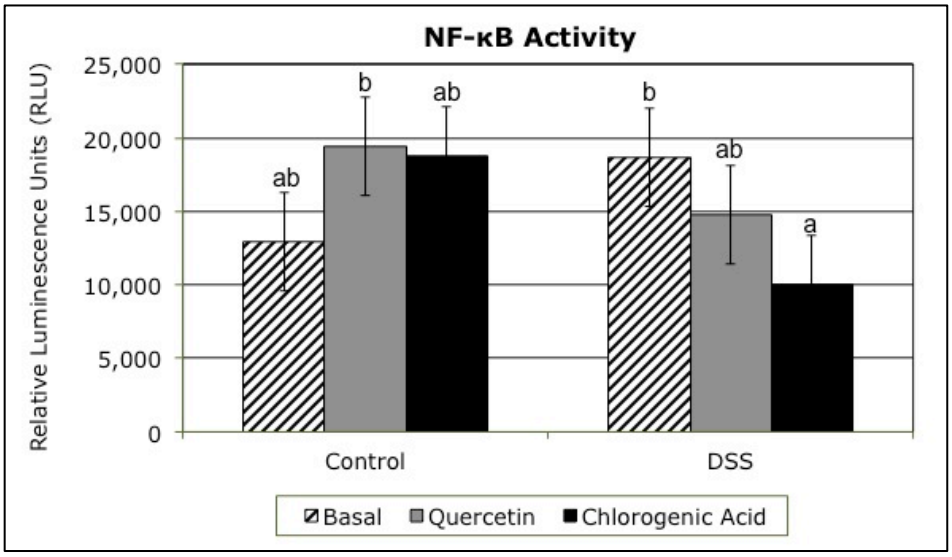


FIGURE 16 Chlorogenic acid significantly reduced NF-κB expression compared to the basal diet, in DSS-treated animals. Means not sharing a common superscript are significantly different ($p<0.05$). Data are LS means ±SEM, n=8-11 rats/diet. See Appendix B for actual values.

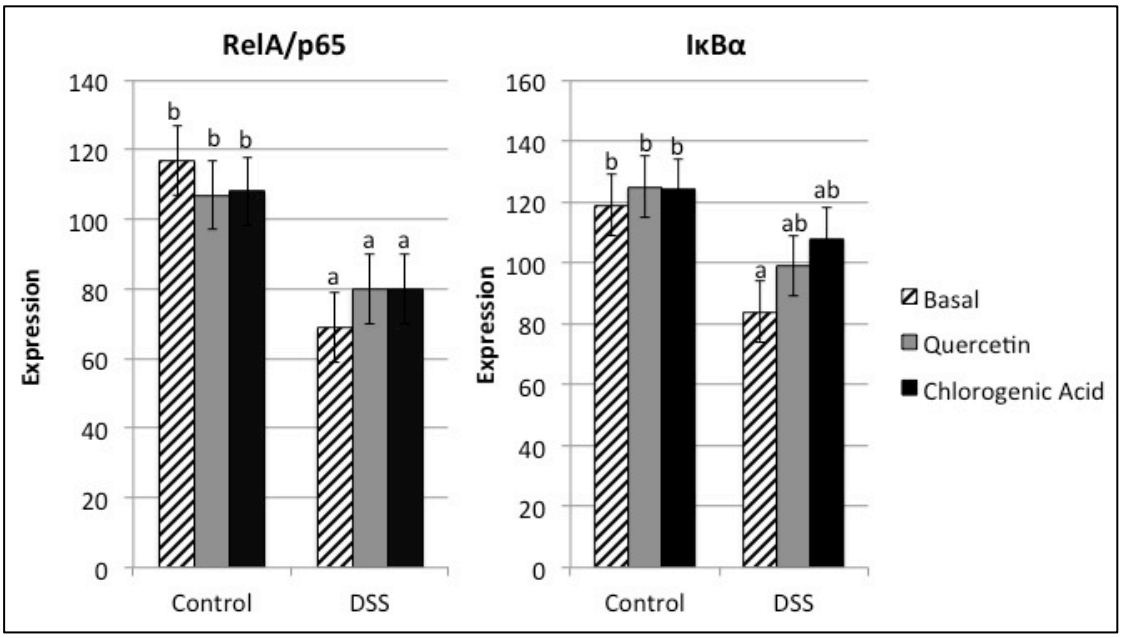


FIGURE 17 DSS significantly reduced RelA/p65 mRNA levels in all diet groups ($p<0.05$). IκBα mRNA levels were significantly reduced in DSS-treated animals fed the basal diet ($p<0.05$), but experimental diets maintained IκBα mRNA expression. Means not sharing a common superscript are significantly different ($p<0.05$). Data are LS means ±SEM, n=7-8 rats/diet. See Appendix B for actual values.

Expression of pro-inflammatory cytokines downstream of NF-κB was also measured (**Fig. 18**). Contrary to what we expected, 2 wk after the last DSS treatment COX-2 expression in animals fed basal and quercetin diets was reduced ($p<0.01$) relative to the control rats. Animals fed the basal diet had significantly higher levels of IL-1 ($p<0.01$) and IL-12 ($p<0.05$) expression with DSS treatment. Expression levels of COX-2, IL-1, and IL-12 were not impacted by DSS treatment for animals fed chlorogenic acid diet. IL-1 expression was also not impacted with DSS treatment for animals fed quercetin diet. TNF-α, IL-6, and TGF-B expression was not affected by either diet or DSS treatment (**Table 2**). Given this data, DSS-induced inflammation may be directly impacted by IL-1 and IL-12 expression, two cytokines under the transcriptional control

of NF-κB, which can be mitigated in part by quercetin or almost completely by chlorogenic acid.

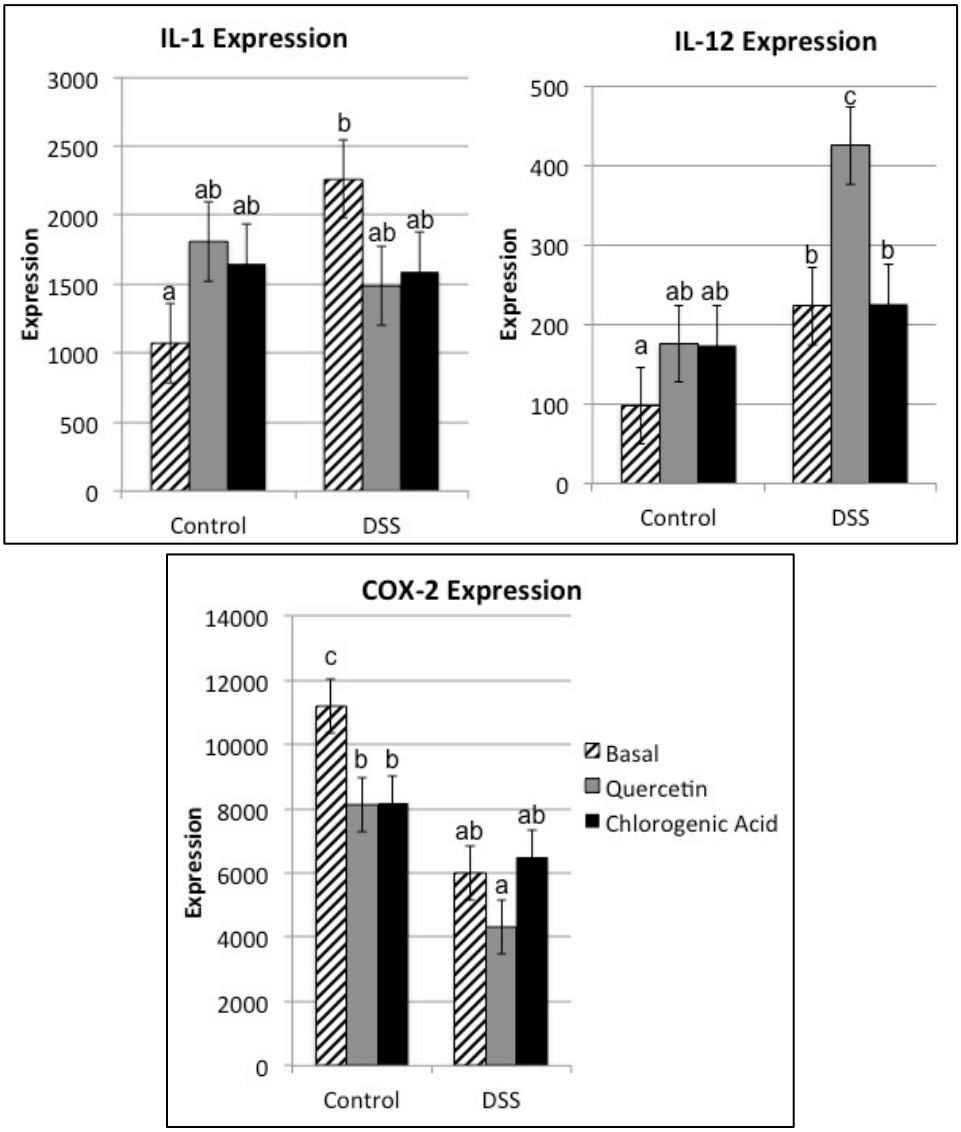


FIGURE 18 DSS increased mRNA expression of IL-1 and IL-12 and reduced expression of COX-2 in animals fed the basal diet. Means not sharing a common superscript are significantly different ($p<0.05$). Data are LS means ±SEM, n=7-8 rats/diet. See Appendix B for actual values.

TABLE 2 Expression of pro-inflammatory cytokines downstream of NF-κB[1]

	Basal Diet		Quercetin Diet		Chlorogenic Acid Diet	
	Control	DSS	Control	DSS	Control	DSS
IL-6	752±168^{a}	544±168^{a}	923±168^{ab}	1,365±180^{b}	828±168^{a}	606±168^{a}
TGF-B	152±18^{a}	147±18^{a}	179±18^{a}	177±20^{a}	161±18^{a}	170±18^{a}
TNF- α	1,804±354^{a}	2,090±354^{ab}	2,596±354^{ab}	2,991±378^{b}	2,543±354^{ab}	1,662±354^{a}

[1]Values are LS means ± SEM. Means not sharing a common superscript differ ($p<0.05$). n=7-8 rats/group.

Toll like receptor pathways

DSS reduced expression of TLR-4 in animals fed the basal diet, but did not significantly alter expression of TLR-2, TLR-9, and TLR-5 (**Table 3**). Animals fed a chlorogenic acid diet had decreased expression of TLR-9 ($p<0.01$) with DSS-treatment, a potentially protective TLR. Another protective TLR, TLR-2, had increased expression with quercetin supplementation in non-DSS treated compared to those consuming the basal diet ($p<0.05$).

TABLE 3 Expression of TLRs, MyD88, and Tollip[1]

	Basal Diet		Quercetin Diet		Chlorogenic Acid Diet	
	Control	DSS	Control	DSS	Control	DSS
TLR-2	3.9±1.0^{a}	6.6±1.0^{ab}	6.9±1.0^{b}	7.7±1.7^{b}	6.2±1.0^{ab}	7.7±1.0^{b}
TLR-4	61.4±5.5^{a}	30.3±5.5^{b}	55.0±5.5^{a}	34.9±5.9^{b}	55.7±5.5^{a}	36.5±5.5^{b}
TLR-5	30.1±3.6^{ab}	25.3±3.6^{ab}	31.2±3.6^{ab}	22.7±3.8^{a}	35.1±3.6^{b}	26.1±3.6^{ab}
TLR-9	7.0±1.2^{abc}	4.7±1.2^{ab}	7.6±1.2^{bc}	7.9±1.3^{bc}	8.9±1.2^{c}	3.9±1.2^{a}
MyD88	99.7±8.4^{c}	60.4±8.4^{a}	104.8±8.4^{c}	70.5±8.9^{ab}	91.8±8.6^{bc}	74.2±8.4^{ab}
TOLLIP	58.1±4.2^{c}	42.7±4.2^{a}	57.0±4.2^{bc}	45.1±4.4^{ab}	56.7±4.2^{bc}	46.8±4.2^{abc}

[1]Values are LS means ± SEM. Means not sharing a common superscript differ ($p<0.05$). n=7-8 rats/group

While some TLRs are thought to be protective, all TLRs activate NF-κB through MyD88 transduction molecules. Treatment with DSS significantly reduced MyD88 expression in basal and quercetin diets ($p<0.05$) (**Table 3**). Tollip, a regulatory molecule, inhibits MyD88-induced activation of NF-κB. Tollip expression was significantly reduced with DSS treatment in animals fed a basal diet ($p<0.05$) (**Table 3**). Chlorogenic acid maintained MyD88 expression levels with DSS treatment, while both quercetin and chlorogenic acid maintained Tollip expression levels.

Tumor necrosis factor receptor pathways

Although TNF-α expression was not affected by diet or treatment, TNFR expression was altered. TNFR-1, the pro-apoptotic TNFR, expression was significantly reduced by DSS treatment in animals fed a basal diet ($p<0.05$) (**Fig. 19**). However, animals fed quercetin and chlorogenic acid diets had comparable levels of TNFR-1 between DSS and non-DSS treated animals. In DSS-treated animals, RIP kinase (RIPk) expression was comparable across diets (See Appendix B). Downstream effects of RIP include NF-κB activation and apoptosis via activation of caspase-8, -10, and -3. This suggests that experimental diets may be able to maintain levels of TNFR-1-programmed cell death and/or necrosis. TNFR-2 expression, which promotes cell survival and proliferation, was significantly increased by DSS treatment in animals fed quercetin ($p<0.05$).

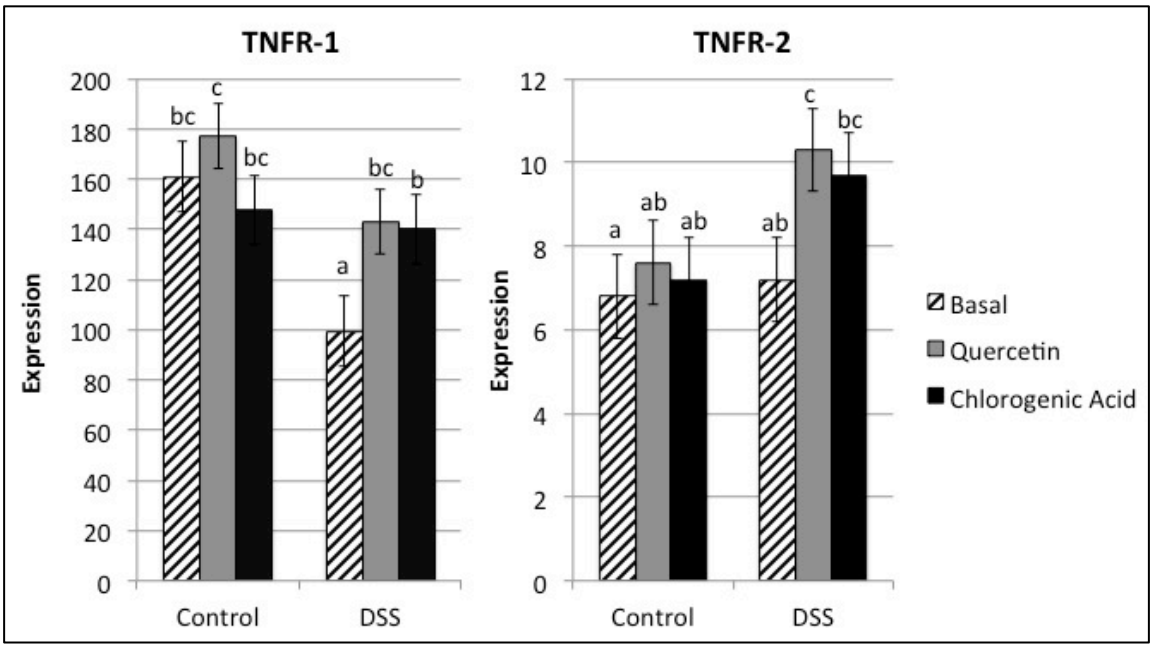


FIGURE 19 DSS significantly reduced mRNA expression of TNFR-1 in animals fed a basal diet. Quercetin and chlorogenic acid maintained expression of TNFR-1 expression. mRNA expression of TNFR-2 was elevated in DSS-treated animals on the quercetin diet. Means not sharing a common superscript are significantly different ($p<0.05$). Data are LS means ±SEM, n=7-8 rats/diet. See Appendix B for actual values.

Proliferation in the distal colon

No significant differences were observed with DSS treatment for proliferative zone (PZ), proliferative index (PI), or crypt height (CH) (**Table 4**). DSS treated animals fed chlorogenic acid diet had shorter crypt heights compared to DSS-treated animals receiving the basal diet.

TABLE 4 Proliferation in the distal colon[1]

	Basal Diet		Quercetin Diet		Chlorogenic Acid Diet	
	Control	DSS	Control	DSS	Control	DSS
CH	22.8±1.0^{ab}	24.8±1.0^{b}	20.9±1.0^{a}	22.2±1.1^{ab}	21.3±1.0^{a}	21.9±1.0^{a}
PI	16.6±2.4^{ab}	15.0±2.4^{a}	20.0±2.4^{ab}	20.0±2.7^{ab}	22.2±2.4^{b}	18.0±2.4^{ab}
PZ	39.0±2.5^{a}	37.5±2.5^{a}	41.3±2.5^{a}	39.3±2.8^{a}	43.8±2.5^{a}	40.6±2.5^{a}

[1]Values are LS means ± SEM. Means not sharing a common superscript differ ($p<0.05$). n=8-10 rats/group.

Discussion

Diets rich in fruits and vegetables are associated with a reduced risk of many diseases, including colon cancer and IBD (2, 3, 22). Treatment regimens relying upon pharmaceuticals and biological therapies are currently inefficient in that they are often ineffective in approximately half of subjects (57). Plums have been shown to have anti-cancer (dried plums) and anti-inflammatory (plum phenolic extract) properties (23, 58). Quercetin and chlorogenic acid, found in stonefruits in high concentrations, have demonstrated anti-inflammatory properties in acute colitis (6, 7). However, the ability of these bioactive compounds to combat chronic colitis has not been investigated.

We used a chronic colitis model (3% DSS for 48 h, 2 wk separation, 3 cycles) to investigate the damaging effects of DSS and possible protective mechanisms of quercetin and chlorogenic acid. The DSS-treated animals presented with symptoms similar to human UC: blood in the stool and diarrhea (increased fecal moisture content) (61). Although weight loss is also a symptom of UC and a characteristic of acute colitis, the rats had no significant differences in weight throughout the study (65, 66). Similarly, decreased intake is observed in acute colitis (66). No decreases in intake were observed

with DSS treatment, possibly due to the low dose of DSS used and that treatment only lasted 48 h. Non-DSS treated animals fed quercetin consumed less than animals on the basal diet (Table 1). Because intakes for other time points (wk 3 and 9) were not significantly different between diet and treatment groups, it is not apparent why this difference exists. With the severity of the disease state observed (fecal observations) in DSS-treated animals, it was a unique observation to have comparable intakes between non-DSS and DSS treated animals.

Changes in the microbial population (numbers, activities, distributions) is thought to be a contributing factor to UC (1). Shifts toward more pathogenic bacteria or decreased production of beneficial molecules, like butyrate, could increase inflammation (32, 39). Diet has been shown to alter microbial populations and activities, and since chlorogenic acid and quercetin are metabolized by the microbiota, it is possible these bioactive compounds can influence the microbiota, as well as their fermentation patterns (51-53, 59). However, experimental diets were unable to mitigate the DSS-induced increases in total SCFA concentrations. Altered fecal concentrations of SCFA, including butyrate, have been reported in UC (39). We observed an increase in SCFA concentrations with DSS treatment, suggesting that DSS did not inhibit fermentation rates of the microbiota. During quiescent stages of UC, butyrate oxidation has reported to be normal (39). Although we did not measure butyrate oxidation, slight decreases in butyrate concentrations during recovery phases (compared to active disease state) could indicate an enhanced uptake and/or utilization compared to active disease state. Although measuring SCFA concentrations in the feces provides a rough estimation of

fermentation patterns, fermentation should have been unaffected. Since DSS causes injury and inflammation in the distal colon and fermentation occurs more extensively in the proximal colon, microbial populations in the proximal colon may not have been affected directly by DSS (42). However, relative concentrations of butyrate and acetate shifted with DSS treatment, suggesting changes in microbial populations, fermentation patterns, or substrate uptake into epithelial cells. Changes to the fermentation patterns could be a result of systemic inflammatory responses caused by the DSS. Analysis of fecal samples from the proximal and distal colon collected at termination could provide more information on how fermentation rates are affected by colitis and by the dietary interventions. In addition, microbial analysis of feces could provide more information on shifts in microbial populations. Shifts in microbial populations could also alter fermentation products found in the lumen.

Since fecal butyrate concentrations were elevated in DSS-treated rats, we assumed butyrate availability in the lumen was not limited for epithelial cells. The high concentrations in the feces could be due to a decreased expression of MCT-1 and butyrate uptake, which has already been demonstrated in DSS-induced colitis (55). Mitigation of DSS-induced decreases in MCT-1 expression by chlorogenic acid and in SLC5A8 expression by both bioactive compounds suggests that uptake may not have been impaired, however it would be necessary to document protein levels by Western blot or immunohistochemical techniques to determine if levels of the transport proteins were maintained.

Butyrate is thought to have anti-inflammatory and anti-cancer properties (39). Although butyrate concentrations and butyrate transport protein expression were not decreased in DSS-treated animals on experimental diets, there was no diet effect on injury or inflammation score. Likewise, both quercetin and chlorogenic acid derivatives have been shown to have anti-inflammatory properties, but no change was observed in injury or inflammation score in our study (6, 7). DSS did significantly increase epithelial injury and colonic inflammation, which mimics human UC (1, 61). The severity of the three exposures to DSS could have caused damage too extensive for experimental diets to compensate for. In addition, hydrocaffeic acid demonstrated to be an anti-inflammatory in an acute model that used cellulose as its fiber source (7). While this acute model used a higher DSS dose (4%), it only had one treatment instead of three. In addition, cellulose in the diet may limit DSS interaction with the epithelium, since it is a non-fermentable fiber and reaches the distal colon, whereas the fermentable fiber pectin used in the current study would have been extensively fermented within the proximal colon. However, gene expression data did suggest that quercetin and chlorogenic acid might function to enhance injury repair molecules and suppress inflammation pathways.

Quercetin also has been shown to enhance epithelial barrier function *in vitro* by increasing expression of claudin-4, a tight junction protein (25). Injury repair molecules can play an important role in maintenance of the epithelial barrier in UC, as well. A decrease in TFF-3, an apical wound-healing molecule, was observed with DSS-treatment in animals fed a quercetin diet. Since TFF-3 is also an injury repair molecule, this is not

considered beneficial. TFF-3 is expressed by goblet cells, suggesting a decrease in number or function of goblet cells in animals fed quercetin (38). Although apical injury repair may be impaired in those fed a quercetin diet, quercetin could enhance wound repair by rebuilding the tissue from the basolateral membrane through enhanced expression of FGF-2. Due to the extensive damage to the epithelium and high levels of fecal moisture content (diarrhea), goblet cells could have decreased in number or function, reducing the level of TFF-3 expression. However, due to the extent of the injury caused by DSS, injury repair deeper in the tissues may be more important than apical repair.

Proliferation is also necessary for maintenance of the epithelial barrier. Previous studies of acute colitis showed that proliferation was increased during the active state of disease (65). Hyper-proliferation, in conjunction with inflammation, can lead to tumor formation (27). Since our animals were terminated during a period of remission, proliferation levels (proliferative index and proliferative zone) were comparable across diet and treatments. If animals were terminated during an active state of disease, changes in proliferation due to diet and/or treatment may have been observed.

Bioactive compounds did affect pathways that regulate the cell cycle. Quercetin and chlorogenic acid mitigated DSS-induced decreases in TNFR-1 expression. In addition, quercetin reduced expression of TNFR-2 in DSS-treated animals. Through RIP and caspase-8, TNFR-1 is able to induce cell death (34). TNFR-2 also plays a role in the cell cycle during inflammation, although it is responsible for cell proliferation and survival, rather than cell death (31). TNFR mRNA expression data would suggest that

DSS-treated animals on a quercetin diet would have decreased levels of proliferation, although these changes were not observed. Despite the changes in TNFR expression, we cannot conclusively discuss downstream changes. Western Blot or immunohistochemical methods could elucidate protein levels of TNFR-1 and -2. In addition, TNFR must be stimulated by TNF-α or other ligands in order to trigger downstream biological responses (34). Our study showed no increase with diet or DSS treatment in TNF-α expression, which could be the reason why no change was observed in proliferation rates.

Both TNFR-1 and -2 also function to activate NF-κB, which is not only anti-apoptotic, but also promotes expression of pro-inflammatory cytokines (31). NF-κB is also activated through TLR pathways and MyD88 transduction molecules (21, 30). Chlorogenic acid significantly decreased NF-κB activation in DSS treated animals when compared to the basal diet. When evaluating the effect of chlorogenic acid on NF-κB-related expression, we observed maintenance of IκBα, MyD88, and Tollip expression. Quercetin mitigated DSS-induced decreases in Tollip and IκB expression. Since dysregulation in NF-κB activation occurs in UC, chlorogenic acid and quercetin may be able to maintain regulation of NF-κB activation by suppressing DSS-induced changed in NF-κB regulatory molecules. Proper regulation of NF-κB in epithelial cells could be protective in colitis (2, 27, 32).

Due to the high level of inflammation in chronic colitis, an elevation in NF-κB was expected. The elevation in NF-κB activity in the basal diet with DSS treatment was not significant. Expression of NF-κB-associated molecules (RelA/p65 and IκBα) was

reduced by DSS treatment, however. The reduced expression of NF-κB could be why NF-κB activation was not elevated as we expected. Downstream of NF-κB, expression of pro-inflammatory cytokines was affected by both diet and treatment. DSS-treated animals on the basal diet showed increases in IL-1 and IL-12. Quercetin has been shown to inhibit inflammation in acute DSS-induced colitis by suppressing NF-κB activation (6). Quercetin was able to mitigate DSS-induced increases in IL-1, a severe pro-inflammatory cytokine and activator of NF-κB. Hydrocaffeic acid, has also shown to alleviate DSS-induced inflammation in acute colitis by suppressing expression of pro-inflammatory cytokines (7). In our study, expression levels of COX-2, IL-1, and IL-12 were comparable between non-DSS and DSS treated animals on the chlorogenic acid diet. Quercetin and chlorogenic acid may function through TLR and NF-κB pathways to influence expression of pro-inflammatory cytokines.

Although elevated COX-2 levels were expected with DSS treatment, reduced levels of COX-2 were actually observed. COX-2 can promote inflammation and tumorigenesis, but can also be protective when expressed in epithelial cells (27). COX-2 may contribute to the decreased paracellular permeability to bacterial translocation into the lamina propria (27). The reduced expression of COX-2 with DSS treatment in animals fed basal and quercetin diets could be harmful, especially since dysbiosis and impaired integrity of the epithelium occurs in UC. Quercetin did reduce COX-2 expression in non-DSS treated animals, as occurred in a prior experiment using the AOM model of colon cancer (15).

Conclusion

The ability of quercetin and chlorogenic acid to down-regulate expression of pro-inflammatory cytokines may not be the only mechanism of protection in UC. Altering the luminal environment and uptake of SCFA, in addition to enhancing injury repair molecule expression could protect the epithelium in colitis. These compounds could also impact the cell cycle, although no difference was observed in proliferation. Further studies should be completed to examine these compounds during the different stages of colitis. More significant differences could have been observed if animals were terminated immediately following the third DSS exposure, for example. We did not choose this termination time point due to the severity of the disease state and the thought that, at that time, there would be little or no intact mucosa. Also, if given a longer recovery period these bioactive compounds could have continued to function through the previously described pathways to induce a more rapid recovery. Staggering termination time points would reveal the most conclusive data to how quercetin and chlorogenic acid prevent injury and inflammation and enhance injury repair. Lastly, further studies should examine these compounds in levels comparable to those found in Western and/or Mediterranean diets. These compounds could also be examined together in order to identify any additive or synergistic effects. Our data suggests potential benefits of supplementing the diet with foods rich in quercetin and chlorogenic acid, like stonefruits. However, further investigation is needed before this data can be translated to clinical trials.

CHAPTER III

SUMMARY AND CONCLUSIONS

Summary

Pharmaceuticals and biological therapies for ulcerative colitis (UC) are currently inefficient in that they are often ineffective in approximately half of subjects (57). Dietary interventions increasing the amount of phytochemical-rich fruits and vegetables are associated with a reduced risk of many diseases, including colon cancer and IBD (2, 3, 22). A diet rich in phytochemicals, like quercetin and chlorogenic acid, is thought to be protective due to their anti-cancer, anti-oxidant, and anti-inflammatory properties. In chronic inflammatory diseases, reducing inflammation can not only alleviate symptoms, but also protect against carcinogenesis. Proper regulation of the cell cycle is also important for maintenance of the epithelial barrier, wound healing, and prevention of hyperplasia. Previous studies have elucidated possible mechanisms by which quercetin and chlorogenic acid function *in vitro* and *in vivo,* but only in acute colitis models.

Inflammation associated with UC and DSS-induced colitis is controlled through the NF-κB pathway. Although NF-κB activity was not significantly increased by DSS in animals fed the basal diet, DSS treatment reduced expression of NF-κB subunits and regulatory molecules (RelA/p65, IκBα, MyD88, Tollip). The reduced expression of NF-κB and related molecules could be why NF-κB activation was not elevated. To verify the reduction in NF-κB activation is caused by a reduced expression of NF-κB-associated

molecules, protein levels could be measured. Altered levels of expression of these molecules could suggest a dysregulation of NF-κB activation, which can exacerbate NF-κB-mediated inflammation. This study showed that quercetin maintains Tollip and IκBα expression and chlorogenic acid maintains expression of MyD88, Tollip, and IκBα. This suggests that quercetin and chlorogenic acid may preserve the healthy regulation of NF-κB activation. MyD88 and Tollip are involved in TLR pathway. With DSS treatment, we did not observe increased expression of TLR. On the contrary, TLR-4 expression was decreased by DSS treatment. Increased levels of TNF-α have shown to increase expression of TLR-4 *in vitro* (37). Since TNF-α expression was not altered, this could be the reason an increase in TLR-4 expression was not observed.

Downstream of NF-κB, elevated expression of pro-inflammatory cytokines (IL-1 and IL-12) was observed with DSS exposure in animals fed the basal diet. IL-1 stimulates further activation of NF-κB through positive feedback and also injures the protective mucosa (20). Quercetin was able to mitigate DSS-induced increases in IL-1, while chlorogenic acid mitigated DSS-induced changes in expression of COX-2, IL-1, and IL-12. By suppressing expression of IL-1, quercetin and chlorogenic acid may have been able to inhibit NF-κB activation and maintain NF-κB regulation.

While NF-κB could exacerbate inflammation through increased activity in macrophages, activated NF-κB could play a protective role by promoting cell repair and integrity of the epithelial barrier (1, 20, 21). Since the mucosal scrapings contain a large portion of epithelial cells, the decreased activity of NF-κB observed could reflect an inhibition of protective mechanisms, as well. For example, reduced levels of COX-2

expression were observed with DSS treatment in animals fed a basal diet. In epithelial cells, COX-2 expression limits bacterial translocation into the lamina propria (27). Allowing for increased permeability of the paracellular pathways could exacerbate immune and inflammatory responses. Quercetin, the positive control diet, did reduce COX-2 expression in non-DSS treated animals, as previously demonstrated in an AOM model (15).

Other protective mechanisms include SCFA transport, TLR-mediated protection, and enhanced expression of injury repair molecules. Quercetin increased TLR-2 expression, a molecule that has downstream anti-inflammatory effects (increased TFF-3 expression) (38, 39). TFF-3, an apical injury repair molecules was decreased in DSS-treated animals fed the quercetin diet. This indicates that increased TLR-2 expression does not necessarily reflect TLR-2 stimulation. Quercetin may enhance wound healing, however, through increased expression of FGF-2, a basolateral membrane repair molecule. In addition, chlorogenic acid may be protective in a non-colitis state. Although the mechanism is unclear, chlorogenic acid reduced injury score in non-DSS treated animals.

Lastly, sufficient energy sources (i.e. butyrate) are needed for a proper functioning epithelium. DSS increased butyrate concentrations in the feces, but no diet effect was observed. The increased SCFA concentrations in the feces could be due to an increased production and/or a decreased uptake. In addition, changes in the relative concentrations of SCFA could be due to changes in bacterial populations. Chlorogenic acid was able to mitigate the DSS-induced decreases in both MCT-1 and SLC5A8, while

quercetin only showed benefit for SLC5A8 expression. These bioactive compounds may preserve SCFA transport that is usually impaired in UC.

Conclusions

The involvement of these compounds in inflammatory, substrate transport, and injury repair pathways could elucidate specific protective mechanisms and provide further support that a diet rich in phytochemicals can be not only beneficial to reduce cancer risk, but also to alleviate inflammation in UC. Quercetin may enhance wound healing (FGF-2) and maintain levels of SCFA transport (SLC5A8), while functioning as an anti-inflammatory (IL-1). Chlorogenic acid may maintain levels of SCFA transport (SLC5A8 and MCT-1) and function as an anti-inflammatory (IL-1 and IL-12), as well. Both compounds may have the ability to prevent dysregulation of NF-κB pathways through maintenance of RelA/p65, MyD88, Tollip, and IκBα expression. Together, in stonefruits, these compounds could target multiple protective mechanisms. However, these mechanisms should be further investigated by measuring protein levels in the epithelial cells.

Future Research

Results from this study provide merit for further investigation of supplementing the diet with foods rich in quercetin and chlorogenic acid. While quercetin and chlorogenic acid affected expression of some of the same molecules, they also had unique effects. If these compounds were studied together in one diet, it is possible they could have additive or synergistic effects. In order to study these compounds together in one diet, it would also be necessary to use levels more reflective of those found in the Western and/or Mediterranean diets. Since chlorogenic acid was effective at a low dose, there is potential that chlorogenic acid could have mitigated injury and inflammation scores at a higher concentration. Since quercetin levels were extremely high, a lower dose should be investigated, to see if protective mechanisms are still active at lower doses.

Our study suggests that quercetin and chlorogenic acid may play a role in both NF-κB and TLR pathways. However, in order to form a confident conclusion of which pathways are impacted by these compounds, further analysis of gene expression and protein levels is needed. In addition, some of the expected outcomes (increased NF-κB activation and COX-2 expression) were not observed. Since these outcomes were observed in acute colitis, altering the experimental design could reveal more prominent changes in inflammatory pathways. This could be achieved by staggering termination points. For example, terminating animals in order to obtain tissue samples during active disease state and throughout the recovery process.

Although this study showed no diet effect on injury and inflammation scores, gene expression data suggests that quercetin and chlorogenic acid may have benefits if supplemented in the diet. However, further investigation of the additive and synergistic effects of these compounds during active colitis and recovery periods is necessary before these results can be translated to clinical trials.

LITERATURE CITED

1. Seril DN, Liao J, Yang G-Y, Yang CS. Oxidative stress and ulcerative colitis-associated carcinogenesis: studies in humans and animal models. Carcinogenesis. 2003;24:353-62.

2. Chan AT GE. Primary prevention of colorectal cancer. Gastroenterol. 2010 Jun;138:2029-43.e10.

3. Steinmetz KA, Potter JD. Vegetables, fruit, and cancer prevention: a review. J Am Diet Assoc. 1996;96:1027-39.

4. Tomas-Barberan FA, Gil MI, Cremin P, Waterhouse AL, Hess-Pierce B, Kader AA. HPLC-DAD-ESIMS analysis of phenolic compounds in nectarines, peaches, and plums. J Agric Food Chem. 2001 Oct;49:4748-60.

5. Vizzotto M, Cisneros-Zevallos L, Byrne DH. Large variation found in the phytochemical and antioxidant activity of peach and plum germplasm. J Am Soc Hortic Sci. 2007;132:334-40.

6. Comalada M, Camuesco D, Sierra S, Ballester I, Xaus J, Galvez J, Zarzuelo A. In vivo quercitrin anti-inflammatory effect involves release of quercetin, which inhibits inflammation through down-regulation of the NF-kappaB pathway. Eur J Immunol. 2005 Feb;35:584-92.

7. Larrosa M LC, Vivoli E, Pagliuca C, Lodovici M, Moneti G, Dolara P. Polyphenol metabolites from colonic microbiota exert anti-inflammatory activity on difference inflammation models. Mol Nutr Food Res. 2009;53:1044-54.

8. Yang Y, Gallaher DD. Effect of dried plums on colon cancer risk factors in rats. Nutr Cancer. 2005;53:117-25.

9. Olthof MR, Hollman PC, Katan MB. Chlorogenic acid and caffeic acid are absorbed in humans. J Nutr. 2000;131:61-71.

10. Dilis V, Trichopoulou A. Antioxidant intakes and food sources in Greek adults. J Nutr. 2010 Jul;140:1274-9.

11. Noratto G, Porter W, Byrne DH, Cisneros-Zevallos L. Identifying peach and plum polyphenols with chemopreventative potential against estrogen-independent breast cancer cells. J Agric Food Chem. 2007;57:5219-26.

12. Shanely RA, Knab AM, Nieman DC, Jin F, McAnulty SR, Landram MJ. Quercetin supplementation does not alter antioxidant status in humans. Free Radic Res. 2010 Feb;44:224-31.

13. Turner ND, Paulhill KJ, Davidson LA, Chapkin RS, Lupton JR. Quercetin suppresses early colon carcinogenesis partly through inhibition of inflammatory mediators. Acta Hortic. 2009;841:237-42.

14. Vanamala J, Leonardi T, Patil BS, Taddeo SS, Murphy ME, Pike LM, Chapkin RS, Lupton JR, Turner ND. Suppression of colon carcinogenesis by bioactive compounds in grapefruit. Carcinogenesis. 2006 Jun;27:1257-65.

15. Warren CA, Paulhill KJ, Davidson LA, Lupton JR, Taddeo SS, Hong MY, Carroll RJ, Chapkin RS, Turner ND. Quercetin may suppress ray abberant crypt foci formation by suppressing inflammatory mediators that influence proliferation and apoptosis. J Nutr. 2008;139:101-5.

16. Molodecky NA SI, Rabi DM, Ghali WA, Ferris M, Chernoff G, Benchimol EI, Panaccione R, Ghosh S, Barkema HW, Kaplan GG. Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. Gastroenterol. 2012;142:46-52.

17. Cosnes J G-RC, Seksik P, Cortot A. Epidemiology and natural history of inflammatory bowel diseases. Gastroenterol. 2011;140:1785-94.

18. CDC. Inflammatory bowel disease (IBD); 2011. Available from: http://www.cdc.gov/ibd/ - epidIBD.

19. Nakano S, Ohara S, Kubota T, Saigenji K, Hotta K. Compensatory response of colon tissue to dextran sulfate sodium-induced colitis. J Gastroenterol. 1999 Apr;34:207-14.

20. Atreya I AR, Neurath M F. NF-kappaB in inflammatory bowel disease. J Intern Med. 2008 Jun;263:591-6.

21. Karrasch T, Jobin C. NF-kappaB and the intestine: friend or foe? Inflamm Bowel Dis. 2008 Jan;14:114-24.

22. Reif S KI, Lubin F, Farbstein, Hallak A, Gilat T. Pre-illness dietary factors in inflammatory bowel disease. Gut. 1997;40:754-60.

23. Tan AC KI, Ramzam I, Zabaras D, Sze DMY. Potential antioxidant, antiinflammatory, and proapoptotic anticancer activities of Kakuda plum and Illawarra plum polyphenolic fractions. Nutr Cancer. 2011;63:1074-84.

24. Zhou YH YJ, Liu YL, Teng XJ, Ming M, Lv P, An P, Liu SQ, Yu HG. Effects of *Ginkgo biloba* extract on inflammatory mediators (SOD, MDA, TNF-alpha, NF-kappaBp65, IL-6) In TNBS-induced colitis in rats. Med Infl. 2006;2006:1-9.

25. Amasheh M SS, Amasheh S, Mankertz J, Zeitz M, Fromm M, Schulske JD. Quercetin enhances epithelial barrier function and increases claudin-4 expression in Caco-2 cells. J Nutr. 2008;138:1067-73.

26. Rao CV DD, Simi B, et al. Inhibitory effect of caffeic acid esters on azoxymethanie-induced biochemical changes and aberrant crypt foci formation in rat colon. Cancer Res. 1993;53:4182-8.

27. Karin M, Greten F. NF-kappaB: linking inflammation and immunity to cancer development and progression. Nat Rev Immunol. 2005 Oct;Immunology. 5:749-59.

28. Jobin C SR. The IKB/NF-KB system: a key determinant of mucosal inflammation and protection. Am J Physiol Cell Physiol. 2000;278:C451-C62.

29. Egger B B-EM, MacDonale TT, Inglin R, Eysselein, Buchler MW. Characterisation of acute murine dextran sodium sulphate colits: cytokine profile and dose dependency. Dig. 2000;62:240-8.

30. Rogler G BK, Vogl D, Page S, Hofmeister R, Andus T, Knuechel R, Baeuerle PA, Scholmerich J, Gross V. Nuclear factor-kB is activated in macrophages and epithelial cells of inflamed intestinal mucosa. Gastroenterol. 1998;115:357-69.

31. Mizoguchi E MA, Takedatsu H, Cario E, De Jong YO, Ooi CJ, Xavier RJ, Terhorst C, Podolsky DK, Bhan AK. Role of tumor necrosis factor receptor 2 (TNFR2) in colonic epithelial hyperplasia and chronic intestinal inflammation in mice. Gastroenterol. 2002;122:134-44.

32. Sartor R. Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative colitis. Gastroenterol Hepatol. 2006;3:390-407.

33. Alex P ZN, Nguyen T, Gonzales L, Chen TE, Conklin LS, Centola M, Li X. Distinct cytokine patterns identified from multiplex profiles of murine DSS and TNBS-induced colitis. Inflamm Bowel Dis. 2009;15:341-52.

34. Chan FK SJ, Bixby JG, Felices M, Zheng L, Appel M, Orenstein J, Moss B, Lenardo MJ. A role for tumor necrosis factor receptor-2 and receptor-interacting protein in programmed necrosis and antiviral responses. J Biol Chem. 2003;278:51613-21.

35. Drexler SK FB. The role of toll-like receptors in chronic inflammation. Int J Biochem Cell Biol. 2010;42:506-18.

36. Salcedo R WA, Cardone M, Jones Y, Gyulai Z, Dai R, WAng E, Ma W, Haines D, O'hUigin C, Marincola FM, Trinchieri G. MyD88-mediated signaling prevents developmet of adenocarcinomas of the colon: role of interleukin 18. J Exp Med. 2010;207:1625-36.

37. Cario E. Bacterial interactions with cells of the intestinal mucosa: toll-like receptors and NOD-2. Gut. 2005;54:1182-93.

38. Podolsky DK GG, Eyking A, Cario E. Colitis-associated variant of TLR2 causes impaired mucosal repair because of TFF3. Gastroenterol. 2009;137.

39. Hamer HM JD, Venema K, Vanhoutvin S, Troost FJ, Brummer RJ. The role of butyrate on colonic function. Aliment Pharmacol Ther. 2008;27:104-19.

40. Krieg AM. Therapeutic potential of Toll-like receptor 9 activation. Nature Reviews. 2006;5:471-84.

41. Katakura K LJ, Rachmilewitz D, Li G, Eckmann L, Raz E. Toll-like receptor 9-induced type I IFN protects mice from experimental colitis. J Clin Invest. 2005;115:695-702.

42. Rose DJ, DeMeo MT, Keshavarzian A, Hamaker BR. Influence of dietary fiber on inflammatory bowel disease and colon cancer: importance of fermentation pattern. Nutr Rev. 2007 Feb;65:51-62.

43. Steenholdt C AL, Pedersen G, Hansen A, Brynsov J. Expression and function of toll-like receptor-8 and Tollip in colonic epithelial cells from patients with inflammatory bowel disease. Scand J Gastroenterol. 2009;44:195-204.

44. Becker C FM, Neurath MF. TGF-beta as a T cell regulator in colitis and colon cancer. Cytokine and Growth Factor Rev. 2006;17:97-106.

45. Henningsson A, Bjock I, Nyman M. Short-chain fatty acid formation at fermentation of indigestible carbohydrates. Scand J Food Nutr. 2001;45:165-8.

46. Galvez J, Rodriguez-Cabezas ME, Zarzuelo A. Effects of dietary fiber on inflammatory bowel disease. Mol Nutr Food Res. 2005 Jun;49:601-8.

47. Wong JM, de Souza R, Kendall CW, Emam A, Jenkins DJ. Colonic health: fermentation and short chain fatty acids. J Clin Gastroenterol. 2006;40:235-43.

48. Berggren AM BI, Nyman M. Short-chain fatty acid content and pH in caecum of rats given various sources of carbohydrates. J Sci Food Agric. 1993;63:397-406.

49. Zoran DL, Barhoumi R, Burghardt RC, Chapkin RS, Lupton JR. Diet and carcinogen alter luminal butyrate concentration and intracellular pH in isolated rat colonocytes. Nutr Cancer. 1997;27:222-30.

50. Nancey S, Moussata D, Graber I, Claudel S, Saurin J-C, Fourie B. Tumor necrosis factor-alpha reduces butyrate oxidation *in vitro* in human colonic mucosa: a link from inflammatory process to mucosal damage? Inflamm Bowel Dis. 2005;11:559-66.

51. Selma MV EJ, Tomas-Barberan T. Interaction between phenolics and gut microbiota: role in human health. J Agric Food Chem. 2009;57:6485-501.

52. Ramos FA TY, Shirotori M, Kawaguchi Y, Tsuchiya K, Shibata H, Higuti T, Tadokoro R, Takeuchi M. Antibacterial and antioxidant activities of quercetin oxidation products from yellow onion (*Allium cepa*) skin. J Agric Food Chem. 2006;54:3551-7.

53. Bosscher D BA, Pieters L, Hermans N. Food-based strategies to modulate the composition of the intestinal microbiota and their associated health effects. J Physiol Pharm. 2009;60:5-11.

54. Murakami A AH, Terao J. Multitargeted cancer prevention by quercetin. Cancer Lett. 2008;269:315-25.

55. Thibault R DCP, Daly K, Bourreille A, Cuff M, Bonnet C, Mosneir J, Galmiche J, Shirazi-Beechey S, Segain J. Down-regulation of the monocarboxylate transporter 1 is involved in butyrate deficiency during intestinal inflammation. Gastroenterol. 2007;133.

56. Luhrs H, Gerke T, Muller J, Melchar R, Schauber J, Boxberger F, Scheppach W, Menzel T. Butyrate inhibits NF-kappaB activation in lamina propria macrophages of patients with ulcerative colitis. Scand J Gastroenterol. 2002;37:458-66.

57. Clark M CJ, Feagan BC, Fedorak RN, Hanauer SB, Kamm MA, Mayer L, Regueiro C, Rutgeerts P, Sandborn WJ, Sands BE, Schreiber S, Targan S, Travis S, Vermeire S. American Gastroentergological Association Consesus Development Conference on the use of biologics in the treatment of inflammatory bowel disease, June 21-23, 2006. Gastroenterol. 2007;133:312-39.

58. Yang Y GD. Effect of dried plums on colon cancer risk factors in rats. Nutr Cancer. 2005;53:117-25.

59. Kajiura T TT, Sakata S, Sakamoto M, Hashimoto M, Suzuki H, Suzuki M, Benno Y. Change of intestinal microbiota with elemental diet and its impact on therapeutic effects in a murine model of chronic colitis. Dig Dis Sci. 2009;54:1892-900.

60. Clifford MN. Chlorogenic acid and other cinnamates--nature, occurence, and dietary burden. J Sci Food Agric. 1999;79:362-72.

61. Kawada M, Arihiro A, Mizoguchi E. Insights from advances in research of chemically induced experimental models of human inflammatory bowel disease. World J Gastroenterol. 2007 Nov 14;13:5581-93.

62. Gaudio E, Taddei G, Vetuschi A, Sferra R, Frieri G, Ricciardi G, Caprilli R. Dextran sulfate sodium (DSS) colitis in rats. Dig Dis Sci. 1999;44:1458-75.

63. Jia Q, Lupton JR, Smith R, Weeks BR, Callaway E, Davidson LA, Kim W, Fan Y-Y, Yang P, et al. Reduced colitis-associated colon cancer in Fat-1 (n-3 fatty acid desaturase) transgenic mice. Cancer Res. 2008 May 15;68:3985-91.

64. Leonardi T VJ, Taddeo SS, Davidson LA, Murphy ME, Patil BS, Wang N, Carrol RJ, Chapkin RS, Lupton JR, Turner ND. Apigenin and naringenin suppress colon carcinogenesis through the abberant crypt stage in azonxymethane-treated rats. Exp Biol Med. 2010;235:710-7.

65. Renes IB VM, Van Nispen D, Taminiau JA, Buller HA, Dekker J, Einerhand AWC. Epithelial proliferation, cell death, and gene expresion in he experimental colitis: alterations in carbonic anhydrase I, mucin MUC2, and trefoil factor 3 expression. Int J Colorectal Dis. 2002;17:317-26.

66. Larrosa M Y-GM, Selma MV, Gonzalez-Sarrias A, Toti S, Ceron JJ, Tomas Barberan F, Dolara P, Espin JC. Effect of a low dose of dietary resveratrol on colon microbiota, inflammation and tissue damage in a DSS induced colitis rat model. J Agric Food Chem. 2009;57:2211-20.

APPENDICES

EXPERIMENTAL PROTOCOLS

TABLES OF RESULTS

APPENDIX A

EXPERIMENTAL PROTOCOLS

Preparation of Diets

Weighing/Preparing diet components

Goals:

1. Measure diets into appropriate tubs and freezer bags
2. Arrange in order
3. Seal and store in freezer, if necessary
4. Record diet lot numbers
5. Document process and take pictures

Supplies:

1. Make sure there are adequate diet components
2. Test compounds (Quercetin anhydrase, Q4951-10G, Sigma; Chlorogenic acid min. 95% titration, C3878-5G, Sigma)
3. Adequately clean and label tubs and freezer bags for diet components
   Tub specs: contains up to 20 kg of diet
   Gallon sized Ziploc bags and outer bags
4. Arrange manpower
5. Weighing scales (2): 50 g-5 kg and >50 g
6. Measuring tubs and scoops (& spatulas)
7. Stirring tool
8. Adequate labeled diet bags and outer bags
9. Colored tapes
10. Sharpies
11. Aluminum foil
12. 5 L jugs for oil (2-3)
13. Cart, tub for ice, and ice
14. Gloves, bench papers, napkins
15. Diet specification sheets
16. Mixing bowl and mixer (2)
17. Labeled vials and specimen cups for diet samples

Location: Kleberg basement (diet mixing room)
Manpower: At least 2

Procedure:

1. Transport diet components from storage and/or freezer to mixing room
2. Bring supplies to mixing room
3. Weigh out diet components (according to specification sheet) in the following order
   1st—Dextrose (tub)
   2nd—Casein (tub)
   3rd—Pectin (tub)
   4th—Mineral mix |
   5th—Vitamin mix | all in
   6th—Methionine | one tub
   7th—Choline* |
4. Arrange tubs that belong to a diet group together; within diet groups, arrange into bowl number
5. Store tubs containing mineral/vitamin/methionine/choline mix in freezer
6. Record diet lot numbers
7. Seal, label, and store any excess diet components
8. Clean up

*Note: Choline is kept in a desiccator because it absorbs moisture easily. It will only be taken out when it is time to measure choline. Once measured, the choline needs to be mixed in with the salt/vitamin mix to ensure dryness.

Diet Component	**Company**	**Catalog Number**	**Lot Number**
Corn Oil NF	DYETS	401150	14058
Dextrose	Harlan	160190	08157
Casein- High Protein	Harlan	160030	08088
DL-methionine	Harlan	10850	08046
Vitamin Mix, AIN-76A	Harlan	40077	255073
Mineral Mix, AIN 76	Harlan	170915.pwd	255578
Pectin ISS 150 Citrus (Coyote)	Gum Tech	68779	08G3F001Z
Choline bitartrate	Harlan	30190	08258
Quercetin anhydrase	Sigma	Q4951-10G	118K0888
Chlorogenic acid min. 95% titration	Sigma	C3878-5G	127K640

Mixing diets

Goals:

1. Measure oil needed for the day
2. Mix diet components into each diet groups
3. Package and double-seal into freezer bags
4. Store diet bags in freezer
5. Document process and take pictures

Supplies: (see Preparing/weighing diet components)

Start time: 8am

Location: Kleberg basement (diet mixing room)

Manpower: at least 4

Procedure:

1. Get cart, 2-3 5 L jugs, tub big enough to fit jugs and ice, aluminum foil, bench papers, scissors, sharpies, napkins, weigh scale, plastic beaker.
2. Go to Dr. Chapkin's lab to measure out corn oil, where it is also stored. When done, fill remaining barrel of oil with nitrogen gas, seal, date, and store back in freezer. Record lot number.
3. Cover jugs with foil, label, and put on ice.
4. Bring 2 big stainless steel mixing bowls from lab to basement.
5. Measure out test compounds, add to mineral/vitamin, methionine/choline mix and mix well.
6. Start with Bowl 1 of a diet group, mix by hand diet components in the following sequence:
   1st—Pectin
   2nd—Mineral/vitamin/methionine/choline mix (this mix was prepared day 1) (if experimental diet, mineral mix should have quercetin or chlorogenic acid already mixed in Step 5)
   3rd—Casein
   4th—Dextrose
7. Fit bowl onto electric mixer, cover, and mix dry components for 5 min at #1 setting.
8. Still at setting #1, start mixer for 10 min. During this time, pour oil into mix in 3 separate quantities and times. To get all oil out from jug, use diet to mop it up.
9. Scrape sides of bowl and blade, mix for 5 min more.
10. Lower bowl, scrape sides of bowl and blade. Put bowl back into position and mix for 10 min at #2 setting.
11. Scrape diet off blade, lower and remove bowl. Take a sample of diet mix from four locations in the bowl (send off to analysis).

12. Scoop diet into labeled bags and double-bag. Store in labeled plastic tubs in freezer.
13. Repeat above diet process for all diet groups.
14. Clean up.

Notes:

Before starting on a new diet group, remember to wash bowls and scoops, and change gloves to prevent cross-contamination.

Bowls and scoops need to be sprayed with 70% EtOH after washing and drying.

The dry diet components are in powder form so dust clouds form when pouring; pour slowly to reduce dust.

Mineral mix, vitamin mix, and methionine are stored in the freezer, so put back in freezer after measuring out into tubs.

The mineral/vitamin/methionine/choline mix might be lumpy, so it is best to use gloved hands to break up the lumps and mix uniformly.

Diet samples are taken after mixing for analysis of composition. The small vials are used to collect samples throughout the feeding period and at the end.

Diet component	**Basal Diet**	**Quercetin Diet**	**Chlorogenic acid diet**
Dextrose	51.06%	50.61%	51.01%
Casein	22.35%	22.35%	22.35%
DL-methionine	0.34%	0.34%	0.34%
Mineral mix	3.91%	3.91%	3.91%
Vitamin mix	1.12%	1.12%	1.12%
Choline bitartrate	0.22%	0.22%	0.22%
Pectin	6.00%	6.00%	6.00%
Lipid-corn oil	15.00%	15.00%	15.00%
Quercetin	-	0.45%	-
Chlorogenic acid	-	-	0.05%

Tissue collection at termination

Termination
Rats were euthanized by CO_2 asphyxiation followed by cervical dislocation, and disruption of the diaphragm through a midline incision. The colon is resected, fecal contents removed and rinsed with PBS. Then two 1 cm sections are removed for histological preparations and the remainder is scraped for mucosa. See below for tissue and sample preparation and storage.

4% PFA Solution
PFA solution was mixed a day before the killing. 50 ml of 10X PBS was diluted to 400 ml with ddH_2O, and then 200 mL of 20% PFA was added. PFA solution was refrigerated.

Cassetting
Before starting, put a small specimen cup (150 mL) with PFA solution (80 mL) (see above) in an ice bucket. Put 80 mL 70% EtOH in another small specimen cup (150 mL). Work first with the colon tissue that is in the PFA weight boat. Take the colon tissue with tweezers from the external side of the tissue (be very careful to touch it minimally and try not to touch the mucosa side of the tissue) and place it on the sponge in the PFA cassette; the mucosa side should be exposed. Stretch the tissue with the tweezers, trying not to damage the mucosa side. Close the cassette and place it in the cup with PFA solution. Start timer for 4 hours. Take the colon tissue from EtOH weight boat and do the same as the PFA, but place it in the 70% EtOH cup.

PFA/EtOH changing solutions for PFA fixed tissues
Place the cassette with the colon tissue in PFA solution and start timer for 4 hours. After this time, rinse the cassette with 50% EtOH and place it again in the cup filled with 50% EtOH. Change EtOH solution every 20 minutes. After the 4^{th} change of 50% EtOH, use 70% EtOH for 3 changes. Refrigerate the cup after the last solution change.

Mucosal scrapings and mRNA sample preparation
After fecal material was removed from each colon (proximal and distal), it was washed twice with RNase free Phosphate Buffered Solution (PBS). Mucosa was scraped from the colon on an RNase free surface (on ice) and transferred to an RNase free homogenization tube along with 500 µL of Denaturation solution (Ambion). Scraped mucosa was homogenized for at least six strokes and then transferred to a 2 mL eppitube for storage at -80°C.

Mucosal scrapings and protein aliquots preparation
Scraped mucosa was placed into a homogenizer along with 400 µL of buffer (see below), homogenized for at least 7 strokes (on ice) and transferred into a 2 mL eppitube. The sample was aspirated through a 23-gauge needle twice then incubated on ice for 30 min

prior to centrifugation at 15,000 g for 20 min. The supernatant was transferred and the volume split among aliquot tubes, then placed in a -80°C freezer for storage.

Protein buffer

Protein buffer (10 mL) was mixed on the day of the kill, just prior to use. The protein buffer contained: 1 mL 500 mM Tris-HCl (pH 7.2, Sigma, T1503), 2.5 mL of 1.0 M sucrose (Sigma, S9378), 100 µL 200 mM EDTA (pH 7.6, Sigma, ED4SS), 100 µL 100 mM EGTA (pH 7.5, Aldrich, 23453-2), 1.25 µL 0.4 M NaF (Sigma, S6521), 4.554 mL H2O, 1 mL 10% Triton X-100 (Sigma, T6878), 100 µL 10 mM orthovanadate (Sigma, S6508), 400 µL Protease Inhibitor (Sigma, P8340).

Scoring for Injury and Inflammation

PFA fixed tissues were stained with H&E and scored for injury and inflammation by a pathologist (63).

Inflammation Score

0: presence of occasional inflammatory cells in the lamina propria
1: increased numbers of inflammatory cells in the lamina propria
2: confluence of inflammatory cells, extending into submucosa
3: transmural extension of the infiltrate

Epithelial Injury Score

0: no mucosal damage
1: discrete lympho-epithelial lesions
2: surface mucosal erosion or focal ulceration
3: extensive mucosal damage and extension into deeper structures of the bowel wall

Fecal Dry Matter Protocol

1. Label aluminum pans. Use pressure tool (burnisher) to "carve" number on tab.
2. Dry pans in oven at 60°C over weekend.
3. Remove pans from oven using tongs and place in a desiccator to cool. The pans may be stacked for storage.
4. Never touch the pans with hands. Use only tongs or forceps.
5. Remove one pan at a time, weigh, and record the weight of each pan.
6. Weigh the pellet(s) in each pan, keeping a written record of which animal # corresponds with each pan.
7. Dry fecal material for 72 h and 60°C.
8. Move the pans with dried pellets to the vacuum desiccator. Remember to use tongs and do not stack the tins.
9. Allow samples to cool in vacuum desiccator overnight.
10. Move pans and pellets to plain desiccator one at a time with tongs. Do not stack.
11. Weigh and record each sample, one at a time.
12. Return pans with pellets to 60°C over for overnight.

13. Repeat steps 8-11 until pellets weigh the same as the previous weight recorded.

% dry matter= g dry wt/ g wet wt * 100%

PCNA methodology

Adapted by Leigh Ann Piefer (finalized May 17, 2011)
Protocol completed by Dr. Andy Ambrus (Texas A&M, Vet Med, WO#10468)

1. Deparaffinize slides
   xylene----------5 min (3 times)
[allow slides to dry and circle sections with pap pen]
   100% EtOH--------5 min (2 times)
   95% EtOH---------3 min (2 times)
   70% EtOH---------3 min
   distilled H_2O ----------3 min

   Place citrate buffer (Reveal Decloaker) in a plastic coplin jar. Using a water bath, heat citrate buffer to 97° C.
   Leave slides in citrate buffer for 45 minutes.
   Remove coplin jar and cool for 20 minutes at room temperature.
   >>Transfer slides to ddH_2O for 3 minutes

2. Remove endogenous peroxidase activity
   3% H_2O_2 for 30 min
   20 mL of 30% H_2O_2 +180 mL methanol

3. Wash in PBS
   5 min, 3 times.

4. Prepare Vectastain Blocking Serum by adding
   3 drops of stock Normal Serum (yellow label) to 10 mL of PBS in yellow mixing bottle.

5. Cover each section with Vectastain Blocking Serum. Let stand for 20 min.
   Save remaining Vectastain Blocking Serum for step 9.

6. Wash with PBS
   Cover each section with PBS, 2 min, 3 times

7. Add anti-PC10 (1:100) to each section, completely covering each section (~200 μL). For the negative control slide use PBS instead of anti-PC10. Let stand for 1 hour.

8. Wash with PBS
Cover each section with PBS, 2 min, 3 times

9. Prepare Vectastain biotinylated anti-mouse IgG.
Add 25 μl of stock biotinylated antibody to 5 ml of serum solution from step 4, Mix Gently.

10. Cover each section with Vectastain biotinylated anti-mouse IgG. Let stand for 45 min.

11. Prepare ABC reagent in advance to be used in step 13. The ABC reagent is prepared by adding
-2 drops of reagent A (gray label) to 5 ml PBS in the ABC Reagent gray mixing bottle.
- Mix gently.
-Add exactly 2 drops of Reagent B (gray label) to the same mixing bottle,
-Mix immediately, and allow ABC Reagent to stand for about 30 minutes before use.
*This should be done in a darkened area.

12. Wash with PBS
Cover each section with PBS
2 min, 3 times

13. Apply Vectastain ABC reagent that has been prepared in step 11. Cover each section and let stand for 30 min.

14. Wash with PBS
2 min, 3 times

15. Prepare DAB solution
Place 5.0 mL of distilled deionized water in a 15 mL tube
Add 2 drops of Buffer Stock Solution and mix well
Add 4 drops of DAB Stock Solution and mix well
Add 2 drops of Hydrogen Peroxide Solution

16. One slide at a time, cover sections with DAB solution. Incubate until staining develops (approximately 50-60 s). Rinse with ddH_2O and place in distilled deionized water. Repeat for each slide.
Discard DAB solution and initial rinse water into hazardous waste bottle. (DAB is a mutagen).

17. Wash with ddH_2O for 5 min.

18. Deactivate all DAB materials (glassware, pipet tips, used stock vials, stir bars) and used DAB solutions with bleach overnight, and flush with excess water in drain next day (20 minutes)

19. Counterstain with filtered Hematoxyline < 1 sec.

20. Rinse with H_2O briefly to remove excess counterstain, then wash with H_2O 4 min, 1 times.

21. Dehydrate slides:
    1 x 1 min 70% EtOH.
    1 x 1 min 95% EtOH.
    1 x 1 min 100% EtOH.
    3 x 2 min Xylene.

22. Apply permount and cover glass. Use the wet mounting method. Work quickly to keep tissues moistened. Apply slight pressure to remove any air bubbles.

23. Allow slides to dry over night before cleaning with xylene and examining at 40x.

Reagents:

1. Phosphate buffered saline (PBS)
2. Vectastain ABC Elite Kit -- mouse IgG
    source: Vector Lab.
    Cat #: PK-6102, 1 ml/ea.
    contains: blocking serum
        biotinylated antimouse IgG
        avidin-biotin complex
3. PCNA monoclonal antibody, Purified
    source: COVANCE Signet lab
    Cat #: SIG-3523, 1ml/ea
4. Diaminobenzidine or DAB Substrate Kit for Peroxidase
    source: Vector
    Cat #: SK-4100
5. Harris hematoxylin
    Source: Sigma-Aldrich
    Cat#: HHS
6. Reveal Decloaker
    Source: BioCare Medical

Critical steps prior to staining:
1. tissue fixation in 70% ethanol.

2. processing and baking of tissue at temperatures not exceeding 50° C.
3. keep slides moist at all times during the staining procedure.
4. include one positive and one negative control slide in each batch of slides stained.

Mucosal mRNA Isolation

Totally RNA Kit (Applied Biosystems #AM1910)

Tissue must be removed using RNase free conditions. All disposables, glassware and reagents must be RNase free.

1. Measure and record volume of lysate (200-250 uL)
2. Bring all volumes of lysate up to 500 μL using Denaturation Solution (Applied Biosystems, AM 8540G) FLICK tubes to mix
3. In hood, add 1 ½ vol. (starting volume= 500 uL) (750 uL) phenol/$CHCl_3$:IAA (lower phase) (use fresh tip each time)
4. Shake vigorously for 1 min.; store on ice for 5 min.
5. Prepare Heavy PLG tubes by centrifuging for 30 s at 14000 X g @ 18-20ºC (face joint outwards)
6. Transfer everything to prepared Phase Lock Gel Tube (Heavy 2 mL, version- Cat # 2302830)
7. Centrifuge 16,000 x g, 5 min., 18-20°C
8. Use one tip to "clear" the top milky layer of contaminants
9. Transfer upper phase to new 2.0 ml eppitube. If there is no supernatant add 250 μL more of phenol/CCH_3 :IAA and re-centrifuge
10. Add 1/10 vol. (~40-45 uL) sodium acetate; (Remember to check approx. vol.) Mix by shaking or inversion for about 10 s

11. Add 1 vol ACID: phenol/$CHCl_3$ #2 (Starting volume=500 uL). Do not add more than one starting volume even if the volume of the lysate is greater than the starting volume (lower phase=remember new tips)
12. Shake vigorously for 1 min.; store on ice for 5 min.
13. Prepare Heavy PLG tubes by centrifuging for 30 sec at 14000 X g @ 18-20ºC (face joint outwards)
14. Transfer to new Phase Lock Gel tube (Heavy 2 mL version- Cat # 2302830)
15. Centrifuge at 16,000 x g, 5 min at 18-20°C
16. Transfer upper phase to new tube 2.0 mL eppitube
17. Measure and record the volume (~300 uL)
18. Add an equal volume (measured in previous step) isopropanol; mix well (invert or flick)
19. Store at -20°C, at least 30 min. to overnight
20. Centrifuge at 14,000 x g, 15 min. at 4°C. Discard supernatant (pour off into phenol waste)
21. Wash pellet with 300 µL of 70% ethanol; flick for ~0.5-3 min
22. Centrifuge for 5-10 min at low speed (~3000 X g or 7,500 rpm in a microfuge) at RT or 4°C. Discard supernatant
23. Wash pellet with 300 µL of 70% ethanol; flick for ~0.5-3 min
24. Centrifuge for 5-10 min at low speed (~3000 X g or 7,500 rpm in a microfuge) at RT or 4°C
25. Carefully remove supernatant with pipette and discard. Respin briefly, remove supernatant with fine tip pipette.
26. Cover w/ Kim wipe and air dry for ~ 5 min.

27. Re-suspend mRNA pellet in 50 µL Nuclease Free Water (Applied Biosystems, AM9937)
28. Heat to 55° for ~8 min and vortex occasionally for all mRNA to go into solution.

DNase treatment:

1. To mRNA, add 0.1 vol (~5 uL) 10X DNase I buffer (Ambion DNA free kit, Applied Biosystems, AM1906) and 2 µL DNase. Mix gently and incubate at 37°C for 20-30 min.
2. Add 0.1 vol (~5 uL) DNase inactivation reagent (after resuspending it). Incubate at RT for 2 min, flicking a few times during incubation.
3. Centrifuge tube ~1.5 min 10,000 g 4°C. Remove supernatant to new tube, leaving reagent behind.
4. Place ~5 µL of sample in a small eppitube and store at -80°C. This sample will be used for mRNA purity analysis on the Agilent 2100 Bioanalyzer.
5. Add 0.1 volume (~5 µL) 0.1 mM EDTA in DEPC treated water (Applied Biosystems, AM9912). Store at -80°C.

SUPPLIES and REAGENTS:

Applied Biosystems

ToTally RNA Kit	AM1910
Nuclease Free Water	AM9937
0.1 EDTA in DEPC treated water	AM9912
Denaturation Solution	AM8540G

Quantifying mRNA in samples

1. Turn on computer and NanoDrop 1000 spectrophotometer (ThermoScientific, Wilmington, DE). Verify that NanoDrop 1000 is plugged in properly to the computer.
2. Open ND-1000 software. Select “Nucleic Acid.”

3. Lift the sample arm. Put ddH_2O on a KimWipe, then use the moistened KimWipe to clean the upper and lower pedestals.
4. Load 1 µL of ddH_2O on the lower pedestal, lower the sample arm, and click OK. This will calibrate the spectrophotometer.
5. Clean the pedestals as described in # 3.
6. Change Sample Type to "RNA-40."
7. Load 1 µL of ddH_2O and click "BLANK." This will calibrate the spectrophotometer again.
8. Clean the pedestals as in # 3.
9. Type sample name in "Sample ID" field. Load 1 µL of sample on the lower pedestal and lower the sample arm. Click "Measure."
10. Allow the run to complete and the curve for the sample to appear. Clean the pedestals as in # 3.
11. Repeat Steps 9 and 10 for all samples, making sure the pedestals are cleaned before each run.
12. Load 1 µL of ddH_2O and click "BLANK."
13. Click "Show Report."
    a. Concentrations are reported as ng/µL
    b. Purity can be estimated using the 260/280 ratio (should be ~2.0)
    c. Observing the 260/280 ratio and the curves could show potential contaminants. Questionable samples should be re-run before sending samples to be analyzed for purity using the Nano Chip.

Measuring purity of mRNA

1. Put ~5 µL of each sample in a labeled eppitube.
2. Place samples in a piggyback rack and place on ice. Deliver samples to Evelyn in Dr. Chapkin's lab.
3. Evelyn will run the samples on a NanoChip on an Agilent 2100 Bioanalyzer using complementary software.
    a. Make sure Bioanalyzer is connected to computer and power source.
    b. Turn on Bioanalyzer, make sure indicator light is green.
    c. Start Agilent 2100 Bioanalyzer software.
    d. Select Assay>RNA>mRNA nano
    e. Prepare samples, buffer, and nano chip. (See RNA 6000 Nano LabChip kit)
    f. Place chip in Bioanalyzer and close lid.
    g. Click "Start" above the chip icon.
    h. Ensure that sample names are all entered in the "Sample Information" tab.
    i. Change File Prefix and click "Start."
    j. When the run is finished, clean as indicated by the See RNA 6000 Nano LabChip kit.
    k. Print Data.

i. RNA Integrity Number (RIN)
>9 is optimal
>8 is acceptable, if no errors are seen on the curve

NOTES:
• Prepare and run chips within 10 minutes. Longer chip preparation times may lead to evaporation of buffers and to bad chip performance.

• Vortex chips for appropriate 1 minute (not required for protein chips). Improper vortexing can lead to poor results.

• Do not force the chip into the receptacle of the Agilent 2100 Bioanalyzer. Proper placement of the chip should not require force. Improper placement of the chip could damage the electrode assembly when you close the lid. Check whether the chip selector is in the correct position.

• Do not touch wells of the chip. The chip could become contaminated, leading to poor measurement results.

• Do not leave any wells of the chip empty or the assay will not run properly. Add 1 µL of sample buffer to each unused sample well.

Creating cDNA
Invitrogen Superscript™ III Reverse Transcriptase (18080-400, Invitrogen)

1. Get ice, two 2 mL tubes, PCR plate
2. Add the following components to an RNase free 2 mL tube, label "RT1."
    a. Hexamers
    b. Oligo dT
    c. Annealing Buffer
    For 48 reactions:

RT1	Kit amount (mL)	# animals (over estimate)	Master amount (µL)
Hexamers	0.25	55	13.75
Oligo dT	1	55	55
Annealing Buffer	1	55	55
Total			123.75

3. Mix well by flicking/vortexing and put RT1 on ice.
4. Label each microcentrifuge tube with Sample Name, Date, and cDNA.
5. To each sample well, add
    a. 2.25 µL from RT1

   b. 700 ng mRNA
      (700ng)/(ng/µL from NanoDrop)= X µL of mRNA sample
   c. Bring volume up to 8 µL/well with nuclease free H_2O
      8 µL – 2.25 µL RT1 – X µL mRNA= µL nuclease free H_2O
6. Seal the card, making sure all edges are sealed

7. Centrifuge CR422 (Program 11: 184 mm radius)
   a. 1.5 min at 4°C
   b. 1,000 rpm
8. Turn on thermocycler and load program "LER1." Make sure it is set for 96 well plate.
   a. Heat mixture to 65°C for 5 min
9. Incubate on ice for at least 1 min.
10. While heating/incubating on ice, prepare RT2. Label tube "RT2" and add the following:
    a. 2X buffer
    b. SSIII RT/RNase block
       For 48 animals:

RT2	Kit amount (mL)	# animals (over estimate)	Master amount (µL)
2X Buffer	10	55	550
SSIII RT/RNase block	2	55	110
Total			660

11. To each sample, add 12 µL of RT2
12. Seal the card and centrifuge (Program 11: 184 mm radius)
    a. 1.5 min at 4°C
    b. 1,000 rpm
13. Load program "LER2" on the thermocycler.
    a. 25°C for 10 min
    b. 50°C for 50 min
    c. 85°C for 5 min (inactivate reaction)

*Verify purity/concentrations of cDNA by using Quantifying mRNA in samples protocol on page 80, except set "Sample Type" to DNA (default).

TaqMan Microfluidic Array Cards
TaqMan® Custom Arrays (434229, Applied Biosystems)

1. Get ice and cold plate.
2. Place PCR plate on cold plate.
3. Record which samples are in each well.
4. Add the following to each well
   a. 20X Primer/Probe mix
   b. 2X TaqMan Universal Master Mix, No AmpErase UNG (4324018, Applied Biosystems)

c. RNase free water
d. cDNA

	1 reaction	# animals (over estimate)	Master amount (µL)
20X Primer/Probe mix	1	55	55
2X TaqMan Universal Master Mix	10	55	550
RNase free water	8	55	440
Total			1,045

5. Add 19 µL of Master Mix made in #4 to each well
6. Add 1 µL of cDNA to each well, keeping track of which sample goes into which well.
7. Remove TaqMan Microfluidic Array Card (4342249, Applied Biosystems) from the refrigerator and remove from packaging.
8. Load 20 µL of sample into each well. Pipette slowly to allow air to exit the well and to avoid bubbles.
9. Wrap loaded cards in foil and place on ice. Take cards upstairs to Dr. Chapkin's lab and centrifuge (1,200 rpm, 2 min)
10. Seal the card.
    a. Position the sealer/carriage
    b. Insert card into sealer, foil side up and snap into place
    c. Push the carriage across the sealer in the direction of the arrows **DO NOT MOVE THE CARRIAGE BACK BEFORE REMOVING THE TAQMAN ARRAY
    d. Remove the card and return the carriage to starting position
11. Use scissors to trim off the fill reservoirs
12. Re-wrap in foil and store at 4°C until further analysis. Cards should be run within 4-8 h of preparation.
13. Turn on 7900HT and the computer, and open SDS 2.4 software.
    a. The TaqMan Array Microfluidid Card Thermocycling Block must be installed
14. File>New
    a. Assay= $\Delta\Delta C_T$ (RQ)
    b. Container=384 Wells TaqMan Low Density Array
    c. Click OK
15. File>Import
    a. *Use the CD provided with the custom TaqMan Array
    b. Navigate to the SDS Setup File
    c. Click Import
16. Save the SDS Plate document
    a. File>Save As

b. .sds or .sdt
c. Click Save

17. Open the SDS plate document, if not already open
18. Select the Instrument tab
    Default thermocycling conditions have been loaded from the CD
19. Select the Real-Time tab
    a. Click "Connect to Instrument"
    b. Click "Open/Close"
    c. Remove card from foil and place card in the card holder, barcode facing toward you
    d. Click "Open/Close"
    e. Click "Run"
20. To remove card once the run is complete, click "Open/Close"
21. Disconnect the instrument from the computer. Save data in SDS file on flash drive. Shut down all equipment.

Analyzing mRNA expression data

1. Open SDS 2.4
    a. Load SDS files
    b. View amplification plots for each gene
        i. Verify smooth curves and when it crosses the threshold
    c. View errors for each animal and gene
        i. View Troubleshooting help page to determine which errors merit exclusion of the data
2. Open RQ Manager 1.2.2
3. File>New Study
    a. Load all SDS files
    b. Select all animals, including their duplicate runs
    c. Select 18S as the Endogenous Control Detector
    d. Click Analyze
4. Export data to excel and calculate gene expression ($2^{(40-C_T)}$)

Measuring SCFA concentrations in the fecal material

1. Powder the samples
    a. grind in a chilled (liquid nitrogen) mortar and pestle
2. Powdered samples were weighed (~0.30 g) and mixed with 1 mL internal standard solution (2-ethylbutyric acid in 70% ethanol)
3. Vortex and incubated overnight at 4°C. To isolate SCFA,
4. Vortex again, then centrifuge at 15,000 rcf for 30 m at 4°C
5. Remove the supernatant
    a. To 100 µL supernatant, add 100 µL 70% ethanol (excess supernatant stored at -20°C)

   b. Add 200 µL internal standard solution (heptanoic acid in 70% ethanol)
   c. Right before analysis, add 20 µL H_3PO_4
6. Turn on GC
   Varian 3900 GC fitted with an HP-FFAP 30 m, 0.53 mm i.d. capillary column (Agilent, CA). 1 m of deactivated glass capillary precolumn (Supelco, Bellmonte, PA) was in place as a guard column.
7. Turn on the computer
   a. Ensure there are no error in communication between Varian CP3841 Autoinjector, the GC, and the computer
8. Load Program (STFRT) and place samples into the Auto Injector. Label Sample names appropriately (be sure to change the vial number for each sample)
   a. At the beginning of each run and after every 4 samples, place a vial containing 70% EtOH
   b. Ensure that wash vials are full and the caps are intact
   c. The GC conditions were programmed as follows: injector temperature, 200°C; detector temperature, 300°C; column flow, 7.0 mL/min He; make-up flow, 23 mL/min nitrogen. Oven temperature increased from 60°C to 220°C by a triple gradient over 51 min.
9. 1 µL was then injected
10. Data were integrated and plotted using the Varian Start Workstation Program (Agilent)
11. Fecal production and moisture content data will be used to calculate SCFA concentrations on a dry weight basis and 24-h SCFA excretion.

% dry matter= g dry wt/ g wet wt * 100%

$$\frac{\mu\text{mol SCFA}}{\text{g wet wt}} \times \frac{\text{g wet wt}}{\text{g dry wt}} = \frac{\mu\text{mol SCFA}}{\text{g dry wt}}$$

$$\frac{\text{average g feces (stale)}}{24\text{ h}} \times \frac{\%\text{ dry matter (stale)}}{} = \text{g fecal production (dry wt)/ 24 h}$$

$$\frac{\mu\text{mol SCFA}}{\text{g dry wt}} \times \frac{\text{g fecal production (dry wt)}}{24\text{ h}} = \frac{\mu\text{mol SCFA}}{24\text{ h}}$$

Coomassie Protein Assay

Reagents

Coomassie Plus Protein Assay Kit (23236, Pierce, Rockford, IL)
contains: Coomassie Plus (Bradford) Assay Reagent,
Albumin (BSA) Standard Ampules (2 mg/ml)

Procedure:

1. Prepare BSA standards:

Vial	Volume of dH_20	Volume/Source of BSA	Final BSA Concentration
A	3,555 µL	45 µL of Stock	25 µg/mL
B	6,435 µL	65 µL of Stock	20 µg/mL
C	3,970 µL	30 µL of Stock	15 µg/mL
D	3,000 µL	3,000 µL of vial B dilution	10 µg/mL
E	2,500 µL	2,500 µL of vial D dilution	5 µg/mL
F	1,700 µL	1,700 µL of vial E dilution	2.5 µg/mL

2. Prepare samples, buffer and standards in 1:800 dilutions in a 2 mL eppitube.

Type	Sample Source	µL of Sample Source	µL of diluent
Standards A-F	Sample buffer*	0.625	499.375 of BSA standards
Sample	Mucosa sample	0.625	499.375 of dH_2O
Blank	Sample buffer*	0.625	499.375 of dH_2O

*The sample buffer was used from the particular set (during kills). In the case of more than one set run per plate, the buffer was divided into equal portions and added to the blank or standard.

3. Pipette 150 µL standard, sample, and blank into each appropriate well. The standards, blank, and samples were run in triplicate.

4. Add 150 µL Coomassie Plus Reagent to each well and mix with plate shaker for 30 s.

5. Incubate samples at RT for 10 minutes.

6. Measure absorbance (A595) on plate reader.

7. Subtract the mean blank reading from each of the sample and standard replicates to find their means.

TransAM NF-κB p65 (101016, Active Motif, Carlsbad, CA)

1. Prepare buffers for appropriate number of wells

Buffer	Reagent	1 well
Complete Lysis Buffer	DTT	0.11 µL
	Protease Inhibitor	0.23 µL
	Lysis Buffer AM2	22.2 µL
		22.5 µL total
Complete Binding Buffer	DTT	0.07 µL
	Herring sperm DNA	0.34 µL
	Binding buffer AM3	33.4 µL
		33.8 µL total
1x Wash Buffer	Distilled Water	2.025 mL
	10x wash buffer AM2	225 µL
		2.25 mL total
1x Antibody binding buffer	Distilled water	157.5 µL
	10x Ab Binding Buffer AM2	17.5 µL
		175 µL total
Chemiluminescent Working Solution	Chemi Reagent	18.8 µL
	Reaction Buffer	37.5 µL
		56.2 µL total

2. Add 30 µL Complete Binding Buffer to each well to be used. (We did not complete the competitive binding step)

3. *Sample Wells*: Add 20 µL of sample diluted in Complete Lysis Buffer (4 µg of protein/sample)
Positive control Well: Add 1.25 µg of the provided Jurkat nuclear extract diluted in 20 µL of Complete Lysis Buffer (0.5 µg of nuclear extract in 19.5 µL of Complete Lysis Buffer)
Blank Well: Add 20 µL of Complete Lysis Buffer per well.
Blank and Buffer Well: Add 0.33 µL of set A buffer with 19.67 µL of Complete Lysis Buffer for a total of 20 µL.

4. Cover and incubate for 1 h at RT with mild agitation.

5. Wash each well 3 times with 200 µL 1x wash buffer.

6. Add 50 μL of diluted NF-κB antibody (1:10,000 dilution in 1x antibody binding buffer) to each well.

7. Incubate for 1 h without agitation at RT. Repeat wash steps.

8. Add 50 μL of diluted HRP-conjugated antibody (1:10,000 dilution in 1x antibody binding buffer) to each well.

9. Incubate for 1 h without agitation. Place working solution at RT. Wash 4 times with 1X wash buffer.

10. Add 50 μL RT working solution to all wells. Minimize exposure to light.

11. Read luminescence in plate reader. For best results, read at several different sensitivities. The results for this assay were read at 180 sensitivity.

12. Subtract the "blank and buffer" reading from all of the wells.

APPENDIX B

TABLES OF RESULTS

Body weight over time

TABLE B-1 Body weight (g) measured throughout the study[1]

Appx. Day	Basal Diet		Quercetin Diet		Chlorogenic Acid Diet	
	Control	DSS	Control	DSS	Control	DSS
0	65.9±2.6	67.1±2.5	65.1±2.6	65.5±2.5	65.7±2.6	66.9±2.5
18	170.1±4.4	174.3±4.2	167.8±4.4	172.8±4.2	169.0±4.4	172.8±4.2
22	189.6±4.8	193.7±4.6	189.3±4.8	194.3±4.6	190.6±4.8	192.2±4.6
36	264.9±6.1	265.0±5.8	260.5±6.1	268.6±5.8	264.7±6.1	264.6±5.8
43	281.8±6.4	272.8±6.1	271.2±6.4	278.0±6.1	279.0±6.4	270.9±6.1
50	315.3±7.8	308.5±7.4	308.4±7.8	323.2±7.4	311.1±7.8	313.4±7.4
60	340.3±8.5	333.1±8.1	322.0±8.5	342.1±8.1	335.5±8.5	340.2±8.1
64	345.8±9.2	335.6±8.8	333.4±9.2	346.6±8.8	339.8±9.2	346.4±8.8

[1] Values are LS means ± SEM. No significant differences were observed. n=10-11 rats/group.

Fecal moisture content over time

TABLE B-2 Fecal moisture content [(g dry matter/g wet weight)*100%] over time on fresh samples[1]

Time point	Basal Diet		Quercetin Diet		Chlorogenic Acid Diet	
	Control	DSS	Control	DSS	Control	DSS
PreDSS1	48.3±1.0^{ab}	48.6±1.0^{ab}	48.8±1.0^{ab}	50.4±0.9^{b}	47.0±1.0^{a}	48.6±1.0^{ab}
Post DSS1	49.1±1.5^{a}	66.1±1.5^{b}	50.4±1.5^{a}	64.5±1.5^{b}	48.1±1.6^{a}	69.2±1.5^{b}
Pre DSS 2	49.6±1.3^{ab}	57.0±1.2^{c}	51.1±1.3^{b}	57.3±1.3^{c}	46.9±1.4^{a}	57.5±1.3^{c}
Post DSS2	48.1±1.8^{a}	62.1±1.8^{b}	52.4±1.8^{a}	63.5±1.8^{bc}	47.6±1.8^{a}	68.5±1.8^{c}
Pre DSS3	50.5±1.6^{b}	56.7±1.6^{c}	49.6±1.6^{ab}	57.3±1.5^{c}	45.9±1.6^{a}	58.4±1.5^{c}
Post DSS3	47.2±1.7^{a}	67.5±1.6^{b}	48.6±1.7^{a}	64.3±1.5^{b}	47.0±1.6^{a}	66.2±1.6^{b}
Pre-Kill	50.8±1.9^{a}	56.6±1.7^{b}	49.8±1.9^{a}	58.8±1.8^{b}	47.3±2.0^{a}	59.4±1.7^{b}

[1] Values are LS means ± SEM. Means with superscripts without a common letter differ ($p<0.05$). n=8-11 rats/group.

SCFA Concentrations on wet weight basis

TABLE B-3 Acetic acid concentrations (μmol/g wet weight feces) on wet weight basis[1]

	Basal Diet		Quercetin Diet		Chlorogenic Acid Diet	
Time point	Control	DSS	Control	DSS	Control	DSS
PreDSS1	12.9±0.8^{a}	13.7±0.8^{a}	12.7±0.8^{a}	13.0±0.8^{a}	13.2±0.8^{a}	17.6±0.8^{b}
Post DSS1	16.2±1.4^{ab}	21.4±1.4^{c}	13.7±1.4^{a}	17.2±1.4^{a}	13.2±1.4^{a}	18.4±1.4^{bc}
Pre DSS3	12.5±1.2^{a}	18.2±1.2^{b}	12.1±1.2^{a}	16.1±1.2^{b}	11.9±1.2^{a}	22.7±1.2^{c}
Post DSS3	13.0±1.3^{a}	21.4±1.2^{c}	11.6±1.3^{a}	17.8±1.2^{b}	12.3±1.3^{a}	23.8±1.2^{c}
Pre-Kill	11.6±1.1^{a}	15.8±1.1^{b}	12.7±1.1^{a}	18.4±1.1^{b}	10.6±1.1^{a}	18.0±1.1^{b}

[1] Values are LS means ± SEM. Means with superscripts without a common letter differ ($p<0.05$). n=10-11 rats/group.

TABLE B-4 Propionic acid concentrations (μmol/g wet weight feces) on wet weight basis[1]

	Basal Diet		Quercetin Diet		Chlorogenic Acid Diet	
Time point	Control	DSS	Control	DSS	Control	DSS
PreDSS1	4.5±0.4^{a}	4.5±0.4^{a}	4.3±0.4^{a}	4.7±0.4^{a}	4.6±0.4^{a}	5.4±0.4^{a}
Post DSS1	5.0±0.4^{ab}	6.1±0.4^{b}	4.8±0.4^{a}	5.0±0.4^{ab}	4.9±0.4^{a}	5.4±0.4^{ab}
Pre DSS3	3.1±0.6^{a}	6.0±0.5^{b}	3.5±0.6^{a}	5.4±0.5^{b}	3.6±0.6^{a}	6.7±0.5^{b}
Post DSS3	3.6±0.4^{a}	5.5±0.4^{b}	2.7±0.4^{a}	5.3±0.4^{b}	3.0±0.4^{a}	6.4±0.4^{b}
Pre-Kill	2.9±0.5^{a}	5.5±0.4^{b}	3.3±0.5^{a}	5.4±0.4^{b}	2.4±0.5^{a}	6.0±0.4^{b}

[1] Values are LS means ± SEM. Means with superscripts without a common letter differ ($p<0.05$). n=10-11 rats/group.

TABLE B-5 Isobutyric acid concentrations (μmol/g wet weight feces) on wet weight basis[1]

	Basal Diet		Quercetin Diet		Chlorogenic Acid Diet	
Time point	Control	DSS	Control	DSS	Control	DSS
PreDSS1	0.86±0.1^{ab}	0.87±0.1^{ab}	0.77±0.1^{a}	0.86±0.1^{ab}	1.0±0.1^{b}	0.93±0.1^{ab}
Post DSS1	0.94±0.1^{cd}	0.72±0.1^{ab}	0.82±0.1^{bc}	0.64±0.1^{a}	1.1±0.1^{d}	0.61±0.1^{a}
Pre DSS3	0.81±0.1^{ab}	0.85±0.1^{ab}	0.75±0.1^{a}	0.82±0.1^{ab}	0.85±0.1^{ab}	0.93±0.1^{b}
Post DSS3	0.77±0.04^{bc}	0.73±0.04^{b}	0.74±0.04^{b}	0.59±0.04^{a}	0.88±0.04^{c}	0.71±0.04^{ab}
Pre-Kill	0.75±0.05^{ab}	0.85±0.05^{bc}	0.70±0.05^{a}	0.92±0.05^{c}	0.76±0.05^{ab}	0.88±0.05^{bc}

[1] Values are LS means ± SEM. Means with superscripts without a common letter differ ($p<0.05$). n=10-11 rats/group.

TABLE B-6 Butyric acid concentrations (μmol/g wet weight feces) on wet weight basis[1]

	Basal Diet		Quercetin Diet		Chlorogenic Acid Diet	
Time point	Control	DSS	Control	DSS	Control	DSS
PreDSS1	5.0±0.4^{ab}	4.9±0.4^{a}	5.2±0.4^{ab}	5.5±0.4^{ab}	5.9±0.4^{b}	5.9±0.4^{b}
Post DSS1	5.5±0.5^{a}	6.8±0.5^{b}	5.8±0.5^{ab}	5.7±0.5^{ab}	5.8±0.5^{ab}	6.1±0.5^{ab}
Pre DSS3	4.8±0.4^{ab}	5.3±0.4^{b}	5.1±0.4^{ab}	4.8±0.4^{ab}	4.3±0.4^{a}	6.5±0.1^{c}
Post DSS3	4.2±0.4^{a}	5.1±0.3^{a}	4.5±0.4^{a}	4.7±0.3^{a}	4.4±0.4^{a}	4.9±0.3^{a}
Pre-Kill	3.9±0.3^{a}	4.5±0.3^{ab}	4.8±0.3^{b}	5.3±0.3^{b}	3.9±0.3^{a}	4.4±0.3^{ab}

[1] Values are LS means ± SEM. Means with superscripts without a common letter differ ($p<0.05$). n=10-11 rats/group.

TABLE B-7 Isovaleric acid concentrations (μmol/g wet weight feces) on wet weight basis[1]

	Basal Diet		Quercetin Diet		Chlorogenic Acid Diet	
Time point	Control	DSS	Control	DSS	Control	DSS
PreDSS1	2.1±0.2^{ab}	2.1±0.2^{ab}	1.8±0.2^{a}	2.0±0.2^{ab}	2.5±0.2^{b}	2.2±0.4^{ab}
Post DSS1	2.2±0.1^{bc}	1.4±0.1^{a}	1.9±0.2^{b}	1.2±0.1^{a}	2.6±0.2^{c}	1.2±0.1^{a}
Pre DSS3	1.9±0.1^{ab}	1.8±0.1^{ab}	1.7±0.1^{a}	1.7±0.1^{a}	2.1±0.1^{b}	1.9±0.1^{ab}
Post DSS3	1.8±0.1^{a}	1.3±0.1^{b}	1.7±0.1^{a}	1.1±0.1^{b}	2.1±0.1^{a}	1.2±0.1^{b}
Pre-Kill	1.7±0.1^{a}	1.7±0.1^{a}	1.6±0.1^{a}	1.8±0.1^{a}	1.8±0.1^{a}	1.7±0.1^{a}

[1] Values are LS means ± SEM. Means with superscripts without a common letter differ ($p<0.05$). n=10-11 rats/group.

TABLE B-8 Valeric acid concentrations (μmol/g wet weight feces) on wet weight basis[1]

	Basal Diet		Quercetin Diet		Chlorogenic Acid Diet	
Time point	Control	DSS	Control	DSS	Control	DSS
PreDSS1	1.9±0.1^{a}	1.8±0.1^{a}	1.8±0.1^{a}	1.8±0.1^{a}	2.2±0.1^{b}	2.0±0.1^{ab}
Post DSS1	1.9±0.1^{bc}	1.6±0.1^{ab}	1.8±0.1^{b}	1.3±0.1^{a}	2.1±0.1^{c}	1.4±0.1^{a}
Pre DSS3	1.6±0.1^{a}	1.8±0.1^{ab}	1.6±0.1^{a}	1.7±0.1^{a}	1.7±0.1^{a}	2.0±0.1^{b}
Post DSS3	1.6±0.1^{a}	1.5±0.1^{a}	1.5±0.1^{a}	1.2±0.1^{b}	1.6±0.1^{a}	1.5±0.1^{a}
Pre-Kill	1.4±0.1^{a}	1.6±0.1^{ab}	1.4±0.1^{a}	1.7±0.1^{b}	1.6±0.1^{ab}	1.7±0.1^{b}

[1] Values are LS means ± SEM. Means with superscripts without a common letter differ ($p<0.05$). n=10-11 rats/group.

TABLE B-9 Total SCFA concentrations (μmol/g wet weight feces) on wet weight basis[1]

	Basal Diet		Quercetin Diet		Chlorogenic Acid Diet	
Time point	Control	DSS	Control	DSS	Control	DSS
PreDSS1	27.3±1.4^{a}	27.9±1.3^{a}	26.6±1.4^{a}	27.8±1.3^{a}	29.3±1.4^{a}	34.0±1.3^{b}
Post DSS1	31.7±1.9^{a}	38.0±1.8^{b}	28.8±1.9^{a}	31.2±1.8^{a}	29.7±1.9^{a}	33.2±1.8^{ab}
Pre DSS3	24.6±2.1^{a}	33.9±2.0^{c}	24.7±2.1^{ab}	30.5±2.0^{bc}	24.4±2.1^{a}	40.7±2.0^{d}
Post DSS3	25.0±1.7^{a}	35.5±1.7^{c}	22.8±1.7^{a}	30.6±1.7^{b}	24.2±1.7^{a}	38.5±1.7^{c}
Pre-Kill	22.2±1.7^{a}	30.0±1.7^{b}	24.5±1.7^{a}	33.5±1.7^{b}	21.1±1.7^{a}	32.8±1.7^{b}

[1] Values are LS means ± SEM. Means with superscripts without a common letter differ ($p<0.05$). n=10-11 rats/group.

Concentrations on dry weight basis

TABLE B-10 Acetic concentrations (μmol/g dry weight feces) on dry weight basis[1]

	Basal Diet		Quercetin Diet		Chlorogenic Acid Diet	
Time point	Control	DSS	Control	DSS	Control	DSS
PreDSS1	25.0±1.5^{a}	26.4±1.5^{a}	25.7±1.5^{a}	26.4±1.4^{a}	24.7±1.5^{a}	32.6±1.5^{b}
Post DSS1	31.7±5.5^{a}	65.8±5.5^{c}	28.2±5.5^{a}	49.7±5.5^{b}	25.2±5.5^{a}	65.9±5.5^{c}
Pre DSS3	25.7±3.9^{a}	46.0±3.9^{bc}	24.1±3.9^{a}	37.9±3.7^{b}	22.2±4.1^{a}	55.5±3.7^{c}
Post DSS3	24.9±5.1^{a}	73.4±4.8^{c}	22.4±5.1^{a}	53.4±4.6^{b}	23.3±4.8^{a}	74.0±4.8^{c}
Pre-Kill	23.2±3.6^{a}	38.5±3.2^{b}	24.7±3.6^{a}	45.5±3.4^{b}	20.9±3.8^{a}	45.4±3.2^{b}

[1] Values are LS means ± SEM. Means with superscripts without a common letter differ ($p<0.05$). n=8-11 rats/group.

TABLE B-11 Propionic concentrations (μmol/g dry weight feces) on dry weight basis[1]

	Basal Diet		Quercetin Diet		Chlorogenic Acid Diet	
Time point	Control	DSS	Control	DSS	Control	DSS
PreDSS1	8.9±0.8^{ab}	8.7±0.9^{ab}	7.8±0.9^{a}	9.5±0.8^{ab}	8.5±0.9^{ab}	10.3±0.8^{b}
Post DSS1	9.9±1.0^{a}	17.9±1.0^{c}	10.0±1.0^{a}	14.3±1.0^{b}	9.6±1.0^{a}	17.9±1.0^{c}
Pre DSS3	6.4±1.6^{a}	15.5±1.6^{b}	6.9±1.6^{a}	12.9±1.6^{b}	6.9±1.7^{a}	16.3±1.6^{b}
Post DSS3	6.5±1.2^{a}	18.4±1.1^{c}	5.4±1.2^{a}	15.2±1.1^{b}	5.7±1.1^{a}	19.2±1.1^{c}
Pre-Kill	5.5±1.6^{a}	13.3±1.4^{b}	6.4±1.6^{a}	13.1±1.5^{b}	4.9±1.7^{a}	15.3±1.4^{b}

[1] Values are LS means ± SEM. Means with superscripts without a common letter differ ($p<0.05$). n=8-11 rats/group.

TABLE B-12 Isobutyric concentrations (μmol/g dry weight feces) on dry weight basis[1]

	Basal Diet		Quercetin Diet		Chlorogenic Acid Diet	
Time point	Control	DSS	Control	DSS	Control	DSS
PreDSS1	1.7±0.1^{a}	1.6±0.1^{a}	1.6±0.1^{a}	1.8±0.1^{a}	1.9±0.1^{a}	1.8±0.1^{a}
Post DSS1	1.8±0.1^{a}	2.1±0.1^{a}	1.7±0.1^{a}	1.8±0.1^{a}	2.0±0.2^{a}	2.0±0.1^{a}
Pre DSS3	1.7±0.2^{ab}	2.1±0.2^{b}	1.5±0.2^{a}	1.9±0.2^{abc}	1.6±0.2^{a}	2.3±0.2^{c}
Post DSS3	1.4±0.1^{a}	2.2±0.1^{b}	1.5±0.1^{a}	1.7±0.1^{a}	1.7±0.1^{a}	2.1±0.1^{b}
Pre-Kill	1.5±0.2^{a}	2.0±0.1^{b}	1.4±0.2^{a}	2.3±0.2^{b}	1.6±0.2^{a}	2.2±0.1^{b}

[1] Values are LS means ± SEM. Means with superscripts without a common letter differ ($p<0.05$). n=8-11 rats/group.

TABLE B-13 Butyric concentrations (μmol/g dry weight feces) on dry weight basis[1]

	Basal Diet		Quercetin Diet		Chlorogenic Acid Diet	
Time point	Control	DSS	Control	DSS	Control	DSS
PreDSS1	9.7±0.8^{a}	9.6±0.9^{a}	10.9±0.9^{a}	11.1±0.8^{a}	11.3±0.8^{a}	11.5±0.8^{a}
Post DSS1	10.7±1.7^{a}	21.1±1.7^{c}	11.8±1.7^{ab}	16.5±1.7^{bc}	10.9±1.8^{a}	20.9±1.7^{c}
Pre DSS3	9.7±1.0^{ab}	13.1±1.0^{cd}	10.1±1.0^{a}	11.3±1.0^{bc}	7.9±1.0^{a}	15.8±1.0^{d}
Post DSS3	7.9±1.7^{a}	17.7±1.6^{b}	8.9±1.7^{a}	13.9±1.5^{b}	8.3±1.6^{a}	15.1±1.6^{b}
Pre-Kill	8.0±0.9^{a}	10.8±0.8^{bc}	9.6±0.9^{ab}	13.1±0.8^{c}	8.0±0.9^{a}	11.0±0.8^{bc}

[1] Values are LS means ± SEM. Means with superscripts without a common letter differ ($p<0.05$). n=8-11 rats/group.

TABLE B-14 Isovaleric concentrations (μmol/g dry weight feces) on dry weight basis[1]

	Basal Diet		Quercetin Diet		Chlorogenic Acid Diet	
Time point	Control	DSS	Control	DSS	Control	DSS
PreDSS1	4.1±0.3^{a}	3.9±0.3^{a}	3.8±0.3^{a}	4.1±0.3^{a}	4.7±0.3^{a}	4.4±0.3^{a}
Post DSS1	4.3±0.3^{a}	4.0±0.3^{a}	3.9±0.3^{a}	3.5±0.3^{a}	4.9±0.3^{a}	3.9±0.3^{a}
Pre DSS3	3.8±0.3^{ab}	4.3±0.3^{ab}	3.5±0.3^{a}	4.1±0.3^{ab}	3.9±0.3^{ab}	4.7±0.3^{b}
Post DSS3	3.4±0.2^{ab}	3.8±0.2^{b}	3.4±0.2^{ab}	3.1±0.2^{a}	3.9±0.2^{b}	3.3±0.2^{ab}
Pre-Kill	3.5±0.3^{ab}	4.1±0.3^{abc}	3.2±0.3^{a}	4.4±0.3^{bc}	3.7±0.3^{abc}	4.4±0.3^{c}

[1] Values are LS means ± SEM. Means with superscripts without a common letter differ ($p<0.05$). n=8-11 rats/group.

TABLE B-15 Valeric concentrations (μmol/g dry weight feces) on dry weight basis[1]

	Basal Diet		Quercetin Diet		Chlorogenic Acid Diet	
Time point	Control	DSS	Control	DSS	Control	DSS
PreDSS1	3.6±0.2^{a}	3.4±0.2^{a}	3.6±0.2^{a}	3.7±0.2^{a}	4.2±0.2^{a}	4.0±0.2^{a}
Post DSS1	3.7±0.3^{ab}	4.8±0.3^{d}	3.6±0.3^{a}	3.8±0.3^{bc}	4.0±0.3^{abcd}	4.7±0.3^{cd}
Pre DSS3	3.3±0.3^{a}	4.4±0.3^{bc}	3.2±0.3^{a}	3.9±0.3^{ab}	3.2±0.3^{a}	5.0±0.3^{c}
Post DSS3	2.9±0.3^{a}	4.9±0.3^{b}	3.0±0.3^{a}	3.5±0.2^{a}	3.1±0.3^{a}	4.3±0.3^{b}
Pre-Kill	3.0±0.3^{a}	3.9±0.3^{b}	3.0±0.3^{a}	4.3±0.3^{b}	3.1±0.3^{a}	4.3±0.3^{b}

[1] Values are LS means ± SEM. Means with superscripts without a common letter differ ($p<0.05$). n=8-11 rats/group.

TABLE B-16 Total SCFA concentrations (μmol/g dry weight feces) on dry weight basis[1]

	Basal Diet		Quercetin Diet		Chlorogenic Acid Diet	
Time point	Control	DSS	Control	DSS	Control	DSS
PreDSS1	53.1±2.7^{a}	53.6±2.9^{a}	53.6±2.9^{a}	56.6±2.6^{a}	55.2±2.9^{a}	64.6±2.7^{b}
Post DSS1	62.1±7.6^{a}	115.6±7.6^{c}	59.1±7.6^{a}	89.7±7.6^{b}	56.6±8.0^{a}	115.2±7.6^{c}
Pre DSS3	50.7±6.7^{a}	85.4±6.7^{bc}	49.4±6.7^{a}	72.0±6.4^{b}	45.7±7.1^{a}	99.4±6.4^{c}
Post DSS3	47.1±7.7^{a}	120.4±7.3^{c}	44.5±7.7^{a}	90.8±6.9^{b}	45.8±7.3^{a}	118.1±7.3^{c}
Pre-Kill	44.7±6.1^{a}	72.7±5.5^{b}	48.3±6.1^{a}	82.6±5.8^{b}	42.2±6.5^{a}	82.7±5.5^{b}

[1] Values are LS means ± SEM. Means with superscripts without a common letter differ ($p<0.05$). n=8-11 rats/group.

24 h SCFA concentrations

TABLE B-17 24 h production of acetic acid (μmol/d) on dry weight basis[1]

	Basal Diet		Quercetin Diet		Chlorogenic Acid Diet	
Time point	Control	DSS	Control	DSS	Control	DSS
PreDSS1	10.4±1.1	12.6±1.2^{a}	12.4±1.2^{a}	11.5±1.0^{a}	10.0±1.2^{a}	12.7±1.1^{a}
Post DSS1	14.4±5.1^{a}	47.3±4.8^{b}	15.2±4.8^{a}	42.8±5.4^{b}	11.7±5.1^{a}	49.5±4.8^{b}
Pre DSS3	15.2±3.5^{a}	33.5±3.5^{b}	13.7±3.3^{a}	20.3±3.1^{a}	11.0±3.5^{a}	34.6±3.3^{b}
Post DSS3	12.5±8.7^{a}	64.5±7.7^{b}	10.7±8.2^{a}	49.3±7.4^{b}	10.5±7.7^{a}	61.5±8.1^{b}
Pre-Kill	12.6±3.4^{a}	22.6±3.2^{b}	12.4±3.4^{a}	31.1±3.2^{b}	10.7±3.6^{a}	27.6±3.2^{b}

[1] Values are LS means ± SEM. Means with superscripts without a common letter differ ($p<0.05$). n=8-11 rats/group.

TABLE B-18 24 h production of propionic acid (μmol/d) on dry weight basis[1]

	Basal Diet		Quercetin Diet		Chlorogenic Acid Diet	
Time point	Control	DSS	Control	DSS	Control	DSS
PreDSS1	3.6±0.5[a]	4.1±0.5[a]	3.7±0.5[a]	4.2±0.4[a]	3.4±0.5[a]	4.0±0.5[a]
Post DSS1	4.3±1.5[a]	13.3±1.4[b]	5.4±1.4[a]	11.4±1.5[b]	4.5±1.5[a]	14.1±1.4[b]
Pre DSS3	3.4±1.3[a]	11.4±1.3[c]	3.9±1.3[a]	6.9±1.2[a]	3.3±1.3[a]	10.3±1.3[b]
Post DSS3	3.0±2.2[a]	16.5±2.0[b]	2.6±2.1[a]	13.5±1.9[b]	2.7±2.0[a]	15.8±2.1[a]
Pre-Kill	3.1±1.1[a]	8.3±1.1[b]	3.3±1.2[a]	8.8±1.1[b]	2.5±1.2[a]	9.1±1.1[b]

[1] Values are LS means ± SEM. Means with superscripts without a common letter differ ($p<0.05$). n=8-11 rats/group.

TABLE B-19 24 h production of isobutyric acid (μmol/d) on dry weight basis[1]

	Basal Diet		Quercetin Diet		Chlorogenic Acid Diet	
Time point	Control	DSS	Control	DSS	Control	DSS
PreDSS1	0.68±0.1[a]	0.76±0.1[a]	0.78±0.1[a]	0.78±0.1[a]	0.77±0.1[a]	0.73±0.1[a]
Post DSS1	0.82±0.2[a]	1.47±0.1[b]	0.88±0.1[a]	0.51±0.2[b]	0.90±0.1[a]	1.57±0.2[b]
Pre DSS3	0.94±0.2[a]	1.5±0.2[c]	0.87±0.1[a]	1.0±0.1[ab]	0.80±0.2[a]	1.4±0.1[bc]
Post DSS3	0.76±0.2[a]	1.9±0.2[b]	0.68±0.2[a]	1.5±0.2[b]	0.76±0.2[a]	1.7±0.2[b]
Pre-Kill	0.83±0.2[ab]	1.2±0.1[bc]	0.70±0.2[a]	1.5±0.1[c]	0.81±0.2[ab]	1.3±0.1[c]

[1] Values are LS means ± SEM. Means with superscripts without a common letter differ ($p<0.05$). n=8-11 rats/group.

TABLE B-20 24 h production of butyric acid (μmol/d) on dry weight basis[1]

	Basal Diet		Quercetin Diet		Chlorogenic Acid Diet	
Time point	Control	DSS	Control	DSS	Control	DSS
PreDSS1	4.0±0.4[a]	4.5±0.4[a]	5.2±0.4[a]	4.6±0.4[a]	4.6±0.4[a]	4.5±0.4[a]
Post DSS1	4.8±1.9[a]	15.0±1.8[b]	6.2±1.8[a]	14.0±2.0[b]	4.8±1.9[a]	16.7±1.8[b]
Pre DSS3	5.7±1.0[a]	9.5±1.0[b]	5.8±1.0[a]	6.2±1.0[a]	3.4±1.0[a]	9.4±1.0[b]
Post DSS3	4.1±2.9[ab]	16.7±2.6[c]	4.3±2.8[ab]	12.7±2.5[c]	2.8±2.6[a]	11.8±2.8[bc]
Pre-Kill	4.4±0.9[ab]	6.6±0.8[abc]	4.9±0.9[ab]	8.8±0.8[c]	4.1±0.9[a]	6.7±0.8[bc]

[1] Values are LS means ± SEM. Means with superscripts without a common letter differ ($p<0.05$). n=8-11 rats/group.

TABLE B-21 24 h production of isovaleric acid (μmol/d) on dry weight basis[1]

	Basal Diet		Quercetin Diet		Chlorogenic Acid Diet	
Time point	Control	DSS	Control	DSS	Control	DSS
PreDSS1	1.7±0.2^{a}	1.9±0.2^{a}	1.8±0.2^{a}	1.9±0.2^{a}	1.9±0.2^{a}	1.7±0.2^{a}
Post DSS1	1.9±0.3^{a}	2.8±0.3^{b}	2.0±0.3^{ab}	2.8±0.3^{c}	2.2±0.3^{abc}	3.0±0.3^{c}
Pre DSS3	2.2±0.3^{ab}	3.1±0.3^{b}	2.0±0.3^{a}	2.2±0.3^{ab}	2.0±0.3^{a}	2.8±0.3^{ab}
Post DSS3	1.8±0.3^{ab}	3.1±0.3^{c}	1.6±0.3^{a}	2.6±0.3^{bc}	1.8±0.3^{a}	2.8±0.3^{c}
Pre-Kill	1.9±0.3^{ab}	2.4±0.3^{b}	1.6±0.3^{a}	2.9±0.3^{c}	1.9±0.3^{ab}	2.5±0.3^{bc}

[1] Values are LS means ± SEM. Means with superscripts without a common letter differ ($p<0.05$). n=8-11 rats/group.

TABLE B-22 24 h production of valeric acid (μmol/d) on dry weight basis[1]

	Basal Diet		Quercetin Diet		Chlorogenic Acid Diet	
Time point	Control	DSS	Control	DSS	Control	DSS
PreDSS1	1.5±0.2^{a}	1.6±0.2^{a}	1.7±0.2^{a}	1.6±0.2^{a}	1.7±0.1^{a}	1.6±0.1^{a}
Post DSS1	1.6±0.3^{a}	3.4±0.3^{b}	1.9±0.3^{a}	3.1±0.3^{b}	1.8±0.3^{a}	3.6±0.3^{b}
Pre DSS3	1.9±0.3^{a}	3.1±0.3^{b}	1.8±0.3^{a}	2.1±0.3^{a}	1.6±0.3^{a}	3.0±0.3^{b}
Post DSS3	1.5±0.5^{a}	4.2±0.5^{b}	1.4±0.5^{a}	3.1±0.4^{b}	1.4±0.5^{a}	3.5±0.5^{b}
Pre-Kill	1.6±0.3^{ab}	2.4±0.3^{bc}	1.5±0.3^{a}	2.9±0.3^{d}	1.6±0.3^{ab}	2.6±0.3^{cd}

[1] Values are LS means ± SEM. Means with superscripts without a common letter differ ($p<0.05$). n=8-11 rats/group.

TABLE B-23 24 h production of total SCFA (μmol/d) on dry weight basis[1]

	Basal Diet		Quercetin Diet		Chlorogenic Acid Diet	
Time point	Control	DSS	Control	DSS	Control	DSS
PreDSS1	21.9±2.1^{a}	25.5±2.2^{a}	25.7±2.2^{a}	24.6±2.0^{a}	22.4±2.2^{a}	25.4±2.1^{a}
Post DSS1	27.8±8.5^{a}	83.2±8.1^{b}	31.7±8.1^{a}	75.8±9.0^{b}	26.0±8.5^{a}	88.5±8.1^{b}
Pre DSS3	29.2±6.3^{a}	62.0±6.3^{b}	28.1±6.0^{a}	38.7±5.7^{a}	22.6±6.3^{a}	61.6±6.0^{b}
Post DSS3	23.7±14.4^{a}	107.0±12.9^{b}	21.2±13.6^{a}	82.8±12.3^{b}	21.0±12.9^{a}	97.1±13.6^{b}
Pre-Kill	24.5±5.8^{a}	43.4±5.5^{b}	24.4±5.8^{a}	56.0±5.5^{b}	21.6±6.2^{a}	49.8±5.5^{b}

[1] Values are LS means ± SEM. Means with superscripts without a common letter differ ($p<0.05$). n=8-11 rats/group.

Relative SCFA Concentrations

TABLE B-24 Relative acetic acid concentrations [(µmol acetic acid/µmol total SCFA)*100%)][1]

	Basal Diet		Quercetin Diet		Chlorogenic Acid Diet	
Time point	Control	DSS	Control	DSS	Control	DSS
PreDSS1	47.1±1.4^{ab}	49.4±1.4^{b}	48.0±1.4^{ab}	46.9±1.3^{ab}	45.0±1.4^{a}	50.3±1.4^{b}
Post DSS1	50.4±1.8^{bc}	55.8±1.8^{d}	47.4±1.8^{ab}	54.7±1.8^{cd}	44.9±1.9^{a}	55.9±1.8^{d}
Pre DSS3	50.7±1.0^{ab}	53.3±1.0^{bc}	49.0±1.0^{a}	52.8±1.0^{bc}	48.6±1.1^{a}	55.6±1.0^{c}
Post DSS3	52.3±1.5^{a}	60.7±1.4^{bc}	50.5±1.5^{a}	57.6±1.4^{b}	50.8±1.4^{a}	62.6±1.4^{c}
Pre-Kill	51.5±1.2^{ab}	52.6±1.1^{abc}	50.5±1.2^{a}	55.1±1.2^{bc}	49.6±1.3^{a}	54.7±1.1^{c}

[1] Values are LS means ± SEM. Means with superscripts without a common letter differ ($p<0.05$). n=8-11 rats/group.

TABLE B-25 Relative propionic acid concentrations [(µmol propionic acid/µmol total SCFA)*100%)][1]

	Basal Diet		Quercetin Diet		Chlorogenic Acid Diet	
Time point	Control	DSS	Control	DSS	Control	DSS
PreDSS1	16.8±1.1^{a}	16.0±1.2^{a}	14.7±1.2^{a}	16.7±1.1^{a}	15.1±1.2^{a}	16.0±1.1^{a}
Post DSS1	16.0±1.1^{a}	16.1±1.1^{a}	16.7±1.1^{a}	16.4±1.1^{a}	16.6±1.1^{a}	16.4±1.1^{a}
Pre DSS3	11.8±1.1^{a}	17.5±1.1^{c}	13.9±1.1^{ab}	17.4±1.0^{c}	14.7±1.2^{abc}	16.4±1.0^{bc}
Post DSS3	13.4±1.0^{ab}	15.5±1.0^{bc}	12.0±1.0^{a}	17.5±1.0^{c}	11.9±1.0^{a}	16.3±1.0^{c}
Pre-Kill	12.1±0.8^{a}	17.9±0.7^{b}	13.1±0.8^{a}	15.8±0.8^{b}	11.7±0.8^{a}	18.0±0.8^{b}

[1] Values are LS means ± SEM. Means with superscripts without a common letter differ ($p<0.05$). n=8-11 rats/group.

TABLE B-26 Relative isobutyric acid concentrations [(µmol isobutyric acid/µmol total SCFA)*100%)][1]

	Basal Diet		Quercetin Diet		Chlorogenic Acid Diet	
Time point	Control	DSS	Control	DSS	Control	DSS
PreDSS1	3.1±0.2^{a}	3.0±0.2^{a}	3.0±0.2^{a}	3.1±0.2^{a}	3.4±0.2^{a}	2.9±0.2^{a}
Post DSS1	3.0±0.2^{bc}	1.9±0.2^{a}	2.9±0.2^{b}	2.1±0.2^{a}	3.5±0.2^{c}	1.8±0.2^{a}
Pre DSS3	3.3±0.2^{cd}	2.6±0.2^{ab}	3.0±0.2^{bc}	2.7±0.2^{ab}	3.6±0.2^{d}	2.3±0.2^{a}
Post DSS3	3.1±0.2^{b}	1.9±0.2^{a}	3.3±0.2^{bc}	2.0±0.4^{a}	3.7 ±0.2^{c}	1.8±0.2^{a}
Pre-Kill	3.4±0.2^{bc}	3.0±0.2^{ab}	3.0±0.2^{ab}	2.7±0.2^{a}	3.7±0.2^{c}	2.8±0.2^{a}

[1] Values are LS means ± SEM. Means with superscripts without a common letter differ ($p<0.05$). n=8-11 rats/group.

TABLE B-27 Relative butyric acid concentrations [(µmol butyric acid/µmol total SCFA)*100%)][1]

	Basal Diet		Quercetin Diet		Chlorogenic Acid Diet	
Time point	Control	DSS	Control	DSS	Control	DSS
PreDSS1	18.4±1.0^{a}	17.8±1.1^{a}	20.2±1.1^{a}	19.4±1.0^{a}	20.5±1.1^{a}	17.7±1.0^{a}
Post DSS1	17.5±0.9^{a}	18.3±0.9^{ab}	20.1±0.9^{b}	18.4±0.9^{ab}	19.4±1.0^{ab}	17.9±0.9^{ab}
Pre DSS3	19.8±0.8^{b}	15.8±0.8^{a}	20.4±0.8^{b}	15.9±0.8^{a}	17.4±0.8^{a}	15.9±0.8^{a}
Post DSS3	17.4±1.0^{bc}	14.3±1.0^{a}	19.9±1.0^{c}	15.3±1.0^{ab}	18.2±1.0^{c}	12.8±1.0^{a}
Pre-Kill	18.2±0.8^{a}	14.9±0.8^{b}	20.2±0.8^{a}	15.9±0.8^{b}	18.9±0.8^{a}	13.7±0.8^{b}

[1] Values are LS means ± SEM. Means with superscripts without a common letter differ ($p<0.05$). n=8-11 rats/group.

TABLE B-28 Relative isovaleric acid concentrations [(µmol isovaleric acid/µmol total SCFA)*100%)][1]

	Basal Diet		Quercetin Diet		Chlorogenic Acid Diet	
Time point	Control	DSS	Control	DSS	Control	DSS
PreDSS1	7.7±0.5^{ab}	7.4±0.5^{ab}	7.2±0.5^{ab}	7.3±0.5^{ab}	8.4±0.5^{b}	6.9±0.5^{a}
Post DSS1	7.1±0.5^{b}	3.6±0.5^{a}	6.6±0.5^{b}	4.1±0.5^{a}	8.6±0.5^{c}	3.4±0.5^{a}
Pre DSS3	7.7±0.4^{bc}	5.4±0.4^{a}	7.0±0.4^{b}	5.7±0.4^{a}	8.7±0.4^{c}	4.7±0.4^{a}
Post DSS3	7.4±0.5^{a}	3.4±0.5^{b}	7.6±0.5^{a}	3.6±0.4^{b}	8.6±0.5^{a}	2.9±0.5^{b}
Pre-Kill	7.9±0.5^{cd}	6.0±0.5^{ab}	6.9±0.5^{bc}	5.3±0.5^{a}	8.8±0.5^{d}	5.5±0.5^{a}

[1] Values are LS means ± SEM. Means with superscripts without a common letter differ ($p<0.05$). n=8-11 rats/group.

TABLE B-29 Relative valeric acid concentrations [(μmol valeric acid/μmol total SCFA)*100%)][1]

	Basal Diet		Quercetin Diet		Chlorogenic Acid Diet	
Time point	Control	DSS	Control	DSS	Control	DSS
PreDSS1	6.8±0.3^{ab}	6.4±0.3^{a}	6.8±0.3^{ab}	6.6±0.3^{a}	7.6±0.3^{b}	6.2±0.3^{a}
Post DSS1	6.0±0.3^{b}	4.2±0.3^{a}	6.3±0.3^{bc}	4.4±0.3^{a}	7.0±0.3^{c}	4.2±0.3^{a}
Pre DSS3	6.6±0.3^{a}	5.5±0.3^{b}	6.6±0.3^{a}	5.5±0.3^{b}	7.1±0.3^{a}	5.1±0.3^{b}
Post DSS3	6.3±0.3^{a}	4.2±0.3^{b}	6.8±0.3^{a}	4.0±0.3^{b}	6.8±0.3^{a}	3.7±0.3^{b}
Pre-Kill	6.8±0.3^{cd}	5.6±0.3^{ab}	6.3±0.3^{bc}	5.2±0.3^{a}	7.4±0.5^{d}	5.3±0.3^{a}

[1] Values are LS means ± SEM. Means with superscripts without a common letter differ ($p<0.05$). n=8-11 rats/group.

Post-termination observations

TABLE B-30 NF-κB activity (RLU)[1]

Basal Diet		Quercetin Diet		Chlorogenic Acid Diet	
Control	DSS	Control	DSS	Control	DSS
12,949±3,359^{ab}	18,707±3,166^{b}	19,444±3,359^{b}	14,781±3,166^{ab}	18,807±3,359^{ab}	10,012±2,864^{a}

[1] Values are LS means ± SEM. Means with superscripts without a common letter differ ($p<0.05$). n=8-11 rats/group.

TABLE B-31 Injury and Inflammation Scores (0-3)[1]

	Basal Diet		Quercetin Diet		Chlorogenic Acid Diet	
	Control	DSS	Control	DSS	Control	DSS
Injury	1.0±0.1^{b}	2.1±0.1^{c}	0.8±0.1^{ab}	2.0±0.1^{c}	0.7±0.1^{a}	2.0±0.1^{c}
Inflammation	1.0±0.1^{a}	1.7±0.1^{b}	1.0±0.1^{a}	1.6±0.1^{b}	0.8±0.1^{a}	1.6±0.1^{b}

[1] Values are LS means ± SEM. Means with superscripts without a common letter differ ($p<0.05$). n=9-11 rats/group.

TABLE B-32 Gene (mRNA) expression in mucosal scrapings[1]

	Basal Diet		Quercetin Diet		Chlorogenic Acid Diet	
	Control	DSS	Control	DSS	Control	DSS
MCT-1	333±26.6^{b}	247±26.6^{a}	352±26.6^{b}	229±26.6^{a}	303±26.6^{ab}	256±26.6^{a}
SLC5A8	109±9.9^{b}	73.7±9.9^{a}	111±9.9^{b}	83.8±11^{ab}	106±9.9^{b}	101±9.9^{ab}
FGF-2	98.1±31.9^{a}	182.5±31.9^{a}	139.1±31.9^{a}	306.0±33.1^{b}	172.2±37.3^{a}	175.6±31.9^{a}
TFF-3	1,334±99^{bc}	1,110±99^{ab}	1,405±99^{c}	969±106^{a}	1,428±99^{c}	1,179±99^{ac}
TNFR-1	161.0±13.0^{bc}	99.4±13.0^{a}	177.3±13.0^{c}	143.3±13.9^{bc}	147.8±13.0^{bc}	140.1±13.0^{b}
TNFR-2	6.8±0.9^{a}	7.2±0.9^{ab}	7.6±0.9^{ab}	10.3±1.0^{c}	7.2±0.9^{ab}	9.7±0.9^{bc}
IκBα	119±10^{b}	84±10^{a}	125 ±10^{b}	99±11^{ab}	124±10^{b}	108±10^{ab}
RelA/p65	117±9^{b}	69±9^{a}	107±9^{b}	80±10^{a}	108±9^{b}	80±9^{a}
RIPk	42.4±3.4^{ab}	34.9±3.4^{a}	47.4±3.4^{b}	35.3±3.6^{a}	42.9±3.4^{ab}	38.5±3.4^{ab}
IL-12	98±43^{a}	223±45^{b}	176±48^{ab}	425±46^{c}	175±43^{ab}	227±43^{b}
COX-2	11,189±819^{c}	5,997±793^{ab}	8,113±819^{b}	4,327±848^{a}	8,188±793^{b}	6,512±819^{ab}
IL-1	1,070±268^{a}	2,264±286^{b}	1,808±268^{ab}	1,488±286^{ab}	1,646±268^{ab}	1,589±268^{ab}

[1] Values are LS means ± SEM. Means with superscripts without a common letter differ ($p<0.05$). n=7-8 rats/group.

VITA

Leigh Ann Piefer

PERMANENT ADDRESS
2253 TAMU
College Station, TX, 77843

EDUCATION

Texas A&M University, College Station, TX — Aug 12
Masters of Science in *Nutrition*

Texas A&M University, College Station, TX — May 10
Bachelor of Science in *Nutritional Science*, Minor in *Mathematics*

AWARDS AND HONORS

Graduate Student Oral Competition, Third Place — Mar 12
TAMU Intercollegiate Faculty of Nutrition Research Symposium

Graduate Student Travel Grant Recipient — Mar 12
TAMU Intercollegiate Faculty of Nutrition

Excellence in Research Scholarship — Aug 11
TAMU Intercollegiate Faculty of Nutrition

In-State Tuition Fellowship — Jun 11
TAMU College of Agriculture and Life Sciences

Graduate Student Travel Grant Recipient — Apr 11
TAMU Intercollegiate Faculty of Nutrition

Lechner Scholar Scientific Presentation Grant Recipient — Mar 11
TAMU Walter W. Lechner Estate endowment

Graduate Student Research and Presentation Grant Recipient — Feb 11
TAMU Office of Graduate Studies and the Association of Former Students

ABSTRACTS

- **Piefer L.A.,** Weeks B.R., Carroll R.J., Byrne D.H., Ambrus A., Turner N.D., (2012) Quercetin and chlorogenic acid affect NF-κB activity and gene transcription, but do not mitigate colonic injury in DSS treated rats. *FASEB J.* **26**, 263.6
- **Piefer L.A.,** Stehm R.E., Krenek K.A., Weeks B.R., Carroll R.J., Byrne D.H., Talcott S.T., Turner N.D., (2011) Chlorogenic Acid Reduced Injury and DSS-Induced NF-κB Activation in a Rat Colitis Model. *FASEB J.* **25**, 773.13