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ANTI-INFLAMMATORY EFFECTS OF COCOA SUPPLEMENTATION IN A MICE MODEL OF INFLAMMATORY BOWEL DISEASE

A Thesis in

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by

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ABSTRACT

Inflammatory bowel disease (IBD) afflicts nearly 2.2 million people worldwide. IBD is composed of two main conditions, ulcerative colitis (UC) and Crohn's disease. UC has an incidence of 9–20 cases per 100,000 people per year and a prevalence of 156–291 cases per 100,000 people per year**.** In addition to the decrease in health-related quality of life and increases in hospitalizations, IBD also presents a significant financial burden. In 2014, the estimated direct medical costs were $11-28 billion and the continued increase in prevalence has led to further increases. UC patients suffer from moderate to severe gastrointestinal issues hindering their way of life as it becomes more difficult to absorb and metabolize essential nutrients from their already restricted diet. Patients exhibit increased inflammatory gene expression, cytokine production, oxidative stress, and decreased gut barrier function. Inflammation in UC is a result of antigen-presenting cells in the colonic mucous membrane over-producing pro-inflammatory cytokines. Cocoa has been reported to have antioxidant and anti-inflammation activity. The effects have been linked to cocoa polyphenols, which represent 12–18% of dry weight of cocoa powder. The overall goal of this project was to investigate the dose-dependent beneficial effects of dietary cocoa supplementation on dextran sulfate sodium (DSS)-induced UC in C57BL/6J mice. The DSS-induced mouse model is widely used because it mimics the pathogenesis of UC in humans. The specific objectives of the current study were a) to evaluate the effect of a diet supplemented with cocoa on gross markers of UC and b) to evaluate the effect of this cocoa supplementation on molecular markers of systemic and colonic inflammation.

Male C57BL/6J mice were pre-treated with cocoa supplemented diets (0, 2, 4, and 8% w/w) for two weeks prior to receiving DSS (2% w/v) in drinking water for 7 days to induce ulcerative colitis. Cocoa treatment continued throughout the induction period. Body weight was determined during the DSS induction period. At the end of the study, body weights were reduced

in the 8% cocoa treated mice compared to the control (11.2% lower final body weight, $p < 0.05$). After euthanasia, spleen and liver weight, and colon length were determined. It was found that cocoa-treated mice at all doses had reduced spleen weight compared to DSS-treated control mice (35 – 40% lower). Mice treated with 8% cocoa had 20% lower liver weights. No significant difference in colon length was observed between cocoa-treated mice and DSS-induced controls. Plasma protein levels of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, chemokine ligand 1 (CXCL1), and interferon (IFN)-γ were determined by multiplex ELISA. Colonic mRNA levels of the inflammatory markers, IL-1β, IL-6, IL-10, and TNF-α were determined by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR). Up-regulation of serum markers of inflammation TNF-α, IL-1β, CXCL1, and IFN-γ were seen in the mice that received cocoa intervention compared to DSS-treated mice, as measured by Multiplex ELISA assay. Colonic gene expression of IL-1β, IL-6, IL-10, and TNF-α were not significantly different from DSS-treated control mice. Principal component analysis showed that the mice in the 8% treatment group were significantly different from the other treatment groups, and that this difference was driven mainly by higher levels of plasma TNF-α and IL-1β. Collectively, the results of this study demonstrate that dietary treatment with cocoa was not able to reduce inflammation and may enhance inflammation in the DSS-induced mouse model of UC. Future studies should examine the mechanisms of action underlying the effects observed here.

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ACKNOWLEDGEMENTS

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Chapter 1

Literature Review

Introduction

Cocoa Production

Cocoa originates from the seeds of the cacao tree (*Theobroma cacao*) and is the key ingredient in chocolate manufacturing. The three main cultivars of cacao are *Criollo*, *Forastero*, and *Trinitario* with *Forastero* being the most used for chocolate manufacture. The largest producer and exporter of *Forastero* is Ivory Coast in West Africa. *Criollo* is native to South America. *Criollo* is said to be the cocoa tree used by the Mayas and is deemed as a higher quality cacao variety due to its less bitter taste and more aromatic notes than other beans, from which only 5–10% of chocolate is made. *Trinitario* is a *Criollo* and *Forastero* hybrid and represents 10–15% of cocoa production.[1,2]

Cocoa Processing: Fermentation and Roasting

The quality and flavor of cocoa and cocoa products depend on the steps that take place in cocoa processing including fermentation and roasting.

Fermentation of cocoa beans is crucial for the development of precursors necessary for chocolate flavor, such as free amino acids and reducing sugars. Compounds such as alcohols, aldehydes, and organic acids, among others, are produced during fermentation, contributing to the final flavor profile of chocolate.[3] The quality of fermentation is decided by the amount of these

compounds which are present. Cacao seeds vary in color depending on degree of ripeness, but once the fermentation and drying processes begins these seeds develop a reddish-brown color as the walls of pigmentation cells are broken down.[4] The fermentation process can last anywhere from 2 to 10 days depending on fermentation conditions. The success of cocoa bean fermentation is dependent on the microbial activity of yeasts, lactic acid bacteria, and acetic acid bacteria. Under proper conditions, including both quality of microbial activity and the physiochemical parameters (such as temperature, pH, and available metabolites), fermentation should not exceed 4 days, as any longer can introduce by-product organisms (e.g., bacilli and filamentous fungi) that may be undesirable.[5] After fermentation and prior to shipping, beans are dried to a final moisture content of less than 7.5% (w/w). The stage of drying plays a major role in reducing astringency, bitterness, and acidity, also essential to the development of the characteristic taste and brown coloring of chocolate.[6]

Roasting is an important operation in processing of cocoa beans since it brings about formation of characteristic brown color, mild aroma, and texture of roasted beans, while also being an important pasteurization step for the beans.[6,7] Cocoa beans are typically hot air roasted at temperatures of 120-175°C (250-350°F) for 30-90 min. Roasting converts flavor precursors into flavor compounds, such as ketones and pyrazines, via Maillard reactions. Roasting cocoa beans also leads to a reduction in undesirable volatile components that form during fermentation (e.g., propanoic acid, cyclohexanol, and thiobismethane).[8]

Medicinal History of Cocoa Usage

Texts from the Indigenous, Colonial, and early Modern Periods reveal that cacao has long played important roles as a medicine or a vehicle to administer medicinal products. The use of

cacao by Mesoamerican civilizations dates back at least to 1000 B.C.E.; chromatographic analyses have detected residual cacao on the remains of ancient pottery vessels.[2,9]

There is a rich history behind the development of cacao in relation to spirituality of indigenous peoples and their creation stories that center the idea that cacao was discovered by the gods, coined "kakawa" or "cacao" by the Mayan people of Central America. The word cacao likely originated with the Olmec peoples, who occupied the lowland regions of the eastern Mexican gulf coast. The Aztecs would then adopt cacao as a medicinal food and document its role in the Florentine Codex of 1590 C.E., which is the earliest surviving text known to document cacao and chocolate's usage. Consumed mostly as beverages, cacao was drunk to relieve stomach and intestinal pain. Often, cacao was combined with other ingredients that were documented as having healing capabilities. For example, cacao combined with liquid from the bark of the *Castilla elastica* (silk cotton tree) was said to cure infections while cacao beans ground together with dried maize kernels and *Calliandra anomala* was said to relieve fevers. The Badianus Manuscript (ca. 1552 C.E.), further elaborated on cacao's medicinal uses; medical conditions identified included angina, constipation, tartar removal, indigestion, fatigue, gout, hemorrhoids, and lactation difficulties.[2,9]

Mexican-European interactions would be what led to the introduction of cocoa to the Europeans in the early 1500s C.E. Chocolate, prepared as a beverage, was introduced to the Spanish court in 1544 C.E. Manuscripts produced in Europe and New Spain from the 16th to early 20th century revealed more than 100 medicinal uses for cocoa and/or chocolate[2,9], and cocoa's medicinal capacity has been extensively investigated in the 21st century (**Table 1-1**).

Table 1-1. Research studies that investigate the beneficial health effects of cocoa supplement in models of chronic disorders and chronic inflammation.

Objective	Subject	Duration	Control	Treatment	Outcome	Reference
Improve endothelial function	Overweight adults (n=44)	6 wks (4 wks washout)	Sugar-sweetened cocoa-free placebo beverage	Sugar-free cocoa beverage Sugar-sweetened cocoa beverage	Improved flow-mediated dilation	Njike et al. 2011[10]
Effects on adipose tissue inflammation	Obesity-induced male C57BL/6J mice (n=68)	18 wks	Low-fat diet High-fat diet	HF diet with 8% (w/w) unsweetened cocoa powder	↓ mRNA levels TNF-α, IL-6, iNOS ↓ Protein levels NF-κB ↓ Levels of arachidonic acid in the adipose tissue Ameliorated plasma endotoxin Improved gut barrier function	Gu et al. 2014[11]

Ameliorating obesity-related inflammation	Obesity-induced male C57BL/6J mice (n=126)	18 wks (8 wks cocoa treatment)	Low-fat (10% kcal) HF (60% kcal) diet	HF diet supplemented with 8 % cocoa powder	Reduced body weight gain Reduced the severity of obesity-related fatty liver disease ↓ Serum IL-6 and MCP-1 ↓*Il6*, *Il12b*, *Nos2,* and *Emr1*	Gu et al. 2014[12]
Inhibit ulcerative colitis *in vivo*	Female Wistar rats (n=48)	6 dys 5% DSS-induction; 9 dys 2% DSS-induction	Standard diet	5% cocoa-enriched diet	↓ Serum TNF-α, protein ↓ Colon NO levels	Pérez-Berezo et al. 2012[13]
Inhibit ulcerative colitis *in vivo* and *in vitro*	Female Balb/C mice (n=21-30) LPS-stimulated RAW 264.7 cells	1 wk	No DSS or PCE	500 mg/kg polyphenol-enriched cocoa extract	↓ NO, COX2, STAT1, STAT3, and NF-κB production	Andújar et al. 2011[14]
Observe effects of chocolate consumption on serum lipids and lipid peroxidation	Healthy individuals (n=45)	3 wks	White chocolate (WC)	75g/dy dark chocolate High-polyphenol dark chocolate	↑ Serum HDL cholesterol	Mursu et al. 2004[15]

Improve blood flow (vasomotion)	Heart transplant recipients (n=22)	2 hrs	Control chocolate	Flavonoid-rich dark chocolate (0.27 mg/g catechin & 0.9 mg/g epicatechin; 70% cocoa)	↑ Coronary artery diameter Improved coronary vasomotion ↓ Platelet adhesion	Flammer et al. 2007[16]
Cardioprotective effects	Individuals with congestive heart failure (n=20)	Short-term: 2 hrs Long-term 4 wks	Control chocolate	Flavanol-rich chocolate (0.27 mg/g catechin & 0.9 mg/g epicatechin; 70% cocoa)	Improved flow-mediated vasodilation ↓ Platelet adhesion	Flammer et al. 2012[17]
Improve HDL cholesterol	Type 2 diabetes patients (n=12)	8 wks (4 wks washout)	Chocolate (cocoa butter w/o cocoa solids)	High-polyphenol chocolate (85% cocoa solids)	↑ HDL cholesterol	Mellor et al. 2010[18]
Attenuate progression of Type-2 diabetes	Obese Zucker diabetic rats	6 to 15 wks	Control diet	10% cocoa-rich diet	Prevented beta-cell apoptosis ↑ Bcl-xL ↓ Bax, caspase-3 activity	Fernández-Millán et al. 2015[19]
Observe effects of cocoa diet on gut health	Male Zucker diabetic fatty (ZDF) rats (n = 16)	10 wks	Zucker lean rats; standard diet	10% cocoa-enriched diet	↑ Tight junction protein Zonula occludens-1 (ZO-1)	Álvarez-Cilleros et al 2020[20]

					↓ Colonic mRNA levels of TNF-α, IL-6 and MCP-1	
Prevent colon inflammation	Male Wistar Han rats (n=40)	8 wks	Control diet	12% cocoa-enriched diet	↓ NF-κB, COX2, iNOS Inhibited NF-κB translocation and JNK phosphorylation	Rodríguez-Ramiro et al. 2013[21]
	TNF-α-stimulated Caco-2 cells			Cocoa polyphenol extract (10 μg/ml)		
Inhibit colitis-associated-cancer	Female BALB/c mice (n=50)	62 dys	Control diet	5% or 10% cocoa diet	↓ COX2, iNOS ↑ Nrf2	Pandurangan et al. 2015[22]

Cocoa Polyphenols

Polyphenols

Polyphenols are secondary plant metabolites that protect against ultraviolet (UV) radiation and pathogens. In food, polyphenols may contribute to bitterness, astringency, color, flavor, odor, and oxidative stability.[23]

Polyphenols can be divided into main categories of flavonoids, phenolic acids, stilbenes, and lignans. The most abundant group is flavonoids. The basic skeleton structure of flavonoids is a 3-ring structure that contains two benzene rings (A and B) and a heterocyclic pyran ring (C) (**Fig. 1-1**). Based on their chemical structure, degrees of oxidation and unsaturation on the C ring, flavonoids are then subdivided into subgroups flavonols, flavanols (both flavan-3-ols and flavan-4-ols), flavones, flavanones, isoflavones, and anthocyanidins (**Fig. 1-1**).[24,25]

The bioavailability of phenolic compounds is dependent on the size of the structure as well as the number of hydroxyl groups, and is determined by the extent to which the compound is absorbed and metabolized following ingestion. Absorption can occur by mediated uptake through the intestinal wall via transporters or passive diffusion.[26,27] Two studied transport mechanisms of phenolic absorption are lactase-phlorizin hydrolase (LPH) diffusion or cytosolic β-glucosidase (CBG) transport. LPH is a glucosidase within the small intestine catalyzing the hydrolysis of glycosylated phenolic compounds. CBG-catalyzed hydrolysis involves sodium-dependent glucose transport (SGLT1) across the small intestine.[28–31] For the passive diffusion mechanism, absorption is much higher for lipophilic compounds than for hydrophilic ones, because compounds must pass through lipid bilayer of the membrane. This is the case for aglycones, such as monomeric catechins, which are lower in molecular weight and have a higher lipophilicity.[26,27]

After absorption from the small intestine, Phase II (conjugation) biotransformation, i.e., sulfation, methylation, or glucuronidation, of phenolic compounds can take place in the enterocytes or the hepatocytes, resulting in a series of water-soluble metabolites.[32]

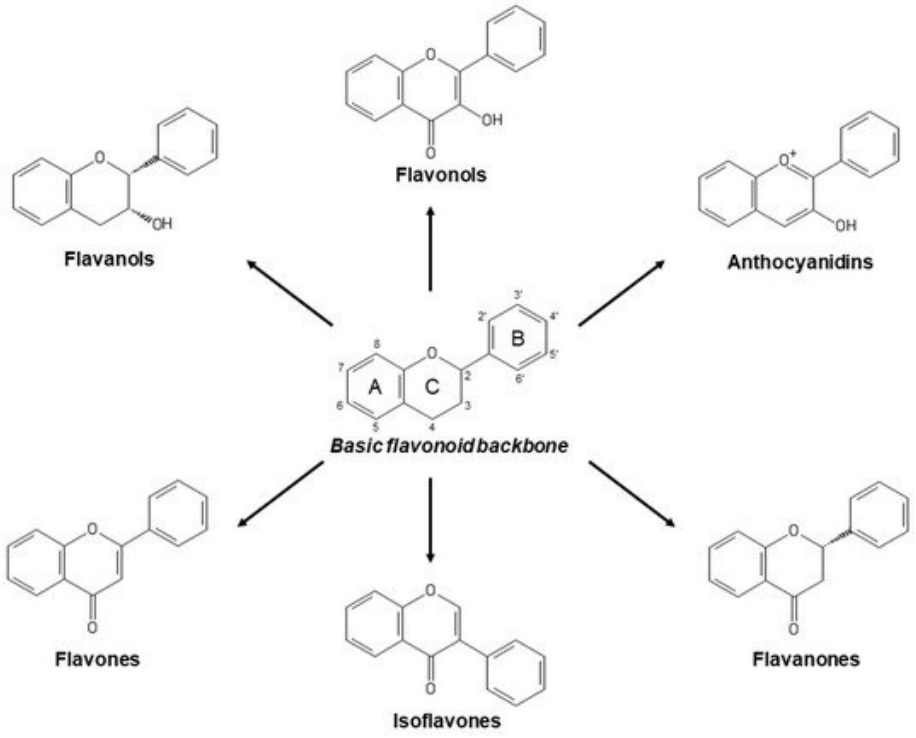

Figure 1-1. Chemical structure of flavonoids.[33]

Flavonoids that are not absorbed from the small intestine transit to the colon, where they are subjected to gut microbial metabolism. Once inside the large intestine, colonic bacteria are known to act enzymatically on the polyphenolic backbone of the remaining unabsorbed polyphenols. The microflora in the colon transforms these unabsorbed polyphenols into low molecular weight phenolic acids, which can be subsequently absorbed.[32,34,35,36]

Polyphenol Content of Cocoa

The putative health beneficial effects of cocoa have been attributed to its polyphenol constituents.[14,37,38] Cocoa contains ~12–18% polyphenols by dry weight.[39] Cocoa contains high amounts of the flavonoids (−)-epicatechin (EC), (+)-catechin and their polymers (proanthocyanidins), while other polyphenols have been found in smaller quantities.[40] Catechins, anthocyanins, and proanthocyanidins (degree of polymerization 1–10 [41,42]) comprise about 37%, 4% and 58% of the total phenolic content, respectively.[43,44]

Metabolism and Pharmacokinetics of Cocoa Polyphenols

Following ingestion, EC and (+)-catechin have absorption rates ranging from 22–55%.[40] Following absorption, these compounds are subject to Phase II metabolism resulting in the formation of O-methylated, O-sulfated, and O-glucuronidated metabolites. Proanthocyanidin dimers (procyanidins B1, B2 and B5) can cross enterocytes but only to 5–10% of the absorption rate of monomers.[45,46] Larger proanthocyanidin polymers do not cross the intestinal barrier and move into the colon where they can be cleaved by colonic bacteria into smaller molecular weight metabolites including phenolic acids and valerolactones which can be absorbed into the bloodstream.[47] Further investigation of the biological activity of non-absorbed, higher molecular weight polyphenols and their microbial metabolites is required to understand the putative anti-inflammatory activity of cocoa polyphenols.[1,33]

The Effects of Cocoa Processing on Phenolic Content and Composition

The consequence in fermented cocoa beans is a decrease of epicatechin concentration to approximately 2–17 mg/g.[2,48] A recent study by Racine et al., found that fermentation decreased

total polyphenol content by 18.3% and flavanols specifically were reduced by 14.4%.[49] Cocoa roasting also leads to loss of total phenolic content and changes in polyphenol chemistry. Typical cocoa roasting occurs between temperatures of 120-135°C (250-275°F) and up to 177°C (350°F) for 30-90 min. One study investigating the degradation of phenolic compounds during the roasting of cocoa found total degradation of epicatechin and loss of ~93% of total phenolic content at 200°C for 50 min.[50,51] Stanley et al., reported that roasting reduced total polyphenol content in a temperature-dependent manner (i.e., -9.7% at 100°C and -39.9% at 190°C). Roasting at 190°C was also found to result in a 98.6% loss of monomeric flavanols (EC).[52] Interestingly, Stanley also discovered that roasting of cocoa beans at 150°C and higher increased the levels of catechin and proanthocyanidin hexamers and heptamers, with catechin increasing by 675% with 10 min roasting at 190°C and proanthocyanidin hexamers and heptamers by 280% and 214%, respectively, by roasting at 190°C for 40 min. Similarly, Payne et al., observed the same phenomenon when cocoa beans roasted at 120°C increased catechin by 696% in unfermented beans and 640% in fermented beans.[53] More specifically, there was an increase of catechin as a result of EC epimerization. Cocoa can also be subjected to alkalization, often referred to as "Dutching", to increase dispersibility, darken color, and reduce acidity. Stanley et al., in a separate study showed that treatment of cocoa powder with NaOH (pH 8, 92°C, 1hr) displayed similar behavior to that of roasting effects as 1) catechin content increased by 40% total and 2) EC and proanthocyanidin content reduced by 23 to 66%.[2,54]

Inflammatory Bowel Disease

Introduction

Inflammatory bowel disease (IBD) is a term grouping two main disorders (Crohn's Disease and ulcerative colitis) that are characterized by chronic inflammation of the gastrointestinal (GI) tract. Crohn's Disease causes chronic inflammation throughout the digestive tract. Ulcerative colitis (UC) is characterized by inflammation localized to the colonic mucosa and can affect both the colon and the rectum.

Ulcerative Colitis (UC)

UC was the first form of IBD to be characterized, dating back to the early 18th and 19th centuries. The term "ulcerative colitis" was coined by physician Sir Samuel Wilks in 1859. In 1909, the Royal Society of Medicine in London held a symposium that focused on UC, which helped physicians and researchers develop a deeper understanding of the condition by discovering commonly experienced symptoms seen in UC cases, evaluating what the common risk factors were, and attempted treatment options. After decades of exploring experimental therapeutic measures, surgical treatments were standardized by the 1930s, with ileostomy and partial/ full colectomy practices remaining as options to this day. In the 1950s, drug therapies including corticosteroids and sulfasalazine, a type of 5-aminosalicylic acid (ASA), were developed. Development of immunomodulators, anti-tumor necrosis factor (TNF) agents, and other drugs followed in the later years to the present day.[55]

Though the disease is incurable, drug therapies are prescribed to UC patients to help provide relief from symptoms including nausea, diarrhea, bloody stool, rectal urgency as well as complications including weight loss caused by malnutrition, fever, eye and vision complications,

and anemia[56]. UC can also increase the risk of developing or exacerbating skin disorders, arthritis, bone weakness, and liver complications.[57,58]

Statistics for ulcerative colitis/ IBD

UC has an incidence (the amount of a population that develops a disease or condition over time) of 9–20 cases per 100,000 people per year and a prevalence (the amount of a population that have a disease or condition at a time) of 156–291 cases per 100,000 people per year.[59] Between 2001–2018, UC prevalence increased by 2.8% annually (**Fig. 1-2**). While overall prevalence was higher in non-Hispanic White people, the rate of increase was greater in other racial groups (e.g., 3.5% in non-Hispanic Black people).[60]

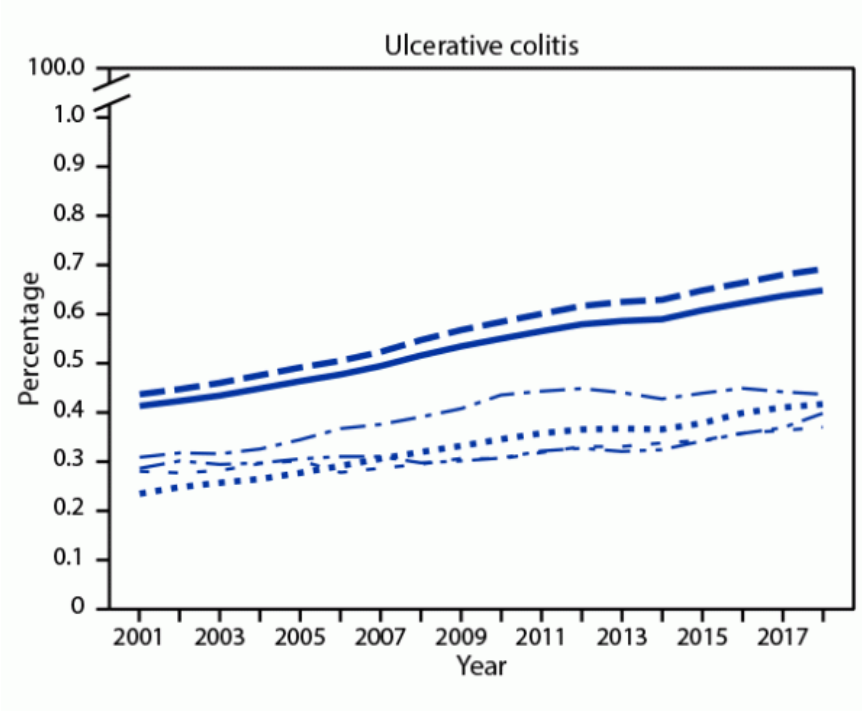

Figure 1-2. Age-adjusted prevalence of ulcerative colitis among Medicare fee-for-service beneficiaries, showing the rate of increase from 2001–2018.[60]

UC patients are at an increased risk of developing colorectal cancer, with risk increasing every 10 years of UC diagnosis: 10 years (2%), 20 years (8%), and 30 years (20–30 %).[61] UC patients in a 2018 survey reported that their lives are impacted by issues such as: 1) being forced to stay in bed (27%), 2) difficulties interacting with loved ones (32%) and 3) worry over IBD worsening over time (55%).[62] Prevalence of mental disorder (anxiety and depression) has an estimated occurrence of 29–35% during disease remission and 60–80% during disease relapse. Mental disorder prevalence in a 16-year cumulative study was estimated at 54% (5390 per 10,000) while the percentage of UC patients with mental disorder was 82%.[63,64]

In 2010, Cohen et al., reported annual per-patient medical costs for UC patients to range from $6127 to $11,477 with hospitalizations accounting for 41–55% of costs due to UC flare-ups and colectomy surgeries. These costs have continued to increase annually by 6%.[65,66] In 2014, the estimated direct medical costs were $11–28 billion and the continued increase in prevalence has led to further increases.[67,68]

Pathogenesis of ulcerative colitis

UC is thought to result from an inappropriate and continuing inflammatory response to gut microbes in a genetically susceptible host (**Fig. 1-3**).

Inflammation in UC is a result of antigen-presenting cells in the colonic mucous membrane over-producing pro-inflammatory cytokines. In this situation, interleukin (IL)-1 receptor antagonist (1RA) cannot counterbalance IL-1 production to inhibit IL-1 binding to the IL-1 receptor (IL-1R) and reduce inflammatory signaling. The IL-1 system consists of cytokines IL-1α and IL-1β, and as IL-1 levels increase during UC progression as a result of colonic

macrophage activation. This leads to activation of IL-1 converting enzyme (ICE) that releases IL-1β into the colonic mucosa.[69]

TNF-α production by colonic mucosa expands pro-inflammatory signaling via binding to TNF receptors (TNFR1 and TNFR2) and activation of transcription factor nuclear factor-κB (NF-κB) which plays an important role in the onset of inflammatory signaling, controlling the transcription of inflammatory genes. In UC the activation (i.e., nuclear translocation) increased NF-κB expression in mucosal macrophages is accompanied by an increase in downstream production of TNF-α, IL-1 and IL-6 and p65 protein subunit of NF-κB, which are found to be commonly up-regulated in the intestinal mucosa epithelium of UC patients.[70–72]

While studies evaluating the roles of TNFR versus TNFR2 in the pathogenesis of colitis in mouse models have yielded conflicting results regarding which receptor is more important, it has been reported that upregulation of TNFR2 is associated with colitis in the dextran sulfate sodium (DSS)-induced mouse model of colitis. This model seems to be independent of TNFR1.[73] Based on the role of TNF-α and the IL-1 family as the main inducers of inflammatory response in UC, they represent attractive molecular targets to inhibit abnormal innate immune activation and colitis development.

T helper 2 (Th2)–secreted IL-4 as an anti-inflammatory cytokine has been found to be lower expressed in the lamina propria of UC patients, supported by genetic studies that found an association between decreased levels of IL-4 and increased risk for disease development. IL-4 T cell activation occurs via the cytokine-binding receptor IL-4Rα/γ common receptor. Activity of IL-4 has been applied to mice models of T-cell receptor alpha chain-deficient mice (TCR -/-) that were treated with anti-IL-4 monoclonal antibody and showed down-regulation of Th2-type mRNA cytokine production. IL-4 has been found to have a direct relationship with IL-10, supported by evidence that states IL-4 enhances IL-10 production and vice versa.[74–76] Anti-inflammatory cytokine IL-10 is produced by monocytes, B cells, and T cells to maintain tissue

integrity and promote intestinal tissue homeostasis through its anti-inflammatory, anti-apoptotic, and tissue regeneration properties. Vasoactive intestinal peptide (VIP) contributes to the maintenance of colon epithelium by stabilizing the expression of IL-10 mRNA in regulatory B cells (Bregs). IL-10 producing Bregs in a model of DSS-induced mice were found to down-regulate Th1 polarization of spleen and mesenteric lymph nodes.[77] The role of IL-10 in gut inflammation has been studied in IL-10 deficient (IL-10 -/-) mice: loss of IL-10 promotes inflammation, as indicated by increased levels of cytokines IL-12 and interferon gamma (IFN-γ). By contrast, suppression of macrophage activation in turn inhibits the production of pro-inflammatory cytokines (e.g., IL-1, IL-6 and TNF-α).[69,78,79]

IL-6 can exhibit both pro-inflammatory and anti-inflammatory behavior. IL-6 acts as a pro-inflammatory signal via binding to soluble IL-6 receptor (sIL-6R) which activates intestinal cells. This protein can also activate anti-inflammatory behavior via promotion by T cell apoptosis and downregulation of primary pro-inflammatory cytokines (such as TNF-α and IL-1β) and promoting the proliferation and expansion of intestinal epithelial cells (IECs) hence maintaining gut homeostasis. However, models of UC usually report that IL-6 influences pro-inflammatory behavior by induction of signal transducer and activator of transcription 3 (STAT3) hence preventing apoptosis.[80–82]

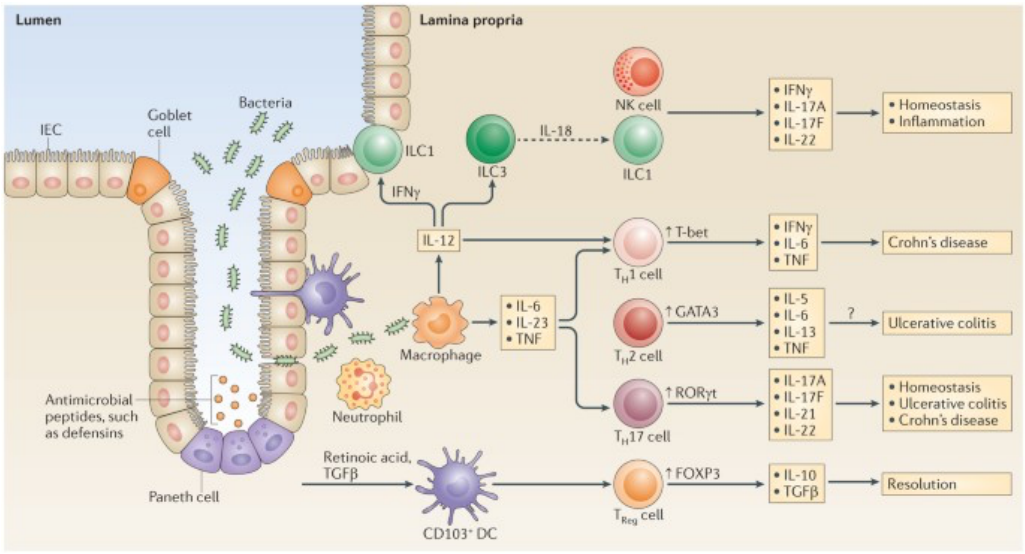

Figure 1-3. Cytokines involved in the pathogenesis of inflammatory bowel disease.[83]

Current treatment options

Subjects with UC require long-term treatment, so finding the most effective treatment option is pertinent for disease management while ensuring little to no impact on the quality-of-life of the patient.

Sulfasalazine (5-ASA), given orally or rectally, is prescribed for IBD management with expected remission rates of 50%, but this medication has high rates of intolerance among patients, causing milder side effects including nausea, abdominal pain, skin rashes or more serious effects such as hepatitis, systemic lupus, or reduced sperm counts. The addition of glucocorticoids (corticosteroids), orally or rectally, is usually prescribed if remission does not occur within two weeks of sulfasalazine usage but cannot be used to maintain remission. Sulfasalazine has been shown to cause both low sperm count and infertility in men, as well as hereditary malformations in offspring.

Thiopurine (an immunomodulator) or anti-TNF agents can be added if subjects do not respond to glucocorticoids. However, thiopurine has been linked to causing hepatoxicity, weight loss, alopecia, anorexia, fatigue, and other adverse effects causing up to a third of patients to stop treatment. Infliximab is the most prescribed anti-TNF agent. The use of anti-TNF agents is associated with adverse effects such as development of hypersensitivity, tuberculosis, and lymphoma.[59,84,85]

Despite disease impact on health-related quality-of-life, 11-46% of patients choose to discontinue medication due to the adverse effects of drug treatment.[86]

Health Disparities in UC and IBD treatment

Disparities are seen in health literacy, suitable treatment, and proper diagnosis in patients of ethnicities other than non-Hispanic white. There has been little research whether there are genetic differences that impact the efficacy of IBD treatment between different ethnic backgrounds. Studies that have investigated the impact of genetic variation amongst different ethnic groups on colitis treatment efficacy have been inconclusive. A multicenter study of pediatric patients with Crohn's Disease, for e.g., from the U.S. and Canada found that African Americans were more associated with a complex disease phenotype but this data in the context of UC is not well investigated or readily available.[87,88]

Though the reasons for race-based disparities in UC treatment are not well determined, these are more often linked to socioeconomic status and healthcare coverage. Sewell et al., revealed findings from a database, collected from 1998 to 2003, African American and Hispanic UC patients were 25-50% less likely to undergo colectomy for UC. Barnes et al., revealed that having health coverage through Medicare was associated with lower colectomy rates compared to private insurance: both African Americans and Hispanics are more likely have coverage through

Medicare or Medicaid.[89,90] Nguyen et al., have reported that among UC patients that do undergo colectomy, the rates of mortality were 3.3 times higher for patients insured through Medicaid compared to those with private insurance.[91] Hospitalization rates for patients who are uninsured have been found to occur at higher rates that insured patients despite having lower rates of elective hospitalization,[92] which could make implications on the quality of medical care for patients who don't have access to afford private insurance.

There is also little to no research on whether there is specific care needed for transgender and gender nonconforming IBD patients. There is no reported 1) knowledge of drug interactions between IBD drugs and hormone therapy drugs or 2) understanding of the role gender-affirming surgeries can have on the potential development of IBD later. Schenker et al., reported two cases of transgender IBD patients, both of whom experienced life-long adverse effects as one patient had observed interactions between prescribed testosterone and their prescribed adalimumab (anti-TNF agent) and developed pancolitis in conjunction with their existing Crohn's Disease. There was no sufficient literature available to confirm this onset was cause by drug interaction but was reported that the patients associated symptoms (bloody diarrhea, abdominal pain) resolved after switching medications.[93]

Experimental Models of Cocoa and Chronic Inflammation

Dextran Sulfate Sodium-induced Mouse Model

Dextran sulfate sodium (DSS) is widely used for the induction of colitis in animal studies *in vivo*. DSS a is water-soluble, negatively charged sulfated polysaccharide. Administration of DSS to animal models via drinking water (1–5% w/v) induces colonic injury, which is characterized by ulceration, inflammation, and shortening, as well as body weight loss. The

mechanism of induction is unclear, but it is generally accepted that intestinal inflammation results in the disruption of the intestinal epithelial monolayer lining.[94–97] Studies have found that DSS-induction causes up-regulation of pro-inflammatory cytokines including, but not excluded to, IL-6, TNF-α, and IL-1β. It has been demonstrated that treatment with anti-inflammatory supplement does not always prove to down-regulate all pro-inflammatory genes equally. For example, Wagner et al., saw significant reductions in colon expression of IL-6 in sulforaphane (SFN) – an anti-inflammatory compound found in cruciferous vegetables – treated mice that were induced with 4% DSS for 7 days. Chemokine ligand 1 (CXCL1), TNF-α, IL-1β, and IL-10 all tended to reduce but were not of significant difference.[96,98] The DSS-colitis model has also provided insight on the importance of mice strain type used in animal models of induced-UC. Studies have revealed differences in UC progression between C57BL/6J and Balb/c mice. Both strains develop colitis with DSS, however, Balb/c mice can recover from sickness fully 4 weeks after removal of DSS treatment whereas C57BL/6J mice continued to exhibit disease symptoms including body weight loss and diarrhea or loose stool up to 5 weeks after DSS removal.[96,99,100]

Anti-inflammatory effects of cocoa *in vitro*

In vitro studies have shown the anti-inflammatory effects of cocoa and cocoa polyphenols. In TNF-α-stimulated Caco-2 human colon cancer cells, a model of GI inflammation, pre-treatment with cocoa polyphenol extract (CPE) (10 μg/ml, supplying 132 nmol-epicatechin, 40 nmol-catechin and 43 nmol-procyanidin B2) led to a reduction in the production of pro-inflammatory IL-8 and down-regulation of cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) enzyme expression.[21] In the same model, hexameric procyanidins were shown to suppress TNF-α-induced NF-κB activation and related pro-inflammatory signaling.[101,102]

Bitzer et al., investigated the anti-inflammatory and GI protective effects of cocoa extracts enriched for different procyanidins (monomers, oligomers, or polymers) using TNFα-stimulated HT-29 human colon cancer cells and DSS-induced Caco-2 cells, respectively. Polymeric procyanidins extracts showed the strongest anti-inflammatory response (i.e., reduction in IL-8 production) and greatest GI protective effect.[103] Finally, treatment of IL-1β-induced Caco-2 cancer cells with cocoa extracts was shown to reduce the production of nitric oxide (NO) by 1.3–fold and prostaglandin E2 (PGE (2)) secretion by 2.2–fold.[104]

Dietary cocoa for prevention and treatment of inflammation *in vivo*

Obesity

Research studies *in vivo* have investigated the effects of dietary cocoa in models of obesity hypothesizing that cocoa will ameliorate obesity-related inflammation, insulin resistance, and inset of fatty liver disease (FLD). Gu et al., found that in high-fat (HF) fed obese mice, cocoa supplementation for 10 weeks (8% diet w/w) reduced obesity-related inflammation. Plasma IL-6 (30.4%) and monocyte chemoattractant protein-1 (MCP-1; 25.2%) were reduced in cocoa-treated mice. A reduction in the expression of pro-inflammatory genes in white adipose tissue (WAT) was also observed.[12]

Gu et al., conducted another experiment in HF-fed obese mice fed a HF diet with 8% (w/w) unsweetened cocoa powder and saw decreased adipose tissue gene expression of TNF-α, IL-6, and iNOS and decreased levels of transcription factor NF-κB.[11] A double-blind, placebo-controlled, pilot clinical trial was conducted in 15 overweight subjects (with borderline criteria of metabolic syndrome) who were given a supplement of cocoa flavonoids (80 mg/day; 4 wks). The authors reported that body weight loss was higher in the subjects assigned to the cocoa

supplement (2.4 kg) when compared to the placebo group (1.7 kg), and that low density lipoprotein-associated cholesterol decreased by 17% while high density lipoprotein-associated-cholesterol increased by 17%.[11]

Diabetes

Type II diabetes (T2D) increases the risk of endothelial cell inflammation which can lead to the development of cardiovascular diseases. Hyperglycemia, or high blood sugar, associated with T2D increases inflammation through NF-κB activation and production of pro-inflammatory cytokines. With evidence that cocoa supplement can decrease markers of inflammation in models of obesity, which raises the risk factor for developing T2D, it could be hypothesized that cocoa supplementation in models of T2D could prove effective. In a model of hyperglycemia-induced arterial inflammation in Zucker diabetic fatty rats (n=16), 10% cocoa-rich diet significantly reduced the expression of IL-6 in aortic tissue, though there were no differences seen in TNF-α levels.[105] Álvarez-Cilleros et al., fed male Zucker rats (10 wks) with either standard diet or cocoa diet (10% w/w) hypothesizing that cocoa would have effect on gut microbiota and found down-regulation of pro-inflammatory cytokines TNF-α, IL-6 and MCP-1 in the colon. There was also up-regulation of tight junction protein Zonula occludens-1 (ZO-1) and mucin glycoprotein which are key in mucus production and maintenance of intestinal barrier function.[20] The evidence from the aforementioned *in vivo* models points to evidence that cocoa supplementation in a mice model of ulcerative colitis could show both preservation of colonic integrity and regulation of inflammation.

Intestinal Inflammation

In vivo studies have also reported results that support cocoa's anti-inflammatory activity in the colon. Rodríguez-Ramiro et al., reported that in an azoxymethane (AOM)-induced rat model of colon carcinogenesis, treatment with cocoa-enriched diets (12% w/w) for 8 weeks reduced NF-κB-p65 activity and suppressed COX-2 and iNOS expression in the colon.[21] Treatment with diets containing 5% and 10% cocoa were also shown to reduce colon expression of NF-κB, COX-2, and iNOS at the gene and protein level in the AOM/ DSS induced mouse model-colitis associated cancer (CAC).[22] In another study, treatment with 500 mg/kg of polyphenol-enriched cocoa extract (PCE) (orally administered) reduced colon IL-6, IL-1β, and TNF-α cytokine production in mice treated with 5% DSS for one week to induce colitis.[106]

Purpose & Significance

Incidence of UC and IBD is rising in both the United States and worldwide, with both the disease itself and the available treatment options having the potential to negatively affect patients' quality of life. In addition, UC increases the risk of developing colon cancer. Based on the studies discussed in this literature review, there is evidence that cocoa can mitigate gastrointestinal inflammation. However, little is known about the dose-dependence of the anti-inflammatory activity of cocoa. The purpose of this research was to establish the dose-dependent anti-inflammatory effects of dietary cocoa in the DSS-induced mouse model of ulcerative colitis. The results of this research, if successful, can aid in dose-selection for future human trials.

Hypothesis and Objectives

Based on the previous studies outlined in my literature review, **I hypothesize that dietary cocoa can dose-dependently reduce the development of ulcerative colitis in DSS-induced mice**.

To test this hypothesis, I propose the following objectives:

1. To determine if cocoa supplementation will dose-dependently reduce gross markers of inflammation in ulcerative colitis, including body weight loss, spleen and liver weights, and colon shortening.
2. To determine if cocoa supplementation will dose-dependently reduce molecular markers of systemic and colonic inflammation.

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Chapter 2

Materials and Methods

Materials

Diets were prepared by Research Diets, Inc. (New Brunswick, NJ) and were based on AIN93G basal diet. Unsweetened, non-alkalized cocoa powder used in diets was provided by Blommer Chocolate Co. (East Greenville, PA). Diets were matched for total energy and macronutrient content. Formulations are detailed in **Table 2-1**. All other reagents were of the highest grade commercially available.

Table 2-1. Composition of mice diets.

	AIN93G	2% Diet	4% Diet	8% Diet
Macronutrient (%*kcal*)				
Protein	15	15	15	15
Carbohydrate	76	76	76	76
Fat	9	9	9	9
Total	100	100	100	100
Energy (kcal/g)	3.8	3.8	3.7	3.6
Ingredient (g)				
Casein	140	140	140	140
L-Cystine	1.8	1.8	1.8	1.8
Corn Starch	495.7	495.7	495.7	495.7
Maltodextrin 10	125	125	125	125
Sucrose	100	100	100	100
Cellulose, BW200[1]	50	46.7	43.3	36.2
Soybean Oil	40	38	35.9	31.5
t-Butylhydroquinone	0.008	0.008	0.008	0.008
Mineral Mix S10022M	35	35	35	35

Vitamin Mix	10	10	10	10
Choline Bitartrate	2.5	2.5	2.5	2.5
Cocoa Powder[2]	0	20.3	41.2	85
Total (g)	1000	1015	1030.4	1062.7

[1]Total fiber content per diet is 5%.
[2]Cocoa powder contains 200 mg/g fiber and 100 mg/g fat.

Animals and Treatment

The mouse experiment described was approved by the Institutional Animal Care and Use Committee (IACUC #45786-1) at the Pennsylvania State University. Male C57BL/6J mice (5 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME) and were allowed to acclimate for 1 week prior to ulcerative colitis (UC) study. All mice were maintained on a 12-hour light/ dark cycle, with access to food and water *ad libitum*. Mice were randomized based on body weight into 4 treatment groups (n = 10 mice per treatment) and were housed 5 per cage into shoebox cages. Mice were maintained on control mouse diet (AIN93G with no cocoa supplementation) or AIN93G diet containing 2%, 4%, or 8% w/w cocoa powder. Mice were treated with experimental diets for 2 weeks prior to UC-induction and for the 7 days during UC-induction. To induce UC, mice were treated with 2% dextran sulfate sodium (DSS) as the sole source of drinking fluid (**Fig. 2-1**). Mice body weights were recorded weekly for the first 2 weeks, and then recorded daily the third week to monitor weight loss caused by UC-induction.

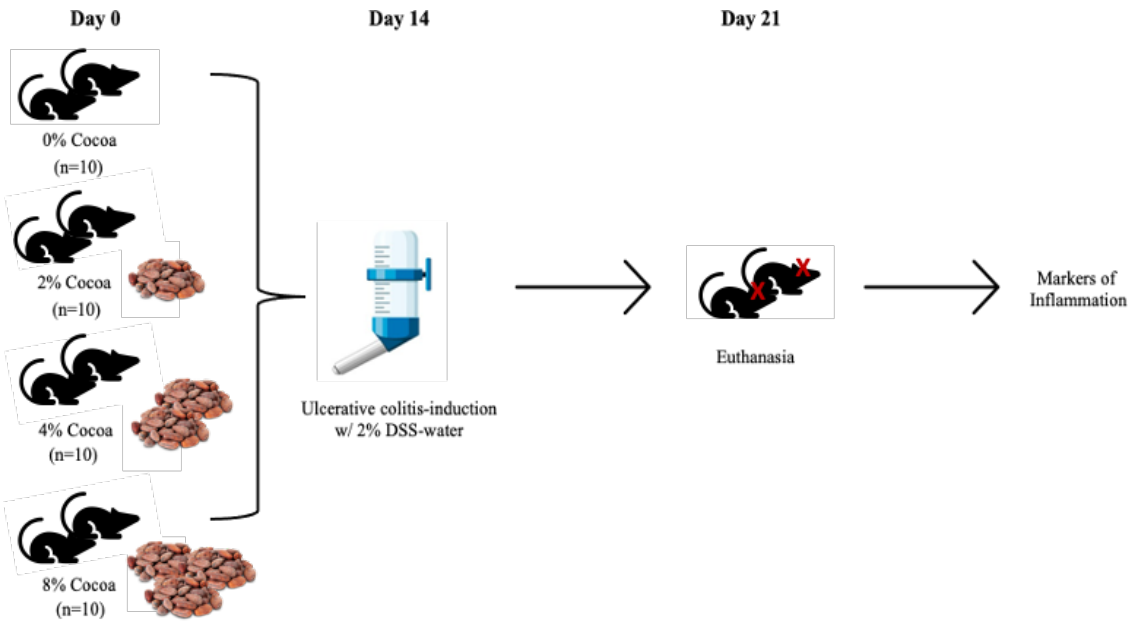


Figure 2-1. Experimental model of cocoa supplemented mice throughout the three-week period.

Blood and tissue collection

At the end of the mouse experiment, mice were euthanized via carbon dioxide asphyxiation and blood was collected via cardiac puncture. Plasma was prepared by centrifugation. Livers and spleens were harvested, rinsed with ice-cold 0.9% saline solution, and weighed. Colons were harvested, rinsed with ice-cold 0.9% saline solution, and the length was measured. Cecum and fecal material were collected. All samples were stored at -20°C prior to analysis.

Plasma Markers of Inflammation

Multiplex enzyme-linked immunoassay (ELISA) was performed to analyze plasma protein levels of a panel of inflammatory biomarkers (V-PLEX Proinflammatory Panel 1 Mouse Kit, Meso Scale Diagnostics, Rockville, MD). Plasma levels of interferon-gamma (IFN-γ), keratinocyte chemoattractant (KC)/human growth-regulated oncogene (GRO), TNF-α, and IL-1β, -2, -4, -5, -6, -10, -12p70 were determined according to the manufacturer's protocol.

RNA Isolation and Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

Total ribonucleic acid (RNA) was extracted using the PureLink RNA Mini Kit (Thermo Fischer Scientific) according to the manufacturer's protocol, with modifications to sample homogenization: colon tissue lysis (600 µL Lysis buffer with 6 µL 2-mercaptoethanol) was performed on ice. Colons were measured (1.5 cm) and cut with sterile equipment in an RNase-free work area. Colon segments were placed into Navy Eppendorf RNA Lysis bead homogenization tubes (Next Advance, Inc., Troy, NY) and homogenized in a Next Advance Bullet Blender® Homogenizer for 5-10 minutes until samples were fully homogenized. All surfaces and materials were decontaminated with Rnase-away. Protocol modifications were also made to RNA isolate collection: 50 µL of Rnase-free water were added to recovery tubes and allowed to incubate for 5 min at room temperature before centrifugation.

RNA was quantified using a Nanodrop OneC UV-Vis Spectrophotometer (Thermo Fischer Scientific). Messenger RNA (mRNA) was reverse-transcribed to complementary DNA

(cDNA) using an RT2 HT First Strand Kit (Qiagen) and the cDNA yield was quantified with a Nanodrop OneC UV-Vis Spectrophotometer (Thermo Fischer Scientific).

Quantitative PCR was performed using a QuantStudio™ 3 Real-Time PCR System (Applied Biosystems). The reaction mixtures consisted of 12.5 µL RT2 SYBR Green ROX qPCR Mastermix, 0.2 µL per forward and reverse primers (**Table 2-2**, 100 µM in IDTE buffer, pH = 8.0), cDNA (1 µg/µL), and Rnase-free water in a 96-well plate. PCR reaction plates were centrifuged for 1 min at 1000 x g (20°C). PCR reactions were incubated at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec and annealing/extension at 60°C for 1 min. Data was recorded and analyzed with Sequence Detector Software (Applied Biosystems). Relative gene expression was determined using the $2^{(-\Delta\Delta Ct)}$ method, where ΔCt = (Ct, target – Ct, reference), with *Actb* as the reference gene and ΔΔCt = (ΔCt, target – Avg ΔCt, control diet).

Table 2-2. Primer sequences for qRT-PCR.

Gene	Product Name	Primer Sequence (5' → 3')
Actb	Beta actin	F: TGT TAC CAA CTG GGA CGA CA R: CTG GGT CAT CTT TTC ACG GT
Il1b	Interleukin 1β	F: GCT GAA AGC TCT CCA CCT CA R: GGC CAC AGG TAT TTT GTC GT
Il6	Interleukin 6	F: CTT CCA TCC AGT TGC CTT CTT G R: AAT TAA GCC TCC GAC TTG TGA AG
Il10	Interleukin 10	F: GCT CTT ACT GAC TGG CAT GAG R: CGC AGC TCT AGG AGC ATG TG
Tnfa	Tumor necrosis factor α	F: CAA ATG GCC TCC CTC TCA T R: CTC CTC CAC TTG GTG GTT TG

Statistical Analysis

Data are presented as mean ± standard deviation (SD). Two-way analysis of variance (ANOVA) with Tukey's post-hoc test was used for body weight comparisons. One-way ANOVA with Tukey's post-hoc test was used for all other data comparisons. Principal Components Analysis (PCA) was used to determine the overall influence of cocoa treatment on the UC-induced mouse model (physiological, cytokine, and gene expression). A PCA biplot was generated to observe the variables that drove the separation amongst the treatment groups. A P-value less than or equal to 0.05 was classified at statistically significant. Analyses were performed using GraphPad Prism 9.0 (San Diego, CA) and Minitab statistical software (State College, PA). PCA was performed using R statistical software (Vienna, Austria), with code presented in **Appendix I**.

Chapter 3

Results

Effects of Cocoa Treatment (CT) on Physiological Parameters

Cocoa treatment prior to induction of colitis had no significant effect on body weight (**Fig. 3-1**). After induction of colitis, cocoa treatment led to a dose-dependent decrease in final body masses compared to control-fed mice. The final body weight of mice treated with 8% w/w cocoa was 11.2% lower than control-fed mice ($p < 0.05$).

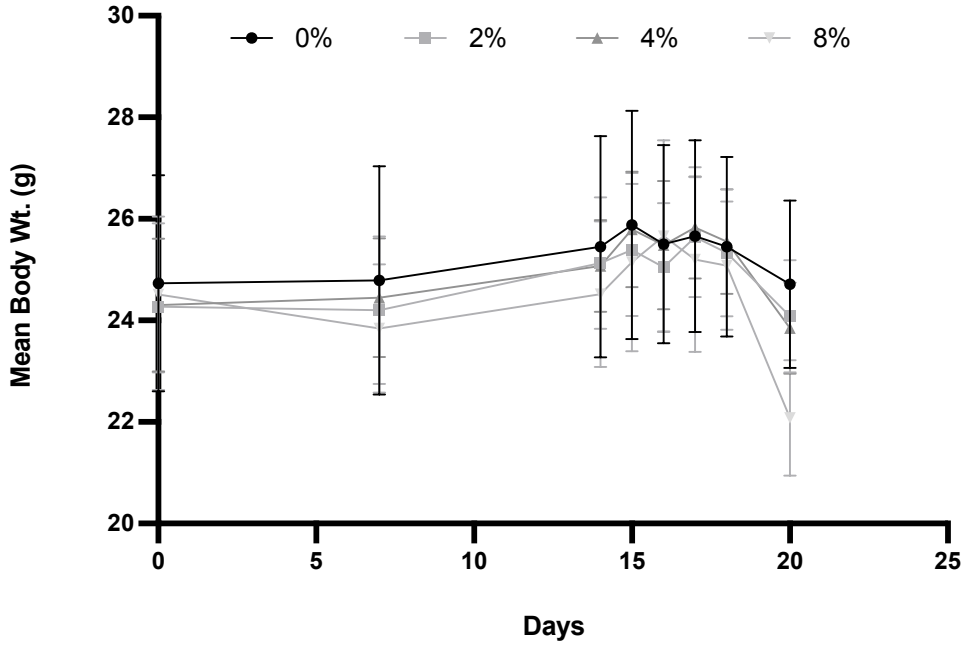


Figure 3-1. Effects of cocoa diet on male C57BL/6J DSS-induced mice. Mean body weights were determined throughout the 3-week study. Data represents the mean ± SD (n = 40). Body weight was higher in all groups compared to 8% cocoa-treated mice ($p < 0.05$) at the 21-day mark.

The spleen and liver weights and colon length were measured. Spleen weight was reduced in all CT groups compared to the control-fed group ($p < 0.05$, **Fig. 3-2**). Cocoa treatments reduced liver weights in a dose-dependent manner. Liver weights in 8% w/w CT mice were 22.9% ($p < 0.05$) lower in mass compared to control-fed mice. There was no significant impact of cocoa supplementation on colon length.

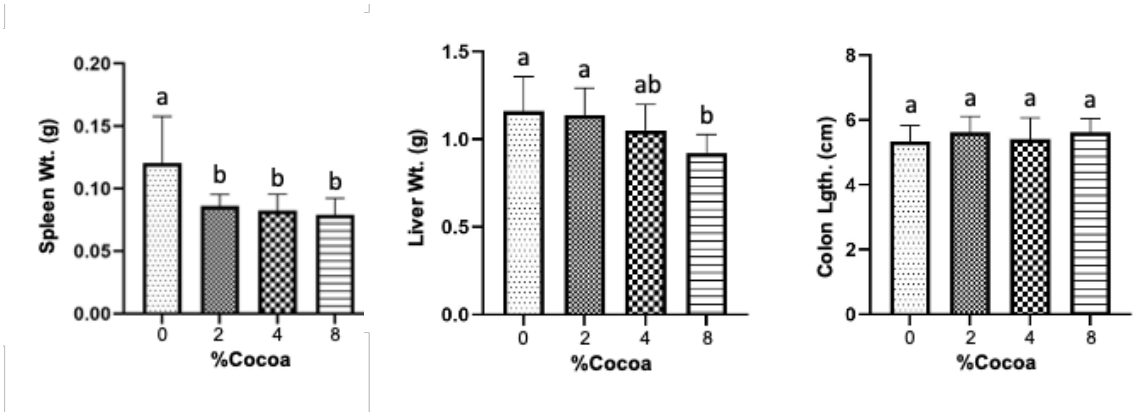
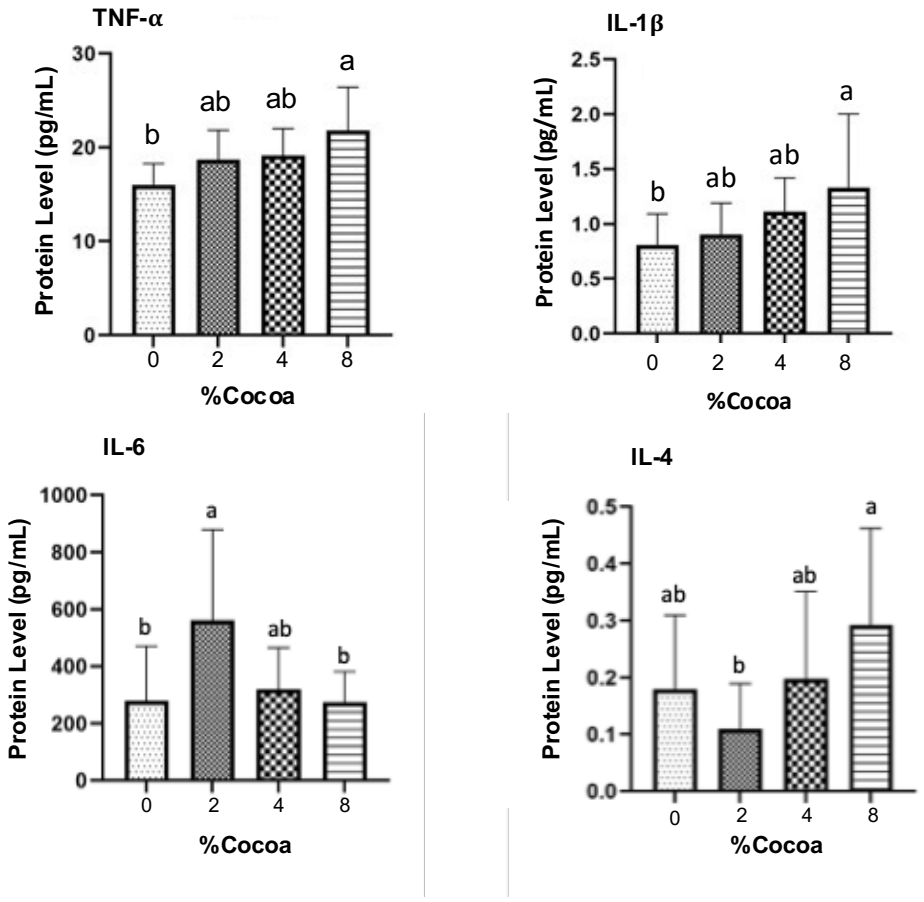


Figure 3-2. Effect of cocoa supplementation on spleen weight, liver weight, and colon length. Data is expressed as mean ± SD. Values that do not share a common superscript letter are statistically different ($p < 0.05$) by one-way ANOVA with Tukey's post-hoc test.

Effect of CT on Plasma markers of Inflammation in DSS-Treated Mice

Protein levels of a panel of inflammatory cytokines were measured in plasma. Plasma levels of TNF-α and IL-1β were significantly increased (1.6–fold and 1.4–fold, respectively) in 8% w/w CT mice ($p < 0.05$, **Fig. 3-3**). These changes appear to be dose dependent. Plasma levels of CXCL1and IFN-γ also appeared to be increased by cocoa treatment in a dose-dependent fashion, but the results were not statistically significant. Plasma IL-6 levels were elevated in the 2% w/w CT mice when compared to the control while plasma levels of IL-4 showed no

significant effect of cocoa treatment when compared to the control-fed group. Plasma levels of IL-2, IL-5, IL-10, and IL-12p70 were not significantly affected by cocoa treatment.



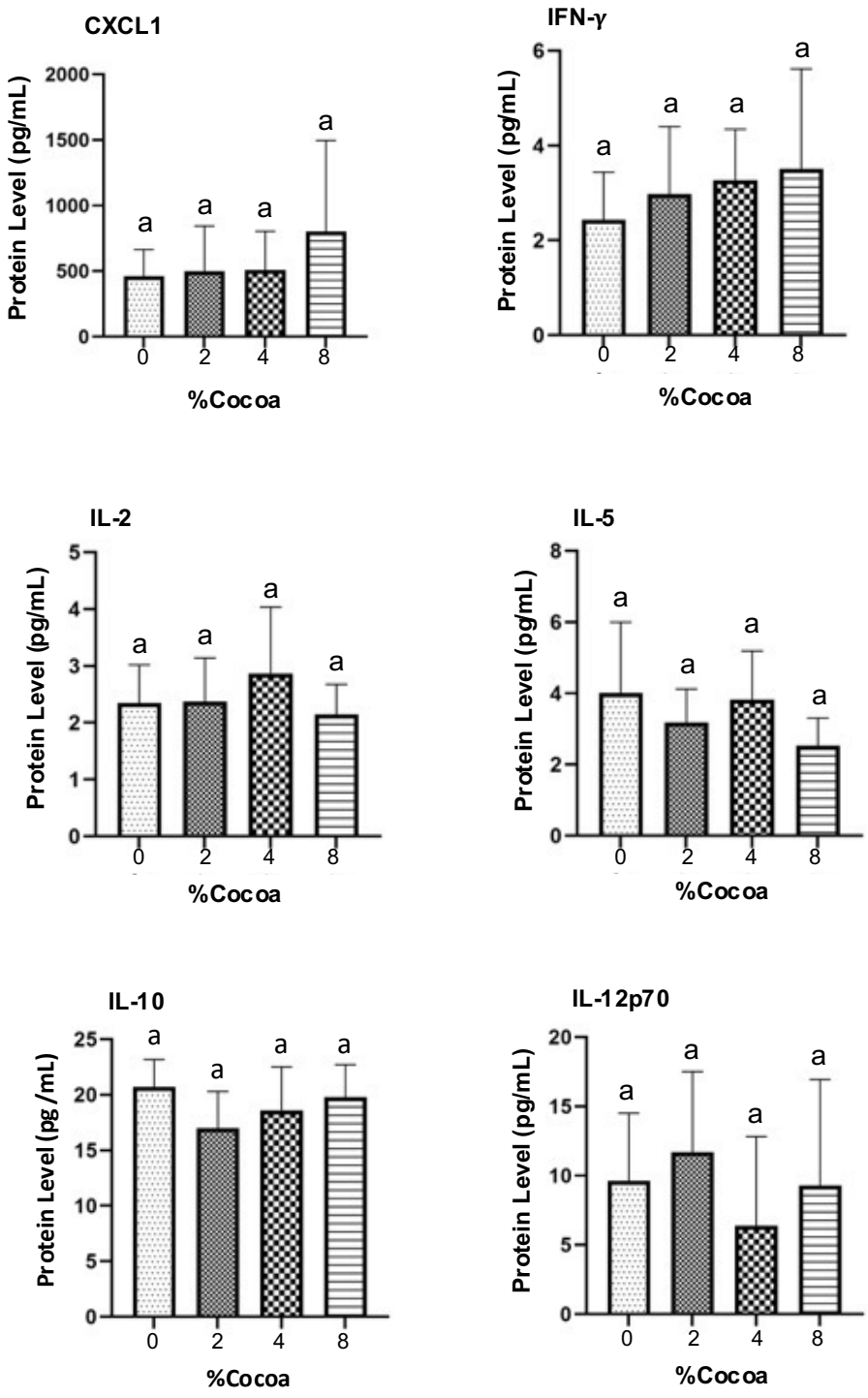


Figure 3-3. Effect of cocoa supplementation on plasma cytokines. Data is expressed as mean ± SD. Values that do not share a common superscript letter are statistically different ($p < 0.05$) by one-way ANOVA with Tukey's post-hoc test.

Impact of CT on Genes Related to Colonic Inflammation in DSS-Treated Mice

Colonic expression of a panel of inflammatory genes was measured (**Table 3-1**). There was no significant effect on any of the genes examined. Colonic mRNA expression of genes of tumor necrosis factor alpha and interleukin 1 beta (*Tnfa* and *Il1b*) was higher in the 8% w/w CT mice versus control-fed mice, with *Il1b* expression having more than a 2–fold increase. However, these changes were not statistically significant. Interleukin -6 and -10 (*Il6* and *Il10*) expression displayed similar behavior with *Il6* having more than a 3–fold increase and *Il10* having a 1.5–fold increase when compared to the control-fed mice. However, these changes were not statistically significant.

Table 3-1. Effect of cocoa supplementation on colonic gene expression of *Tnfa*, *Il1b*, *Il6*, and *Il10*. Data is expressed as mean ± SD. Values that do not share a common superscript letter are statistically different ($p < 0.05$) by one-way ANOVA with Tukey's post-hoc.

Gene	Overall P-value	Control	2% cocoa treatment	4% cocoa treatment	8% cocoa treatment
Tnfa	0.544	1.000^{a} (0.464)	1.682^{a} (2.608)	0.822^{a} (0.524)	1.096^{a} (0.609)
Il1b	0.117	1.000^{a} (0.862)	1.085^{a} (1.682)	0.525^{a} (0.476)	2.432^{a} (2.992)
Il6	0.205	1.101^{a} (1.151)	1.850^{a} (1.884)	1.256^{a} (1.136)	3.860^{a} (5.680)
Il10	0.180	1.000^{a} (1.737)	0.245^{a} (0.286)	0.284^{a} (0.221)	1.485^{a} (2.029)

Principal Components Analysis (PCA) of Gross and Biological Data

Principal components 1 and 2 (PC1 and PC2, respectively) combined of the overall PCA explains ~40% of the variation seen in amongst the data (**Fig. 3-4**). The loading plot shows the

relationship of response variables (vectors) throughout both these dimensions, and the way in which variables influence this treatment behavior based off vector angles and their correlations to either PC1 (x-axis) or PC2 (y-axis). The score plot demonstrates how data point clustering behavior correlates to the observed separations seen amongst treatments. Ellipses represent 95% confidence intervals (CI) with average mean values located in the center. Ellipses that overlap with one another are not statistically different ($p < 0.05$). It can be seen from the score plot that there is no overlap between the control-fed mice and mice given 8% w/w cocoa diet, indicating a statistically significant difference between treatment groups. As seen in the loading plot, the control group is driven by gross markers (spleen, liver, and body weight) and plasma IL-5. This also shows a positive correlation between these variables marked by the proximity of the vectors, meaning all variables follow the same trend (an increase or decrease in value with one another). The 8% w/w group is driven my plasma markers IL-1β, TNF- α, and CXCL1 (KC GRO). This separation can be visualized in the score plot.

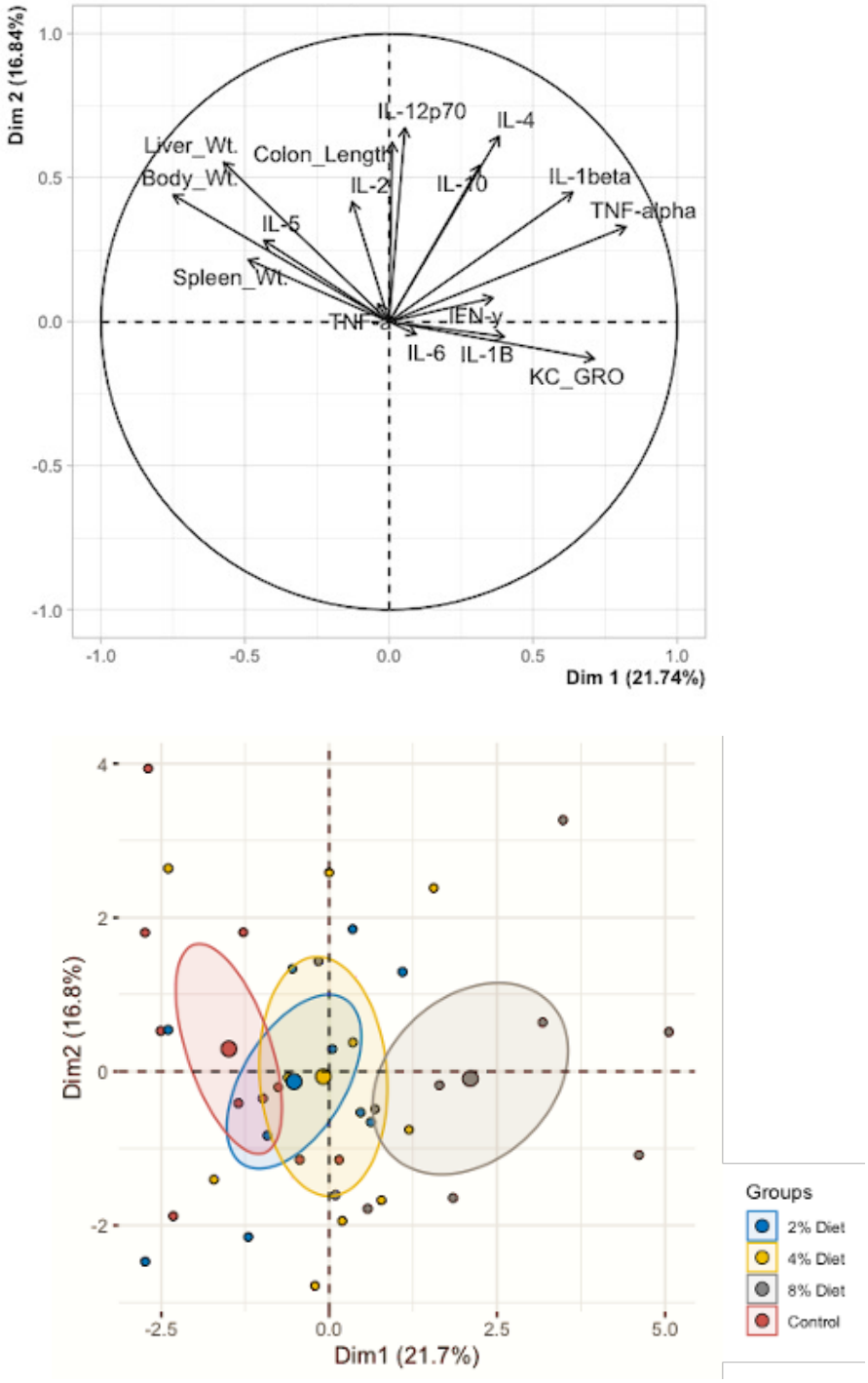


Figure 3-4. Loading plot for overall principal components analysis (PCA) (top) and score plot of PCA showing the distribution of treatment behavior (bottom).

Chapter 4

Discussion

In the present study, the ability of cocoa to mitigate inflammation in the DSS-induced mouse model of UC was investigated. Based on previous studies on the anti-inflammatory effects of cocoa in other models, it was hypothesized that cocoa supplement would dose-dependently reduce gross and molecular markers of colonic and systemic expression of pro-inflammatory biomarkers. Male C57BL/6J mice were pre-treated with 0–8% dietary cocoa for 2 weeks prior to a 1-week DSS-induction.

After 1 week of DSS treatment, mice in all treatment groups lost body weight by the end of the study, as expected based on previous studies with the DSS models. Similar body weight reduction was reported previously in a study by Rodríguez-Ramiro et al., who reported that rats in a model of azoxymethane (AOM)-induced colon carcinogenesis that were fed 12% cocoa diet for 8 weeks showed a 10% loss of body weight[21], when compared to control group. In the present study, the mice weight was significantly reduced in the 8% w/w cocoa-treated group by 11.2% ($p < 0.05$).

In the present study, a reduction in both the liver and spleen weight were observed. The reduction was statistically significant for the spleen mass at all cocoa doses, but only significant at the 8% treatment level for the liver. Increased spleen and liver weights are gross indicators of inflammation.[107] The findings with spleen weight are in agreement with the spleen weight reductions seen with Saadatdoust et al., in their model of AOM/DSS-induced CAC in mice treated with 5% and 10% cocoa.[108] The impact on liver weight has not previously been reported.

Colon shortening is a gross marker of colonic inflammation. In the present study, no effect of cocoa on colon length was observed. Previous studies on cocoa's anti-inflammatory

capacity in a model of UC are limited. Studies in animal models of colon carcinogenesis that have examined cocoa's effect on colon shortening have yielded with some reporting inhibition of colon shortening was seen[106,108,109] while other reports stated that no significant changes were observed.[13,21] Pérez-Berezo et al., reported that treatment of DSS-induced rats with 5% cocoa-enriched diet had no impact on colon shortening.[13] Similarly, Rodríguez-Ramiro found that dietary treatment of AOM-induced-colon carcinogenesis in rats with 12% cocoa for 8 weeks had no significant effect on colon shortening.[21] By contrast, Saadatdoust et al., reported that treatment of AOM/DSS-induced mice with 5% and 10% cocoa for 8-weeks mitigated colon shortening.[108] The lack of consistent findings could be the result of several factors including study-related differences in 1) degree of induced inflammation severity, 2) origin and process history of the cocoa used, 3) varying bioavailability of treatment, 4) animal model, or 5) treatment length and dose.

DSS treatment has been shown to elevate markers of inflammation in the plasma and colon.[13,106] Though Pérez-Berezo saw no effects on colon shortening or improved colitis in mice, cocoa diet in this DSS model reduced serum levels of TNF-α and trended to reduce colonic oxidative activity, signified by reduced iNOS activity and TBARS production in the colon. Similarly, Andújar saw reduced DSS-induced colitis progression in cocoa-fed DSS-treated mice as decreases were seen in production of NO, phosphor-STAT3 (pSTAT3), pSTAT1α, and COX-2 in the colon.[106] However, in the present study, it was found that cocoa supplementation elevated markers of inflammation. Plasma levels of TNF-α and IL-1β were dose-dependently increased. Plasma levels of CXCL1 and IFN-γ also appeared to increase dose-dependently, but the change was not significant. Colonic gene expression shows that *Tnfa*, *Il1b*, and *Il6* levels tended to increase within the 8% cocoa-treated mice but the differences were not significant. PCA was used to gain an overall picture of the changes caused by cocoa in this model and the factors that drove those changes. The overall PCA explains ~40% of the variation seen between the different cocoa

treatments. **Figure 3-4** shows the distribution of physiological and biological measures and influence on treatment behavior and shows a clear separation between the control and 8% treatment groups. The results of the PCA shows that protein levels of TNF-α have the highest correlation in the first dimension, followed by IL-1β and CXCL1. Physiological data is the driver of separation for the effect seen of the control group. TNF-α and IL-1β significantly drove separation between the control and 8% treatment which is in agreement with data trends seen with the 8% cocoa supplement in this present study. The results of the present study contradict the original hypothesis that cocoa has dose-dependent anti-inflammatory activity and indicate instead that cocoa worsens DSS-induced inflammation in mice.

Other studies investigating the beneficial effects of antioxidant foods in DSS-induced colitis have hypothesized that they would exert anti-inflammation and found pro-oxidative behavior at higher doses of treatment. For example, Murakumi et al., found that DSS-induced mice treated with green tea polyphenols (0.5 and 1%) exhibited increased oxidative stress and elevated levels of serum creatine, indicating liver damage. The pro-oxidative behavior was thought to be associated with catechol structure or generation of excess reactive oxygen species through formation of a reactive electrophilic quinone intermediate.[110]

In another study, C57BL/6J mice were treated with diet containing 10% flaxseed oil for 3 weeks before induction with DSS.[111] Similar to the present study, an increase in inflammatory markers (IL-6 and IL-1β) was observed. A potential mechanism of action was thought to be interactions between flaxseed oil bioactives. This claim was supported by a study conducted by Kolar et al., who treated mice colonocyte (colonic epithelial cells) with docosahexaenoic acid (DHA, a polyunsaturated fatty acid derived from fish oil) and butyrate (a short chain fatty acid) and discovered that DHA and butyrate combined increased colonocyte apoptosis, suggesting this treatment was detrimental to mucosal integrity, inducing damage to mucosal lining while also preventing wound healing.

In the present study, the mechanism of enhanced inflammation was not studied. It could be due to the high doses of polyphenols, but it may also be due to interactions between cocoa polyphenols and other dietary components including dietary fiber. Cellulose is the major fiber in the mouse diets used in this study. Polyphenols are known to bind to cellulose fibers, entrapping these bioactive components. This polyphenol–fiber bind creates additional opportunity for unabsorbed polyphenols to reach the colon, where they can potentially help exert anti-inflammatory action.[112,113] There is a possibility that cocoa polyphenols that bind to the insoluble fiber cellulose during digestion could present a mechanism of cocoa's antioxidant action in the colon. The cocoa treatment formulation of this present study decreased total cellulose content with increasing cocoa powder content of the diets. It could be hypothesized that decreased cellulose fiber content in the cocoa diets could be correlated to a lower portion of polyphenols reaching the colon resulting in a reduced capacity of cocoa's protective effects locally in the colon and systemically. As the mechanism of cocoa polyphenol absorption and metabolism is not fully understood, complex ingredient interactions require further investigation.

The present study demonstrated for the first time the cocoa powder supplementation increases colonic mRNA expression of inflammatory genes *Tnfa*, *Il1b*, *Il6*, and anti-inflammatory gene *Il10*, and exacerbates DSS-induced inflammatory bowel disease. The study has several strengths. A dose-response model was employed which helps determine that the expression of inflammatory genes and proteins increased dose-dependently. The dose-response design mimics human relevant doses as 8% w/w cocoa diet is equivalent to 8 tbsp (~59g), or 2 to 4 cups of hot chocolate as one packet of hot cocoa powder can weight 15 to 30g[114]). The cocoa powder used in the mice diets in this study is a commercially available product. This is beneficial because using a commercially available product increases the applicability of the study. Lastly, this study benefits from using the whole food form of cocoa powder as opposed to cocoa isolates, such as cocoa isolated procyanidins, used *in vitro* or cocoa extracts orally administered to mice. Cocoa powder

supplement resembles more closely that of what would be consumed in the human diet. Finally, the DSS-induced mouse model of colitis, and the C57BL/6J mouse strain are widely used in studies of colitis and intestinal inflammation and has been found to accurately mimic the pathogenesis of human inflammatory bowel disease.

There were also limitations to the current study as the polyphenol composition was not determined, so inflammatory behavior cannot be linked to the phenolic profile. The complexity of the macronutrient and micronutrient profile is also a limitation, and there is no information on the effect of these various food ingredients on cocoa's anti-inflammatory capacity, hence further studies are warranted. The influence of food and water consumption was also not recorded, so it cannot be determined what the influence of food versus water intake had on treatment effect. The present study was only performed with male C57BL/6J mice, and it is not known if the outcomes of this study are translatable to what would be observed in a mice model of female C57BL/6J mice. Lastly, this present study examined the effects of cocoa supplementation in a short-term three-week-long study, while other studies have supplementation periods of up to 10 weeks.[20] As ulcerative colitis is a chronic inflammatory disease further studies looking at longer-term cocoa supplementation are warranted.

In conclusion, this investigation demonstrates that cocoa supplementation does not down-regulate the development of DSS-induced ulcerative colitis. Cocoa was found to exacerbate DSS-induced colitis, in contrast from the aforementioned studies[22,106,108,109] discussed in this paper that demonstrated cocoa's ability to improve gross and biological markers of inflammation in other models of chronic inflammation, including obesity and diabetes. Based on the findings of this study, cocoa does not improve colonic inflammation and colonic injury that is associated with ulcerative colitis of inflammatory bowel disease.

Chapter 5

Conclusions and Future Work

Conclusions

The overall hypothesis of this study was that dietary cocoa can dose-dependently reduce the development of ulcerative colitis in DSS-treated mice. This hypothesis was tested using the following objectives.

a. Determining if cocoa supplementation could reduce gross markers of inflammation.

Dietary treatment with cocoa two weeks prior to and during induction of UC with DSS had no effect on the colon shortening compared to DSS-treated control mice. All cocoa-treated mice had significant decreases in total body weight compared to the DSS-treated controls as a symptom disease induction. Spleen weights were shown to have significant decreases in all cocoa treated groups compared to DSS-treated control mice, while liver weights were only decreased in the 8% w/w cocoa diet group. These results show that cocoa treatment reduced inflammation in the liver and spleen, but not in the colon.

b. Determining if cocoa supplementation could reduce systemic markers of inflammation.

Plasma TNF-α and IL-1β levels were dose-dependently increased by cocoa treatment. Plasma levels of CXCL1 and IFN-γ tended to increase with cocoa treatment in a dose-dependent manner, but these changes were not significant. Plasma levels of IL-6, IL-4, IL-2, IL-5, IL-10,

and IL-12p70 were not affected by cocoa treatment. These results show that cocoa failed to improve, and in fact worsened systemic inflammation in DSS-induced mice.

c. Determining if cocoa supplementation could reduce mRNA expression of pro-inflammatory genes in the colon.

It was shown that cocoa-treated mice were not significantly different from control mice in terms of pro-inflammatory gene expression in the colon but, mice in the 8% treatment group tended to have increased expression of some genes.

Overall analysis of the data using PCA displayed separation between the control and 8% treatment group behaviors that was driven by gross markers and serum levels of TNF-α, IL-1β, and CXCL1, respectively. The results of this work show that cocoa supplementation failed to reduce chronic colon inflammation in a mouse model of induced-ulcerative colitis. Further studies are needed to determine if these results translate to other models of UC and IBD.

Future Studies

The apparent pro-inflammatory effects of cocoa powder supplementation in the context of UC which were found in this work need to be examined further. In the current study, only the short-term effects of cocoa supplementation were examined, but as inflammatory bowel disease is a chronic, life-long disease a longer-term study is warranted. Pre-treatment with cocoa could occur for approximately 4 weeks with a 4-week induction/ treatment period for a total 8-week study. This will provide further evidence to clarify if cocoa promotes pro-inflammatory response or determine if the timeframe of the present study was too short for cocoa to exert anti-

inflammatory effects. Increasing the sample size of the treatment groups (n=15-20) would increase statistical power.

Future studies of induced UC where cocoa treatment form is varied could determine if the type of treatment has any effect of bioavailability and efficacy of treatment which could look like cocoa treatment administered as cocoa beverage, cocoa-enriched diet, and cocoa extract.

Evaluating the cocoa fractions of the cocoa diets would be an additional analysis to perform on the cocoa diets used in further studies to discover if a relationship exists between differences in cocoa polyphenol composition and observed treatment effect. An extension of the study could be a model of DSS-induced ulcerative colitis of 4% cocoa-enriched diets examining if treatment effect differs between diets made with the three different dominant cocoa cultivars (*Forastero*, *Criollo*, and *Trinitario*), with HPLC analysis used to determine cocoa phytochemistry and the extent to which phytochemistry predicted changes in inflammatory protein and gene expression. This study could provide insight as to whether 1) treatment effect is influenced by cocoa breed and polyphenol composition and 2) cocoa supplement will upregulate or downregulate systemic and colonic inflammation. Additional short-chain fatty acid (SCFA) analysis on mice fecal samples in future studies would provide insight into colitis severity development as presence of SCFAs, namely acetate, propionate, and butyrate,[115] will indicate whether intestinal homeostasis is maintained or deteriorated with cocoa diet treatment.

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Appendix: R Code for PCA Analysis of the Effect of Cocoa on DSS-induced Colitis

```
# PCA on Kiana's mouse data
# 10 mice each in one of 4 treatments
# control, 2%, 4%, and 8%

# set working directory
library(readxl)
kiana.d <- read_excel("PCA.xlsx",sheet="TMT")
head(kiana.d)
summary(kiana.d)
str(kiana.d)

#convert into dataframe and set factors
kiana.d$TMT <- as.factor(kiana.d$TMT)
kiana.d$Treatment <- as.factor(kiana.d$Treatment)
kiana.d$Cage <- as.factor(kiana.d$Cage)
kiana.d$Mouse <- as.factor(kiana.d$Mouse)
kiana.d$Animal <- as.factor(kiana.d$Animal)

kiana.d <- as.data.frame(kiana.d)
str(kiana.d)
rownames(kiana.d) <- kiana.d$Animal
kiana.d
# run PCA using FactoMineR and factoextra packages
# follow http://www.sthda.com/english/articles/31-principal-component-methods-in-r-practical-guide/112-pca-principal-component-analysis-essentials/
library(FactoMineR)
library(factoextra)

# run PCA on variables, using TMT as grouping variable
# run withouth ID and Treatment columns
# run as correlation matrix
kiana.pca <- PCA(kiana.d[,-c(2,4,5)], quali.sup = 1:2,
                 scale.unit = TRUE,
                 graph = TRUE)

# Eigenvalues and scree plot
fviz_eig(kiana.pca, addlabels = TRUE)  # keep 4 dimensions for 59% expl. variance

# which variables are sign. correlated to PC 1 through PC4
kiana.dimdesc <- dimdesc(kiana.pca, axes = 1:4)
```

```
# PC1
kiana.dimdesc$Dim.1
# TNF-alpha, KC_GRO, IL-1beta, IFN-y, IL4, IL10 all sign. pos. corr
# IL5, Spleen, Boty, Liver all sign. neg corr
# T8 sign. pos corr
# T0 sign. neg. corr

# PC2
kiana.dimdesc$Dim.2
# IL4, IL12p70, ColonL, Liver, IL10, Body, IL1b, IL2, IL5  all sign. pos. corr

#contribution of each var to PCs for first 5 PCs
library("corrplot")
corrplot(kiana.pca$var$contrib, is.corr=FALSE)

# color individual mice by treatment
# PC 1 and PC 2
PCA_biplot <- fviz_pca_ind(kiana.pca,
        axes = c(1,2),
        geom.ind = c('text', 'point'),
        labelsize = 3,
        col.ind = kiana.d$Cage,
        palette = 'jco',
        mean.point = FALSE,
        addEllipses = TRUE,
        ellipse.type = 'confidence',
        legend.title = "Groups")

install.packages("DescTools")
library(DescTools)


#If you use 'custom function', you don't need this lines
#kiana.pca_T0 <- kiana.pca
#kiana.pca_T0$ind$coord <-
kiana.pca_T0$ind$coord[rownames(kiana.pca_T0$ind$coord) %like any% c("C5%", "C6%"),]
#kiana.pca_T0$ind$cos2 <- kiana.pca_T0$ind$cos2[rownames(kiana.pca_T0$ind$cos2)
%like any% c("C5%","C6%"),]
#kiana.pca_T0$ind$contrib <-
kiana.pca_T0$ind$contrib[rownames(kiana.pca_T0$ind$contrib) %like any% c("C5%","C6%"),]
#PCA_biplot_C5 <- fviz_pca_ind(kiana.pca_T0,
#                  axes = c(1,2),
#                  geom.ind = c('text', 'point'),
#                  labelsize = 3,
#                  palette = 'jco',
#                  col.ind = kiana.d$Cage[kiana.d$Cage %like any% c("C5%", "C6%")],
#                  mean.point = FALSE,
#                  addEllipses = TRUE,
```

```
        #                   ellipse.type = 'confidence',
        #                   legend.title = "Groups")

        #Make custom function to create biplot in each Treatment group##
        PCA_subset_fviz_PCA_biplot <- function(PCA_object, Treatment_group){
          if(Treatment_group == "T0"){
            cage_names = c("C5%", "C6%")}
          if(Treatment_group == "T2"){
            cage_names = c("C2%", "C3%")}
          if(Treatment_group == "T4"){
            cage_names = c("C1%", "C8%")}
          if(Treatment_group == "T8"){
            cage_names = c("C4%", "C7%")}
          require(ggplot2)
          require(ggpubr)
          group_PCA <- PCA_object
          group_PCA$ind$coord <- group_PCA$ind$coord[rownames(group_PCA$ind$coord)
%like any% cage_names,]
          group_PCA$ind$cos2 <- group_PCA$ind$cos2[rownames(group_PCA$ind$cos2)
%like any% cage_names,]
          group_PCA$ind$contrib <-
group_PCA$ind$contrib[rownames(group_PCA$ind$contrib) %like any% cage_names,]
          biplot_group_PCA <- fviz_pca_ind(group_PCA,
                                   axes = c(1,2),
                                   geom.ind = c('text', 'point'),
                                   labelsize = 3,
                                   palette = 'jco',
                                   col.ind = kiana.d$Cage[kiana.d$Cage %like any% cage_names],
                                   mean.point = FALSE,
                                   addEllipses = TRUE,
                                   ellipse.type = 'confidence',
                                   legend.title = "Cage")
          biplot_group_PCA <- biplot_group_PCA + guides(shape="none")

          pc12 <- fviz_pca_biplot(group_PCA,
                              axes = c(1,2),
                              geom.ind = 'point',
                              pointshape = 21,
                              fill.ind = kiana.d$TMT[kiana.d$TMT %like% Treatment_group],
                              col.ind = 'black',
                              addEllipses = TRUE,
                              ellipse.type = 'confidence',
                              # color variables by type of measure
                              col.var = factor(c(rep('expression',2),
                                                 rep('multiplex',10),rep('weight-length',4))),

                              legend.title = list(fill = "Treatment", color = 'Measures'),
                              repel = TRUE,
                              ggtheme = theme_classic2(),
```

```
                        title = NULL
            ) +
              ggpubr::fill_palette('jco') +
              ggpubr::color_palette('lancet')
            return(pc12)
            }

          PCA_biplot_T0 <- PCA_subset_fviz_PCA_biplot(kiana.pca, "T0") + ggtitle("Treatment
group = T0")
          PCA_biplot_T2 <- PCA_subset_fviz_PCA_biplot(kiana.pca, "T2") + ggtitle("Treatment
group = T2")
          PCA_biplot_T4 <- PCA_subset_fviz_PCA_biplot(kiana.pca, "T4") + ggtitle("Treatment
group = T4")
          PCA_biplot_T8 <- PCA_subset_fviz_PCA_biplot(kiana.pca, "T8") + ggtitle("Treatment
group = T8")

          #Combine four plots into one object
          library(cowplot)
          plot_grid(PCA_biplot_T0, PCA_biplot_T2, PCA_biplot_T4, PCA_biplot_T8,
                labels = c("A", "B", "C", "D"), label_size = 12)

          ##Making biplot with elipses for each cages
          PCA_subset_fviz_cages_biplot <- function(PCA_object, Treatment_group){
            if(Treatment_group == "T0"){
              cage_names = c("C5%", "C6%")}
            if(Treatment_group == "T2"){
              cage_names = c("C2%", "C3%")}
            if(Treatment_group == "T4"){
              cage_names = c("C1%", "C8%")}
            if(Treatment_group == "T8"){
              cage_names = c("C4%", "C7%")}
            require(ggplot2)
            require(ggpubr)
            group_PCA <- PCA_object
            group_PCA$ind$coord <- group_PCA$ind$coord[rownames(group_PCA$ind$coord)
%like any% cage_names,]
            group_PCA$ind$cos2 <- group_PCA$ind$cos2[rownames(group_PCA$ind$cos2)
%like any% cage_names,]
            group_PCA$ind$contrib <-
group_PCA$ind$contrib[rownames(group_PCA$ind$contrib) %like any% cage_names,]
            biplot_group_PCA <- fviz_pca_ind(group_PCA,
                                    axes = c(1,2),
                                    geom.ind = c('text', 'point'),
                                    labelsize = 3,
                                    palette = 'jco',
                                    col.ind = kiana.d$Cage[kiana.d$Cage %like any% cage_names],
                                    mean.point = FALSE,
                                    addEllipses = TRUE,
                                    ellipse.type = 'confidence',
```

```
                                legend.title = "Cage")
  biplot_group_PCA <- biplot_group_PCA + guides(shape="none")
  return(biplot_group_PCA)
 }

 PCA_biplot_cages_T0 <- PCA_subset_fviz_cages_biplot(kiana.pca, "T0") +
ggtitle("Treatment group = T0")
 PCA_biplot_cages_T2 <- PCA_subset_fviz_cages_biplot(kiana.pca, "T2") +
ggtitle("Treatment group = T2")
 PCA_biplot_cages_T4 <- PCA_subset_fviz_cages_biplot(kiana.pca, "T4") +
ggtitle("Treatment group = T4")
 PCA_biplot_cages_T8 <- PCA_subset_fviz_cages_biplot(kiana.pca, "T8") +
ggtitle("Treatment group = T8")

 #Combine four plots into one object
 library(cowplot)
 plot_grid(PCA_biplot_cages_T0, PCA_biplot_cages_T2, PCA_biplot_cages_T4,
PCA_biplot_cages_T8,
          labels = c("A", "B", "C", "D"), label_size = 12)


 ## HH .. played only with lines up to here !!! Aug-18-2021 HH
 # and did the ANOVAs (lines 125 ff)

 # PC 3 and PC 4
 fviz_pca_ind(kiana.pca,
          axes = c(3,4),
          geom.ind = 'point',
          col.ind = kiana.d$TMT,
          palette = 'jco',
          addEllipses = TRUE,
          ellipse.type = 'confidence',
          legend.title = "Groups")

 # biplot
 library(ggpubr)
 pc12 <- fviz_pca_biplot(kiana.pca,
                  axes = c(1,2),
                  geom.ind = 'point',
                  pointshape = 21,
                  fill.ind = kiana.d$Treatment,
                  col.ind = 'black',
                  addEllipses = TRUE,
                  ellipse.type = 'confidence' ,
                  # color variables by type of measure
                  col.var = factor(c(rep('expression',3), rep('weight-length',5),
                           rep('multiplex',9))))
```

```
                        legend.title = list(fill = "Treatment", color = 'Measures'),
                repel = TRUE,
                ggtheme = theme_classic2(),
                title = NULL))

        +
                ggpubr::fill_palette('jco') +
                ggpubr::color_palette('lancet')

                pc34 <- fviz_pca_biplot(kiana.pca,
                                axes = c(3,4),
                                geom.ind = 'point',
                                pointshape = 21,
                                fill.ind = kiana.d$Treatment,
                                col.ind = 'black',
                                addEllipses = TRUE,
                                ellipse.type = 'confidence',
                                # color variables by type of measure
                                col.var = factor(c(rep('expression',3), rep('weight-
length',5),rep('multiplex',9)),
                                        legend.title = list(fill = "Treatment", color =
'Measures'),
                                        repel = TRUE,
                                        ggtheme = theme_classic2(),
                                        title = NULL) +
                                  ggpubr::fill_palette('jco') +
                                  ggpubr::color_palette('lancet')

                                ggexport(plotlist = list(pc12, pc34),
                                      filename = "Kiana_PCA_biplot.pdf")



        # ANOVA
        # cage is nested in treatment
        # https://rcompanion.org/rcompanion/d_07.html

        library(lme4)
        library(lmerTest)
        tnfa1.lm <- lmer(`TNF-a` ~ TMT + (1|Cage),
                    data = kiana.d,
                    REML = TRUE)
        anova(tnfa1.lm) # NS Tmt
        rand(tnfa1.lm) # NS Cage

        il1b.lm <- lmer(`IL-1B` ~ TMT + (1|Cage),
                    data = kiana.d,
                    REML = TRUE)
```

```
anova(il1b.lm) # NS Tmt
rand(il1b.lm) # NS Cage

bw.lm <- lmer(`Body_Wt.` ~ TMT + (1|Cage),
              data = kiana.d,
              REML = TRUE)
anova(bw.lm) # NS Tmt
rand(bw.lm) # * Cage!

lw.lm <- lmer(`Liver_Wt.` ~ TMT + (1|Cage),
              data = kiana.d,
              REML = TRUE)
anova(lw.lm) # NS Tmt
rand(lw.lm) # NS Cage

sw.lm <- lmer(`Spleen_Wt.` ~ TMT + (1|Cage),
              data = kiana.d,
              REML = TRUE)
anova(sw.lm) # NS Tmt
rand(sw.lm) # * Cage!

cl.lm <- lmer(`Colon_Length` ~ TMT + (1|Cage),
              data = kiana.d,
              REML = TRUE)
anova(cl.lm) # NS Tmt
rand(cl.lm) # * Cage!

ifny.lm <- lmer(`IFN-y` ~ TMT + (1|Cage),
              data = kiana.d,
              REML = TRUE)
anova(ifny.lm) # NS Tmt
rand(ifny.lm) # NS Cage

il10.lm <- lmer(`IL-10` ~ TMT + (1|Cage),
              data = kiana.d,
              REML = TRUE)
anova(il10.lm) # NS Tmt
rand(il10.lm) # NS Cage

il12.lm <- lmer(`IL-12p70` ~ TMT + (1|Cage),
             data = kiana.d,
             REML = TRUE)
anova(il12.lm) # NS Tmt
rand(il12.lm) # NS Cage

il1b.lm <- lmer(`IL-1beta` ~ TMT + (1|Cage),
              data = kiana.d,
              REML = TRUE)
anova(il1b.lm) # NS Tmt
```

```
rand(il1b.lm) # NS Cage

il2.lm <- lmer(`IL-2` ~ TMT + (1|Cage),
               data = kiana.d,
               REML = TRUE)
anova(il2.lm) # NS Tmt
rand(il2.lm) # * Cage!

il4.lm <- lmer(`IL-4` ~ TMT + (1|Cage),
               data = kiana.d,
               REML = TRUE)
anova(il4.lm) # NS Tmt
rand(il4.lm) # NS Cage

il5.lm <- lmer(`IL-5` ~ TMT + (1|Cage),
               data = kiana.d,
               REML = TRUE)
anova(il5.lm) # NS Tmt
rand(il5.lm) # NS Cage

il6.lm <- lmer(`IL-6` ~ TMT + (1|Cage),
               data = kiana.d,
               REML = TRUE)
anova(il6.lm) # *** Tmt!!
rand(il6.lm) # NS Cage

kc.lm <- lmer(`KC_GRO` ~ TMT + (1|Cage),
              data = kiana.d,
              REML = TRUE)
anova(kc.lm) # NS Tmt
rand(kc.lm) # NS Cage

tnfalpha.lm <- lmer(`TNF-alpha` ~ TMT + (1|Cage),
                    data = kiana.d,
                    REML = TRUE)
anova(tnfalpha.lm) # *** Tmt!
rand(tnfalpha.lm) # NS Cage
```