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Exploration Into the Relationship Between Colitis and Depression: A Potential Role for the Aryl Hydrocarbon Receptor

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EXPLORATION INTO THE RELATIONSHIP BETWEEN COLITIS AND DEPRESSION: A POTENTIAL ROLE FOR THE ARYL HYDROCARBON RECEPTOR

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DEDICATION

To my Dad and to my Nanny, for in your deaths this degree was born.

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ABSTRACT

Inflammatory Bowel Disease (IBD) is a group of chronic gastrointestinal disorders with unclear etiology comprised of two defined disorders – Crohn's Disease (CD) and Ulcerative Colitis (UC). IBD leads to chronic pain, socially isolating symptoms, and an overall reduction in patient quality of life. There is currently no cure for IBD. Due to decreased mucous production and weakening of the colonic epithelial lining, gut-microbiota and their metabolites can invade the intestinal lamina propria and circulate systemically, a term known as "leaky gut". Recent studies show a relationship exists between peripheral inflammation, such as IBD, and depression. The gut-brain axis (GBA) acts as a bidirectional communication pathway between the enteric nervous system (ENS) and central nervous system (CNS), and colitis patients have an increased incidence of depression. In the current study, we investigate the relationship of UC and its impact on depressive-like symptoms in mice. Colitis was chemically induced in mice and treated with indole-3-carbinol (I3C), a naturally-occurring aryl hydrocarbon receptor (AhR) ligand our lab previously showed reduced colitis severity. Untargeted metabolomic profiles of over 200 metabolites revealed that during disease induction a potentially neurotoxic and tryptophan-kynurenine (TRP-KYN) pathway metabolite, quinolinic acid (QA), was upregulated in colitis models, but reduced after I3C treatment. As high QA levels are linked to depression, we performed tail suspension tests (TST) over the course of disease and treatment to assess depressive-like behavior in experimental mice. Our results show that colitis- induced mice had altered immobility

times, with results depending on the testing time of day, when compared to healthy controls. These altered immobility times, which are a measure of depressive-like behavior in mice, were reversed upon treatment with I3C. These results suggest that colonic inflammation can lead to altered behavior and increased depressive-like symptoms through dysregulation of the gut metabolome but may be restored after treatment with an AhR ligand like I3C. Collectively, this suggests that activation of AhR can improve colitis-induced symptoms, which include colitis-associated depressive-like behavior.

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LIST OF ABBREVIATIONS

AALAC.....Association for Assessment and Accreditation of Laboratory Animal Care

AhR.....Aryl Hydrocarbon Receptor

ANOVA.....Analysis of Variance

BBB.....Blood Brain Barrier

BDNF.....Brain Derived Neurotrophic Factor

CBD.....Cannabidiol

CD.....Crohn's Disease

CNS.....Central Nervous System

CORT.....Corticosterone

CSF.....Cerebrospinal Fluid

DLAR.....Division of Laboratory Animal Resources

DMSO.....Dimethyl Sulfoxide

DSM-5.....Diagnostic and Statistical Manual of Mental Disorders-5

DSS.....Dextran Sulfate Sodium

ELISA.....Enzyme-Linked Immunosorbent Assay

ENS.....Enteric Nervous System

FMT.....Fecal Microbiota Transplant

GBA.....Gut-Brain Axis

GF.....Germ Free

GI.....Gastrointestinal

GR ..... Glucocorticoid Receptor

GWAS ..... Genome Wide Association Studies

HPA ..... Hypothalamus-Pituitary-Adrenal

I3C Indole-3-Carbinol

IACUC ..... Institutional Animal Care and Use Committee

IBD ..... Inflammatory Bowel Disease

IDO ..... Indoleamine 2,3-Dioxygenase

IL ..... Interleukin

KATs ..... Kynurenine Aminotransferases

KMO ..... Kynurenine 3-Monooxygenase

KYN ..... Kynurenine

KYNA ..... Kynurenic Acid

LPS ..... Lipopolysaccharide

MPO ..... Myeloperoxidase

PCA ..... Principal Component Analysis

PHQs ..... Patient Health Questionnaires

QA ..... Quinolinic Acid

SEM ..... Standard Error Mean

SPT ..... Sucrose Preference Test

TCDD 2,3,7,8-Tetrachlorodibenzo-p-Dioxin

TDO Tryptophan-2,3-Dioxygenase

THC ..... Tetrahydrocannabinol

TNBS ..... Trinitrobenzene Sulfonic Acid

TNF-α.....Tumor Necrosis Factor alpha

Trp.....Tryptophan

TRP-KYN .....Tryptophan-Kynurenine

TST .....Tail-Suspension Test

UC ..... Ulcerative Colitis

CHAPTER 1

INTRODUCTION

Inflammatory Bowel Disease

Inflammatory Bowel Disease (IBD) is a group of chronic gastrointestinal disorders with unclear etiology which cause a reduction in patient quality of life and socially isolating symptoms. Importantly, IBD is a risk factor for early development of colorectal cancer (CRC) which is the third most commonly diagnosed cancer in the United States [1, 2]. It is believed that IBD recognition first emerged in the 1800s in the Western world, approximately in parallel to industrialization. By the mid-twentieth century, IBD was recognized throughout North America and Europe [3]. Since, incidence of IBD has consistently climbed with prevalence in the Western world being estimated at 0.5% of the general population [4]. Beyond the Western world, cases of IBD are increasing in developing nations – with rising prevalence on every continent. IBD has now become a global burden [5].

Colitis, a form of, IBD is comprised of two defined disorders: Crohn's Disease (CD) and Ulcerative Colitis (UC). CD can manifest in any portion of the gastrointestinal (GI) tract in a discontinuous manner, whereas UC is confined to the colon [6]. Symptomatically, both UC and CD often present with diarrhea and abdominal pain. Bloody stool and rectal bleeding are more common in UC than in CD [7]. Moreover, CD displays three broad phenotypes: inflammatory, fibrostenotic, or penetrating disease. It is currently thought that CD begins as inflammatory then progresses to fibrostenotic and penetrating

phenotypes which require non-curative surgical intervention. In contrast, UC does not present in fibrostenotic nor penetrating phenotypes and can be removed surgically through removal of the colon [8]. Lesions are common manifestations in both UC and CD, though ulcers in UC are more superficial whereas lesions in CD often penetrate deeper into the intestinal tissue layers [9]. For human diagnosis, colonoscopy is usually used to diagnose UC, while upper endoscopy is used for CD. Stool samples and blood tests can also be employed to diagnose IBD.

Though incompletely understood, there are environmental, host-lifestyle, and genetic factors that contribute to the onset of IBD. Traditionally, IBD was widely noted in the Western World and lesser in underdeveloped and Eastern nations [10]. Today, IBD has been deemed a global burden with increasing cases on all continents [5]. Population based studies have indicated that there exists a higher incidence of IBD in urban populations [4]. This could be partially due to the "hygiene hypothesis". This hypothesis states that increasing prevalence of chronic inflammatory disorders in developed nations are likely due to a changing microbial environment that has disrupted immunoregulatory circuits which usually quell immune responses [11]. Thus, rearing in a hygienic environment can negatively impact immune development and create susceptibility to immunological diseases later in life. Those in urban and developed nations are less likely to have regular contact with harmless soil bacteria or helminth infections. Thus, their immune systems may be less primed to deal with challenges, subjecting them to chronic inflammation [12].

Additionally, a recent umbrella review of meta-analyses on environmental risk factors for IBD identified several environmental or host-lifestyle factors that increased one's risk for IBD: smoking, urban living, appendectomy, tonsillectomy, antibiotic

exposure, oral contraceptive use, consumption of soft drinks, vitamin D deficiency, and non– *Helicobacter pylori* –like enterohepatic Helicobacter species infection [13]. This same study also identified factors that decreased one's risk for IBD: physical activity, breastfeeding, bed sharing, tea consumption, high levels of folate, high levels of vitamin D, and *H. pylori* infection [13]. Smoking is considered a risk factor for many diseases, but a meta-analysis by Mahid et al. found current smoking to be associated with a low risk of UC but a significantly high risk of CD. The same meta-analysis noted current smoking even had a protective effect on the development of UC when compared with controls, presenting an interesting paradigm of IBD and highlighting differences amongst CD and UC [14]. It is to be noted that many of the existing risk factors for IBD are associated with dietary intake, microbial response, and general lifestyle, thus painting the picture that IBD can be at least partially stimulated by many different aspects of the human experience.

Evidence suggests that genetics also play a role in the onset of IBDs. Genome-wide association studies (GWAS) have been conducted to specify genetic loci involved in the onset of IBDs. A 2012 GWAS found 163 genetic loci associated with IBDs. Of those 163 loci, 110 were shared by both UC and CD, 30 were CD-specific, and 23 were classified as UC-specific [15]. A meta-analysis by Liu et al. noted an additional 38 genetic loci which indicated susceptibility to IBD, bringing the total number of genetic loci associated with IBDs to over 200 [16]. Genes with the strongest associations in humans were involved in the immune response to microbes such as NOD2, IL23R, and ATG16L1 [15]. An estimated 80-90% of the specified genetic loci associated with IBDs are noncoding sequences that likely constitute epigenetic markers, noncoding RNAs, and microRNAs [6, 17]. These findings indicate that IBD has a polygenic process and is not confined to a single locus.

Furthermore, the heritability of IBD has widely been investigated by familial studies between first degree relatives. A recent population-based cohort study found the familial risk of IBD to be higher within generations (sibling-sibling) versus between generations (parent-offspring) – with the highest familial risk being between twins [18]. The coefficient of heritability, a measure of genetic influence on phenotypic variance, is estimated at 25 to 42 for CD and 4 to 15 for UC [19]. Using the coefficient of heritability as a measure in familial studies is nonoptimal in that similar environmental factors between family members are not accounted for, but nevertheless findings indicate that CD is more heritable than UC [19, 20].

In general, IBD is an inflammatory disorder characterized by a multitude of factors including microbial dysbiosis, immune modulation, autonomic dysfunction, and often psychological distress [21]. The intestinal microenvironment contains a multitude of epithelial cells and immune cells in addition the gut microbiome. Microbial dysbiosis has been widely postulated as a crucial factor for developing IBD. Microbial dysbiosis is characterized by a reduction in gut microbial diversity due to a shift in the balance of commensal versus potentially pathogenic microbiota [22, 23]. Normally, gut bacteria are located in the intestinal lumen, but this marked change in gut flora composition can lead to mucous degradation and bacterial invasion into the intestinal epithelial lining leading to increased intestinal permeability and uncontrolled immune activation [24]. Weakening of the intestinal barrier due to microbial dysbiosis leads to uncontrolled bacterial presentation to immune cells in the lamina propria. This uncontrolled immune activation occurs when gut microbiota and their metabolites invade the intestinal lamina propria and circulate systemically, a term known as “leaky gut”. Thus, while exact IBD etiology remains to be

elucidated, the current standing hypothesis is that IBDs are caused by overly active T-cell mediated immune responses to gut-microbes and their subsequent products [8]. Research has confirmed that disturbances of the intestinal barrier can lead to IBD in animal models - specifically in Muc2 deficient mice. Muc2 mutation results in lower goblet cell production of mucous and has been shown to sporadically induce colitis with depleted mucosal barrier and increased bacterial translocation [25].

Current treatment methods for IBD are certainly flawed. For severe presentations, surgical interventions are a last-chance treatment in which portions of the GI tract are removed in a risk associated surgery. Surgery may require subsequent use of colostomy bags, a highly unpreferred intervention by IBD patients due to external alteration to body image [26]. Other treatment for less severe disease include: biologic agents, immunomodulators, steroids, and antibiotics. Biologic agents using monoclonal antibody targeting of tumor necrosis factor-α (TNF-α), such as infliximab, have been a promising treatment method for IBD; however, at least one third of IBD patients are primary non-responders to TNF-α inhibitor induction therapy and effectiveness wanes over time [27]. Immunomodulators, like methotrexate, are sometimes employed along with TNF-α inhibitors. These are also flawed in that they often present severe side-effects and have a 20% patient resistance. Use of steroids, such as corticosteroids, are immunosuppressive and risk onset of secondary infections and severe side-effects after prolonged use. Antibiotic use for mild disease presentation often leads to mild-side effects and is reliant on disease specific response, making this method of treatment flawed as well [28]. Alternative treatment methods for IBD are emerging, including: resveratrol, curcumin,

bromelain, green tea, rutin, pomegranate and cannabinoid receptor ligands like tetrahydrocannabinol (THC) and cannabidiol (CBD) [29, 30].

More specifically, the Busbee lab has focused on a dietary indole in cruciferous vegetables, indole-3-carbinole (I3C), as an investigative IBD treatment method. Previous studies have shown I3C to exhibit anti-carcinogenic, anti-inflammatory, anti-oxidant, and anti-microbial properties [31-34]. In animal models I3C has been shown to ameliorate conditions such as rheumatoid arthritis, myocardial ischemia, and systemic lupus erythematosus [33, 35, 36]. In previous studies from our lab, I3C has been shown to attenuate colitis disease severity, reduce colonic damage, prevent colitis-associated microbial dysbiosis, increase butyrate production (which serves as division fuel for colonic epithelial cells), and increase colonic mucous production [37]. Importantly, I3C is a ligand for the aryl hydrocarbon receptor (AhR). Historically, AhR was first identified when researchers discovered its role in modulating the response to exogenous chemicals such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)—a contaminant of the chemical herbicide Agent Orange [38]. AhR is expressed in many cell types throughout the body, particularly those most relevant to gut homeostasis: colonic epithelial cells and certain immune cells (Th17, ILC3, Treg, Th22) within the gut lamina propria [39]. Studies from other labs have shown that colitis patients often have decreased expression and activation of AhR in the GI tract [38]. Furthermore, global AhR knockout mice develop more severe symptoms as compared to wild type mice [40]. Taken together, these findings indicate AhR to be a primary mode of disease regulation. I3C is our labs chosen treatment method due to mild affinity binding for AhR, which is advantageous because AhR is expressed in many locations throughout the body, and we aim to avoid overstimulation of this receptor.

Additionally, I3C is our chosen treatment method in order to introduce a nontoxic and naturally-occurring mechanism of treatment in an effort to avoid harsh side effects associated with current treatment methods.

Depression

Depression is a broad term referring to a spectrum of disorders and behaviors. Types of depression include, but are not limited to: major depressive disorder (clinical depression), psychotic depression, persistent depressive disorder, and bipolar depression. Some forms of depression may be triggered by psychosocial stressors like traumatic experiences, while some result from biological stressors like chemical imbalances. Symptoms of depression include anhedonia (reduced ability to derive pleasure), diurnal variation, fatigue, loss of appetite, weight loss, insomnia, feelings of worthlessness or guilt, suicidal ideation, and overall depressed mood [41]. Symptoms are not uniform between patients, making depression difficult to identify. It is to be noted that depressive symptoms are different from general, normal mood fluctuations. Depressive symptoms are often intense and recurrent, thus heightening disease burden. According to the World Health Organization, approximately 280 million people worldwide suffer from depression. In 2020, it was estimated that 21 million Americans over the age of 18 experienced at least one major depressive episode. This number constitutes 8.4% of all Americans, indicating major depression to be a social, financial, and health problem [42]. Known risk factors for clinical depression include personal or family history, female gender, alcohol dependence, childhood trauma, older age, recent childbirth, recent stress, low socioeconomic status, and comorbid chronic medical illness [43].

Current diagnostic screenings for depression rely heavily on patient reporting as there are no useful biological diagnostic markers or biological screening tests currently available [43]. A common diagnostic tool for clinical depression is the two-item and nine-item Patient Health Questionnaires (PHQs) – if the PHQ-2 is positive for depression then the PHQ-9 is employed. If PHQ-9 results indicate depression, clinicians use the Diagnostic and Statistical Manual of Mental Disorders-5 (DSM-5) to check criteria for clinical depression. To be considered clinical depressed, patients must exhibit five or more specified symptoms during the same 2-week period and at least one of the symptoms should be either depressed mood or loss of interest or pleasure [44]. This system for clinical depression diagnosis is inherently flawed in that it relies (1) on patients to report symptoms accurately and unbiasedly; (2) relies simply on few subjective criteria; and (3) diagnostics fail to acknowledge "nonmajor" depression or depression-like symptoms that do not quite meet specific questionnaire criteria. Additional problems with the current biomedical understanding of depression exist in that approximately 30% of patients with categorized clinical depression do not achieve remission after treatment with two or more first-line antidepressants and are considered to have treatment resistant depression [45]. These highlighted problems, with current medical understanding and treatment of depression paired with high disease burden and disease prevalence, warrant more research on the topic.

Depression has long been postulated to be linked to inflammatory disorders. The so called "inflammatory hypothesis of depression" refers to the idea that increased inflammation markers like inflammatory enzymes (i.e. myeloperoxidase (MPO), inducible nitric oxide synthase), proinflammatory and anti-inflammatory cytokines, and the phenomenon of oxidative stress can lead to depression [46]. Research has shown that

patients with inflammatory conditions often note higher levels of depression than basal population. These conditions include asthma, meningitis, certain cardiovascular diseases, type 2 diabetes, rheumatoid arthritis, obesity, and importantly IBD [47-51]. Note that in these listed inflammatory conditions, inflammation is stemming from the periphery and not in the brain. Thus, evidence indicates that inflammation in the periphery can cause dysfunction in the brain. Investigators have speculated at this relationship as the brain has been historically considered "immune privileged" which refers to the ability of the central nervous system (CNS) to tolerate immune challenges in the periphery [52]. However, recent findings suggest that this "privilege" is not absolute. The brain has its own highly complex immune regulation system that is closely connected with the peripheral immune system [53, 54].

The exact mechanisms by which peripheral inflammation can lead to altered brain function and ultimately altered behavior have not been elucidated, but viable evidence exists. One particularly relevant aspect is the blood brain barrier (BBB). The BBB lies between the cerebral capillary blood and the interstitial fluid of the brain; it is primarily composed of capillary endothelial cells and the basement membrane, neuroglial membrane, and glial podocytes (projections of astrocytes) [55]. This barrier serves to protect the brain from invasion of pathogenic agents, toxins, foreign invaders, and immune cells [56]. This barrier also serves to regulate the entry of nutrients, vitamins, and ions into the brain [57]. Furthermore, the BBB is highly selective and works to delicately control substance influx into the cerebral blood flow and brain parenchyma. The BBB works to ensure a homeostatic environment within the CNS primarily by regulating volume and composition of cerebrospinal fluid (CSF). This task is accomplished by utilizing tight junctions between

endothelial cells lining the capillaries which permit passage of only select substances, highly specific embedded transport proteins in the basement membrane, and cellular enzymes which function to alter substances simultaneous to passage through the cells [55]. Inasmuch, very select substances can pass the BBB and most large molecules and proteins cannot readily cross this barrier [55]. Though the BBB functions to prevent peripheral entities from affecting the CNS, evidence has shown that under nonoptimal physiological conditions, the BBB can be compromised. One such study in mice found that chronic social stress altered BBB integrity and promoted depression [58]. A detailed list of factors affecting BBB integrity can be found in a review by Zhao et al.; of note, overactivated M1 microglia, proinflammatory cytokines, and excessive glutamate levels which can weaken the BBB and allow for more permeability [59]. These are of note in that they are all related to the involved in various hypotheses of depression and interestingly inflammation.

The Relationship Between IBD and Depression

As discussed above, studies have identified a relationship between inflammation and depression, hence the so called "inflammatory hypothesis of depression". Most relevant to this current study is that a relationship between IBD and depression has been recognized. Studies have shown that a higher incidence of depression in IBD patients exists. A recent meta-analysis on prevalence of symptoms of anxiety and depression in patients with inflammatory bowel disease found that 24% of UC patients also had depression; in active UC this number jumps to 41.3% of patients. This same study found that 24.8% of patients with CD also had depression; in active CD this number jumps to 51% [60]. Note how these numbers are much higher than the national average for depression (8.4%) which further highlights the grounds for a connection between these two

diseases. A recent nested case-control study, including nearly 20,000 patients, found that individuals with IBD had a higher prevalence of depression than matched controls without IBD in the years prior to their diagnosis [61]. Furthermore, one cohort study found that patients with a history of depression were more likely to be diagnosed subsequently with IBD. Interestingly, antidepressant treatment was found to significantly reduce likelihood of subsequent IBD development [62].

The question remains as to precisely what mechanisms contribute to IBD and depression as comorbidities. Maintaining tight control on the intestinal barrier is of crucial importance in that the intestinal barrier is intimately involved with host innate immunity [6]. Due to microbial dysbiosis, the protective mucus layer in the gut lumen gets degraded. This allows for increased colonic epithelial permeability and subsequent invasion of bacteria (normally contained in the lumen) into the lamina propria. This allows presentation to immune cells which ultimately results in immune overactivation. As a result, gut microbiota and their metabolites can circulate systemically, a term known as “leaky gut” [63]. The gut and brain are said to communicate bidirectionally through the gut-brain axis (GBA) [64]. In the GBA, signaling is said to occur through complex interactions of neuroendocrine transmission between the autonomic, enteric, and central nervous system [65]. The GBA is thought to play a large role in the relationship between IBD and depression. Consequences of leaky gut, like increased proinflammatory cytokines and bacterial metabolites, are said to influence the brain through the GBA, although the exact mechanisms remain to be uncovered [66]. One very relevant and scientifically backed idea is that the cytokines and metabolites generated in leaky gut, likely through use of the GBA, circulate systemically and ultimately saturate and invade the BBB to wreak havoc on the

brain [67]. Although the BBB prevents >98% of antibodies and small molecules from entering the brain parenchyma, under inflammatory duress the BBB is compromised allowing for passage of molecules that would not normally be permitted to pass [64, 68]. This in turn more readily allows neuroinflammation and overactivation of microglia, both of which have been linked to depression [69].

Cytokines are small proteins which function to control the growth and activity of immune cells and are major function proteins for inflammatory response to stimuli. Evidence has shown that peripheral cytokines are able to cross the BBB if saturated [70]. Thus, historically, science has recognized cytokine-induced sickness behavior. Studies using lipopolysaccharides (LPS) have found that proinflammatory cytokines that are released in the periphery by activated immune cells act on the brain to induce sickness behavior [71]. Sickness behavior refers to the behavioral changes that develop in sick individuals throughout the course of an infection. These behavioral changes in humans include weakness, malaise, listlessness, inability to concentrate, reduced eating/drinking, lethargy, and often times depression [72]. Since recognition of cytokine-induced sickness behavior, increasing evidence has shown that cytokines access the brain and interact with virtually every domain known to be involved in depression, including neuroendocrine function, neurotransmitter metabolism, and neural plasticity [73].

In recent studies, it has been demonstrated that LPS induced mice show higher levels of TNF-α, IL-1β, and IL-6 in depression related brain areas (hippocampus and prefrontal cortex). Clinical data has shown that when compared to nondepressed individuals, both medically healthy and medically ill patients with clinical depression have been found to have elevated proinflammatory cytokines in both peripheral blood and in

CSF [74, 75]. Therapeutic administration of proinflammatory cytokine interferon-α has been shown to induce depression in up to 50% of patients [74]. Anti-inflammatory cytokines also have been found to modulate the initiation of depressive-like symptoms in murine models. IL-10 knockout mice have shown increased depressive-like symptoms in behavioral testing, but this was reversed with administration of IL-10 [76]. Interestingly, cytokines have been implicated in the pathophysiology of IBD and anti TNF-α agents are currently used as a treatment for IBD. In microbial dysbiosis, mucosal immune cells respond to microbial contact by over production of proinflammatory cytokines. In fact, IL-10 knockout mice have been found to spontaneously develop colitis [77]. Additionally, IBD patients have been shown to have increased circulating IL-1, IL-6, and TNF-α – coincidentally the same proinflammatory cytokines that have been found increased in depression-related brain regions after LPS injection in mice [74, 78-80].

Of specific interest to the Busbee lab is how inflammation-derived depression is related to AhR. AhR is a diverse nuclear receptor which binds to many different ligands. Specifically, the breakdown of tryptophan (Trp), an essential dietary amino acid, provides AhR with many ligands [38]. Dietary Trp is degraded through three pathways: the serotonin pathway, the tryptophan-indole pathway, and the tryptophan-kynurenine (TRP-KYN) pathway via the conversion of indoleamine 2,3-dioxygenase (IDO), using tryptophan-2,3-dioxygenase (TDO) in the liver (Figure 1.1) [81, 82]. Approximately 95% of Trp metabolism is conducted through the TRP-KYN pathway, while the remaining (~5%) is used for indole synthesis and the production of neurotransmitters such as serotonin, which also promotes gut motility [83]. Recent studies have shown Trp metabolism to be related to both inflammation and depression. Decreased bioavailability

of Trp has been noted in clinically depressed patients, suggesting higher turnover of Trp in depressed individuals [84]. Furthermore, clinical studies have shown that TRP metabolism is associated with the severity of IBD [85]. A recent review by Sun et al., concluded that Trp modulates gut-homeostasis via AhR through expression of IL-22 and IL-17 [86]. Previous findings from the Busbee lab has indicated I3C amelioration of colitis via AhR to be IL-22 dependent [37]. Furthermore, one study found that AhR activation with I3C prevented chronic stress-induced depression via suppressing proinflammatory cytokine production and oxido-nitrosative stress in the brain [87]. These findings highlight the relationship between AhR activation, Trp, gut homeostasis, and depression.

More specifically, the major pathway of Trp degradation, TRP-KYN, is implicated in colitis and depression. Through this pathway Trp is degraded to kynurenine (KYN) by the enzyme IDO. AhR regulates expression and activation of IDO and kynurenine 3-monooxygenase (KMO) which are key regulatory enzymes of the TRP-KYN pathway [88]. Studies using LPS to induce inflammation have shown pro-inflammatory cytokines were activated in the brain in response to peripheral administration which in turned enhanced activity of IDO and resulted in increased immobility in tail-suspension-test (TST), a measure of depressive-like behavior [89]. Upregulation of IDO alters T-cell proliferation and survival and higher expression of IDO is present in both IBD and depressed patients [90-92]. Furthermore, increased IDO leads to Trp metabolism being shunted towards the TRP-KYN pathway and away from production of indoles and serotonin. Serotonin is linked to gut homeostasis and is a known mood regulator which has been implicated in both IBD and depression [93, 94]. The product of Trp metabolism with IDO is KYN, which is an agonist for AhR. KYN is further degraded to either anti-inflammatory neuroprotective

kynurenic acid (KYNA) via kynurenine aminotransferases (KATs) or proinflammatory and neurotoxic via quinolinic acid (QA) via KMO. Studies have shown that the delicate balance between KYNA and QA production has a profound influence on the excitability of enteric neurons, which can affect intestinal motor and sensory function [93]. QA has been positively associated with inflammation in the periphery and significantly increased levels of QA have been found in serum of both CD and UC IBD patients [85, 95]. Though studies have shown that QA cannot readily cross the BBB, QA can be locally produced in the brain primarily in microglia [81]. QA is an NMDA receptor agonist; results from other studies suggest NMDA overexcitation to be an important mechanism of depression [96].

The gut microbiota also play a role in Trp metabolism. Of interest, one such study found microbial dysbiosis in mice deficient in caspase recruitment domain 9 (Card9), an IBD susceptibility gene, fail to catalyze Trp into AhR ligands. This resulted in reduced IL-22 release and ultimately to higher susceptibility of mice to dextran sulfate sodium (DSS)-induced colitis [97]. Dysbiosis in the gut microbiome has also been linked to depression. For example, studies have shown that transference of fecal matter from depressed patients into germ free (GF) mice alters behavior resulting in depressive-like symptoms [98]. Trp is degraded by mainly microbes in the gut to produce indoles which are ligands for AhR. Interestingly, these ligands are closely related to I3C and are essential mediators in the GBA [99]. However, gut microbiota also regulate and play a role in the Trp metabolism pathway shunting in all three arms of Trp metabolism. Studies have shown in both GF and antibiotic treated animals that the gut microbiota plays a role in induction of IDO where GF mice had higher serum Trp and lower KYN, which resulted in altered behavior [100]. It has further been demonstrated that the gut microbiota plays a critical role in regulating

host serotonin. Indigenous spore-forming bacteria from the mouse and human microbiota promote serotonin biosynthesis from colonic enterochromaffin cells, which supply serotonin to the mucosa, lumen and circulating platelets [101]. Taken together, these findings have implicated Trp metabolism and AhR activation as possible primary mediators of both IBD and depression. The process by which this occurs is likely through "leaky gut" allowing bacteria to migrate more freely into the bloodstream which results in immune overactivation and stimulation of proinflammatory cytokine production. This subsequently increases expression of IDO, resulting in altered Trp metabolism (in part by gut microbes) and AhR activation. This signals through the GBA resulting in imbalanced QA/KYNA production, impaired function of the BBB, and subsequent neuroinflammation with microglia activation and astrocyte atrophy.

Experimental Intent

In biomedical research, primarily two different chemical agents are used to mimic IBD in murine models for acute forms of colitis. DSS induces intestinal epithelial damage and most closely mimics UC, while 2,4,6-trinitrobenzene sulfonic acid (TNBS) is used to induce damage resembling CD [102]. In current study, we utilize the DSS model to induce colitis symptoms in C57BL/6 mice.

In preliminary studies, our lab conducted an untargeted metabolomics profile on fecal matter from colitis induced mice. Findings showed QA, a Trp metabolite of the TRP-KYN pathway, to be upregulated in diseased mice. Taking these data and findings from other labs into account, our lab sought to investigate the relationship between IBD and depression in context of Trp metabolism and AhR activation. Specifically, we sought to investigate the relationship of depression and UC, a subtype of IBD, and to determine if

AhR is involved in regulating gut inflammation-derived depression. Experiments were designed to provide correlation between colitis status and depressive-like symptoms in C57BL/6 mice. Previous publications from the Busbee lab have shown I3C, a known activator of AhR, to reduce colitis severity [37]. Additionally, publications from other labs have shown I3C to reduce depressive-like behaviors in murine models [87, 103]. Thus, in this experiment we investigate if I3C can ameliorate symptoms associated with colitis-induced depression. Immobility times in the TST reflect despair behavior in mice and immobility was our primary measure of depression as a result from colitis-induced-inflammation [104]. Taking findings from other labs and our own into account, we hypothesized that in colitis induced mice, depressive-like symptoms will be heightened and that treatment with I3C will ameliorate symptoms associated with both conditions.

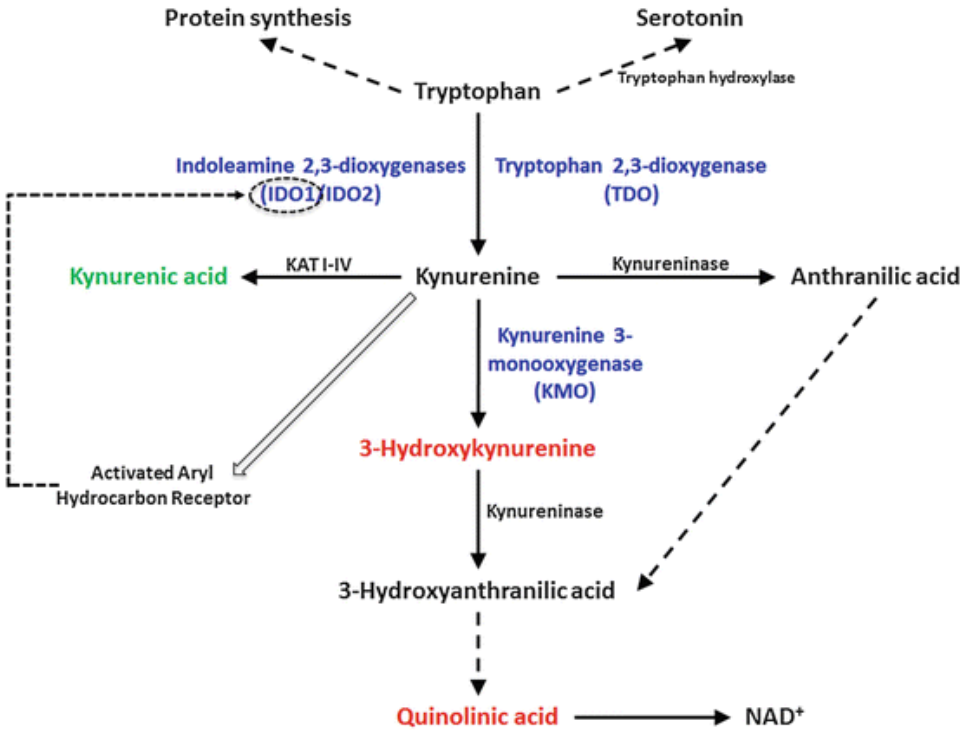

Figure 1.1: *Tryptophan-Kynurenine (TRP-KYN) Pathway*. TRP-KYN pathway as illustrated by Mittal et al. [105]. Approximately 95% of Trp is metabolized by this pathway. Note IDO degrades Trp to KYN. KYN is then either metabolized to neuroprotective KYNA (via KATs) or neurotoxic QA (via KMO).

CHAPTER 2

MATERIALS AND METHODS

Animals

Female and male C57BL/6 mice were purchased from Jackson Laboratory. Experimental mice used were between 10 and 14 weeks of age. All mice were housed at the Association for Assessment and Accreditation of Laboratory Animal Care–accredited (AAALAC-accredited) animal facility at the University of South Carolina School of Medicine. Mice were housed under 12-hour light/12-hour dark cycles. All protocols were followed in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines and in correspondence with Department of Laboratory Animal Research (DLAR) under protocol number 2625-101759-101022. Animals were grouped for experiments as they arrived from the manufacturer to avoid confounding factors for the behavioral assessment. Animals were given free access to food and water at all times. Animals were sacrificed using isoflurane on experiment day 10. Upon sacrifice the colon, blood serum, whole brain, and feces were excised and held in appropriate conditions for further analysis. We conducted 3 trials composed of both males and females (n=5 per group unless specified otherwise). Specific trial information can be visualized in Table 2.1.

Colitis Disease Induction and I3C Treatment Administration

For colitis induction, DSS was purchased from MP Biomedicals (Cat. No 9011-18-1) and introduced into drinking water at a 3% concentration from day 0 to day 6, followed by regular drinking water. For treatment groups, I3C (Sigma, 700-06-1) intraperitoneal

injections were given every other day, starting on the first day of the experiment. Intraperitoneal injections of I3C contained 40 mg/kg I3C in 0.05% dimethyl sulfoxide (DMSO) (Sigma,67-68-4-5)/corn oil (Sigma, C8267) and began approximately 1 hour after introduction of DSS into the drinking water. I3C treatment was given every other day until the experimental endpoint. Vehicle groups in this experiment were given intraperitoneal injections of 0.05% DMSO/corn oil. For disease versus control experiments (composed of two groups only), no injections were given to either group.

Tail-Suspension Test (TST)

All TSTs were conducted using the automated tail suspension BIO-TST5 model from BIOSEB, visualized in Figure 2.1. This apparatus is composed of three chambers, allowing for three mice to be suspended at once. Runs for mice were programmed using the BIOSEB software, with a high-definition camera to capture video and sensors on the suspension hooks to measure energy output. This program featured independent start/stop controls for each of the three mice in a particular run. Animals were brought to the testing site and allowed an hour acclimation period before testing. Subjects were suspended using tape pieces about 2 inches in length. To avoid harm to animals, tape was first placed on human skin to remove some adhesion. Tape was placed at the tail tip through a single wrap method, with tabbing at ends for friendlier removal. Excess tape was attached to the apparatus hook through perforation. Animals were suspended and recorded for 360 seconds (6 minutes) with a 5 second latency period before video capture. Mice were considered to have "beaten" the test if they successfully managed to tail climb onto the seated board atop the apparatus. Tail climbing was considered an exclusion criterion. TST was performed in various ways across our experiments. For our first experimental (trial 1), mice were

subjected to TST on day 0, day 6, and day 9 during light cycle in the morning hours between 10am-1pm. For our second experimental trial (trial 2), mice were subjected to TST on day 9 during light cycle in the morning hours between 10am-1pm. For our final experimental trial (trial 3), mice were subjected to TST on day 9 during dark cycle in the night hours between 12am-3am. Immobility times were analyzed through hand timing of recorded video. Immobility was defined as complete absence of movement and lack of escape behavior (i.e. completely still hanging with no active movements). Immobility times were analyzed using a 120 second (2 minute) latency period and a 240 second (4 minute) latency period. Our lab has not previously employed the use of TST thus solidifying an optimal protocol concerning optimal time for testing and length of latency is still under work. Mice labeled as "control" were only exposed to the TST and stress of handling. These mice were not given any injections nor DSS.

Untargeted Metabolomics

Preliminarily, we performed an untargeted metabolomics approach wherein a large array of metabolites were analyzed in mouse fecal matter using automated linear exchange cold injection system gas chromatography time of flight mass spectrometry (ALEX-CIS GCTOF MS). We conducted untargeted fecal metabolomics in the DSS model of colitis after I3C treatment to gauge metabolic changes in response to colitis. Fecal contents were screened for 220 metabolites. Untargeted metabolomics using ALEX-CIS GCTOF MS was prepped as described in previous reports, and analysis was done using MetaboAnalyst [30].

Assessment of Colitis Disease Parameters

Distal colonoscopy images were taken on day 8 of trial 1 and trial from representative mice using a Karl Storz Tele Pack Vet x LED endoscope designed for small

animals. Colonoscopy scoring was done according to criteria detailed in previous publications [37, 106]. Scoring parameters are detailed in Table 2.2 and include colonic wall transparency, presence of lesions or ulcers, diarrheal stool, and intestinal bleeding. Mice were weighed daily for the duration of the experiment at the same time every day (±1 hour). Percent weight loss was calculated at experiment endpoints. Higher percent weight loss was indicative of colitis induction. Animals were sacrificed on day 10. On day of sacrifice, colons were excised, and colon lengths recorded. Shortened colon lengths were indicative of colitis induction.

Microscopic Analysis of Colonic Tissue

Colon histology was obtained by harvesting distal samples on day of sacrifice from trials 1 and 2. Tissues were fixed in 10% neutral buffered formalin for greater than 24 hours at room temperature at a 20:1 ratio. Processing of the samples was conducted by the University of South Carolina School of Medicine Instrumental Resources Facilities. After fixation, tissue was processed with ethanol dehydration, cleared with xylene, then following such were embedded in paraffin wax. Samples were stained with Harris' hematoxylin and eosin y (H&E). Distal colonic tissues were sliced in 5 μm sections in a transverse orientation to best visualize colonic structure. Images were taken using the Motic Swift Line M3603C light microscope equipped with Motic cam S1 camera. Images were visualized using the Motic Images Plus 3.0 software. Histopathological scoring was then conducted based on previously published reports [37, 107]. Table 2.3 details these scoring parameters which include goblet cell damage, epithelial cell loss, crypt abscess, and inflammation extent. Histological images were edited using Image J software to ethically enhance brightness, contrast, and color balance.

Statistical Analysis

All statistical analyses were conducted using GraphPad Prism software. In order to determine statistical significance, one-way Analysis of Variance (ANOVA) and Tukey's post hoc multiple comparison tests were used when comparing three or more groups. An unpaired two-tailed Students t-test was used for comparison between two groups. For all analyses a P value less than 0.05 was considered significant. Error bars display standard error of mean (SEM) for all figures.

Trial	Sex	Groups	N	TST Days
1	Female	H2O+Vehicle H2O+I3C DSS+Vehicle DSS+I3C Control	5	0, 6, 9 Daytime
	Male	Disease Control	4 3	0, 6, 9 Daytime
2	Female	Disease Control	5	9 Daytime
	Male	H2O+Vehicle H2O+I3C DSS+Vehicle DSS+I3C Control	5	9 Daytime
3	Female	Disease Control	5	9 Nighttime
	Male	Disease Control	5	9 Nighttime

Table 2.1: *Experimental Trials Setup.* Organization and arrangement of experimental trials.

Findings	Criteria	Score
Perianal	None	0
	Yes – diarrhea/ fecal clumps	1
	Yes – blood anal discharge	2
	Yes – rectal prolapse/fistula	3
Wall Transparency	Yes – small and large blood vessels visible	0
	Yes – but most small blood vessels cannot be seen	1
	Not sure – only large blood vessels seen	2
	Not at all – thickened mucosa, blood vessels not seen	3
Intestinal Bleeding	None	0
	Yes – contact bleeding due to endoscopic trauma	1
	Yes – lack of lubricant or excessive endoscopic trauma	2
	Yes – spontaneous bleeding not due to endoscopic trauma	3
Focal Lesions	None	0
	Yes – edematous areas of mucosa	1
	Yes – erosion/reddened areas	2
	Yes – ulcers/tissue sloughing	3

Table 2.2: *Colitis Colonoscopy Scoring Parameters.* Colonoscopy scoring parameters for colitis induction as in other reports [106].

Findings	Criteria	Score
Epithelial loss	None	0
	Loss of <5%	1
	Loss of 5-10%	2
	Loss of >10%	3
Crypt abscesses	Intact crypt	0
	Loss of <10%	1
	Loss of 10-20%	2
	Loss of >20%	3
Inflammation extent	None	0
	Mild	1
	Medium	2
	Pronounced	3
Goblet Cell Damage	None	0
	Yes	1

Table 2.3: *Colitis Histopathological Scoring Parameters*. Histopathological scoring parameters for colitis induction as previously conducted in other reports [107].

Figure 2.1: *BIOSEB BIO-TST5 Apparatus*. BIOSEB BIO-TST5 apparatus used in conducting TSTs for all trials.

CHAPTER 3

RESULTS

Untargeted Metabolomics

In preliminary data, 220 metabolites were screened for in colonic fecal matter with untargeted metabolomics using ALEX-CIS GCTOF MS in wild type control (H2O+Vehicle, H2O+ I3C) and disease mice (DSS+Vehicle) or disease treated mice (DSS+I3C) (n=3 per group). In Figure 3.1, the principal component analysis (PCA) plot shows that the metabolic profile of diseased mice treated with I3C (DSS+I3C) more tightly clustered with the metabolic profile of control mice (H2O+Vehicle, H2O+I3C). Diseased mice had the most dissimilar metabolic profile (DSS+Vehicle). Figure 3.2 shows a volcano plot depicting metabolites significantly up (red) and downregulated (blue) between DSS+I3C versus DSS+Vehicle. Of most importance, QA was found to be significantly downregulated in the DSS model of colitis after treatment with I3C. In line with these findings, Figure 3.3 shows normalized values of QA as found in all four experimental groups (H2O+Vehicle, H2O+I3C, DSS+Vehicle, DSS+I3C). Results showed that QA was detected at its highest amount in DSS+Vehicle. After treatment with I3C, QA levels returned to approximately control levels. A significant difference was found in QA levels between H2O+Vehicle/DSS+Vehicle groups as well as between DSS+Vehicle/DSS+I3C.

Trial 1 Females

Trial 1 females were arranged into five groups – H2O+Vehicle, H2O+I3C, DSS+Vehicle, DSS+I3C, and Control (n=5). In order to verify the efficacy of DSS and

treatment of I3C, mice were weighed daily. Figure 3.4 shows percent weight loss/gain of the five experimental groups in trial 1 females. Results show no significant differences, though diseased mice did overall lose more percent body weight as compared to controls. Further data in Figure 3.5 shows colon lengths in centimeters measured from trial 1 females. Significant differences in colon length were found between H2O+Vehicle/DSS+Vehicle, H2O+Vehicle/DSS+I3C, and DSS+Vehicle/Control.

Representative colonoscopy images from trial 1 females are shown is Figure 3.6. Colonoscopies were scored (Figure 3.7) on a scale 0-12 of (12 being the worst) according to perianal findings, wall transparency, intestinal bleeding, and focal lesions. Significant differences were found between H2O+Vehicle/DSS+Vehicle, H2O+I3C/DSS+Vehicle, and DSS+Vehicle/DSS+I3C. Microscopic evaluation of colonic tissue was conducted, and representative light microscope images of trial 1 females are depicted in Figure 3.8. Distal colonic tissue samples were evaluated and scored on parameters of goblet cell damage, epithelial loss, crypt loss, and inflammation on a scale 0-10 (10 being the worst). Histopathological scores of trial 1 females are visualized in Figure 3.9. Analysis found significant differences between H2O+Vehicle/DSS+Vehicle, H2O+Vehicle/DSS+I3C, H2O+I3C/DSS+Vehicle, H2O+I3C/DSS+I3C, and DSS+Vehicle/DSS+I3C.

Trial 1 females were subjected to TST on experimental days 0, 6 and 9. TST for trial 1 females were conducted during daytime hours. Figure 3.10 and Figure 3.11 show immobility assessed on day 0 in trial 1 female daytime TST using a 2 minute and 4 minute latency, respectively. Analysis revealed no significant differences between groups (H2O+Vehicle, H2O+I3C, DSS+Vehicle, DSS+I3C, and Control). All groups displayed approximately equal average immobility times using both latency periods. Trial 1 females

were subject to daytime TST on experimental day 6; results were analyzed using 2 and 4 minute latency periods (Figures 3.12 and 3.13). Again, no significance was found between groups and all groups were approximately equal in average immobility time using both latency periods. Daytime TST on day 9 for trial 1 females revealed no significant differences using 2 minute and 4 minute latency periods (Figures 3.14 and 3.15). Interestingly, on day 9 all groups displayed similar average immobility times as in days 0 and 6, with the exception of the DSS+Vehicle group. DSS+Vehicle average immobility was found to be lower than all other groups, while DSS+I3C mimicked control groups more closely in day 9 daytime TST for trial 1 females.

Trial 1 Males

Trial 1 Males were arranged into two groups – disease (n=4) and control (n=3). Figure 3.16 shows trial 1 male percent weight loss/gain over the course of the experiment. Significant differences were detected between disease (DSS) versus control (H2O) groups. In line with this finding, Figure 3.17 shows colon lengths in centimeters obtained from trial 2 males. Here we note significant differences in colon length between disease versus control groups.

Trial 1 males were also subjected to TST on experimental days 0, 6 and 9 during daytime hours. Figures 3.18 and 3.19 depict trial 1 male daytime TST for day 0 using a 2 and 4 minute latency period, respectively. Analysis revealed no significant differences between groups (disease versus control). Average immobility times between groups were approximately equal. Day 6 daytime TST of trial 1 males also revealed no significant difference between groups using 2 and 4 minute latency periods (Figures 3.20 and 3.21). It can be noted that the disease group did display slightly higher immobility on average in

trial 1 males day 6 daytime TST, with this difference being more heightened using 4 minute latency. Trial 1 males were further subjected to daytime TST on experimental day 9 with results depicted in Figures 3.22 and 3.23 with 2 and 4 minute latency periods, respectively. Here we note decreased average immobility in trial 1 male disease group using both latency periods. Diseased mice were found to have significantly decreased average immobility in day 9 daytime trial 1 male TST using 4 minute latency period when compared to control mice.

Trial 2 Females

Trial 2 females were arranged into two groups – diseased (n=5) and control (n=5). Figure 3.24 shows percent weight loss/gain of trial 2 females. Significance was shown between disease versus control groups. Partially in line, colon lengths in centimeters are shown in Figure 3.25. Results did not reveal a significant difference, though disease colon lengths trended down as expected in DSS-induced colitis.

Trial 2 females (disease versus control) were subject to TST on experimental day 9 during daytime hours. Immobility times for trial 2 females were analyzed using 2 and 4 minute latency periods and are depicted in Figures 3.26 and 3.27, respectively. Though results did not reveal significant differences, using both latency periods diseased mice had a trend of decreased average immobility in TST as compared to control. These results are similar to those of day 9 daytime TST for trial 1 males.

Trial 2 Males

For trial 2, males were arranged into five groups – H2O+Vehicle, H2O+I3C, DSS+Vehicle, DSS+I3C, and Control (n=5 per group). Figure 3.28 shows percent weight loss/gain of trial 2 males. Significant differences were found between weights of control

(H2O+Vehicle, H2O+I3C, Control) and diseased groups (DSS+Vehicle, DSS+I3C). DSS groups (DSS+Vehicle, DSS+I3C) overall lost the most amount of weight, but I3C did not ameliorate this weight loss. Furthermore, colon lengths from trial 2 males depicted in Figure 3.29 shows significant differences. Significant differences were found between H2O+Vehicle/DSS+Vehicle, H2O+Vehicle/DSS+I3C, H2O+I3C/DSS+Vehicle, H2O+I3C/DSS+I3C, DSS+Vehicle/Control, and DSS+I3C/Control. DSS+Vehicle/DSS+I3C did not reach significance. Colonoscopies were conducted on trial 2 males. Figure 3.30 shows representative colonoscopy images obtained. Colonoscopies were scored in accordance to specified criteria with results shown in Figure 3.31. Significant differences were found between H2O+Vehicle/DSS+Vehicle and H2O+I3C/DSS+Vehicle, but significance was not found between DSS+Vehicle/DSS+I3C. Distal colonic histology was only obtained from H2O+Vehicle and DSS+Vehicle groups. Representative images stained with H&E are depicted in Figure 3.32. Histopathological scoring was conducted on trial 2 male mice and are visualized in Figure 3.33. A significant difference was found in microscopic histopathological scores of disease (DSS+Vehicle) versus control (H2O+Vehicle) trial 2 males.

TST was also conducted on trial 2 males during daytime hours on experimental day 9 only. TST was conducted on all trial 2 male groups (H2O+Vehicle, H2O+I3C, DSS+Vehicle, DSS+I3C, and Control). Much like results noted in day 9 trial 1 females, DSS+Vehicle mice displayed decreased average immobility times in TST using both 2 and 4 minute latency periods (Figures 3.34 and 3.35). DSS+Vehicle trial 2 male mice were found to have a significantly decreased average immobility time in day 9 daytime TST using a 4 minute latency as compared to trial 2 male Control group (Figure 3.35).

Interestingly, DSS+I3C trial 2 male mice had average immobility times which very closely mimicked control groups using both latency periods, again much like trial 1 female daytime day 9 TST.

Trial 3 Females

Trial 3 females were split into two groups – disease (n=5) versus control (n=5). Figure 3.36 shows percent weight loss/gain of trial 3 females. Analysis revealed a significant difference between disease and control groups. In line with this finding, colon length in centimeters from trial 3 females are depicted in Figure 3.37. A significant difference was found between trial 3 female colitis-induced mice and control groups with diseased having shorter colon lengths on average.

Trial 3 females (disease versus control) were subjected to TST during night hours of experimental day 9 only. Results were analyzed using 2 and 4 minute latency periods. In Figure 3.38, trial 3 female day 9 nighttime TST data is depicted using a 2 minute latency period. Analysis revealed a significantly increased average immobility time in TST for diseased trial 3 females versus control. In line with this finding, Figure 3.39 depicts results from trial 3 female day 9 nighttime TST with a 4 minute latency period. Analysis indicated that diseased trial 3 females displayed significantly increased average immobility time as compared to control trial 3 females.

Trial 3 Males

Trial 3 males were arranged into two groups – disease (n=5) and control (n=5). Percent weight loss/gain can be visualized in Figure 3.40. A significant difference was found between trial 3 male disease versus control groups. However, Figure 3.41 reveals that trial 3 males did not have significant differences in colon lengths in disease versus

control groups. Though differences did not reach significance, trial 3 male disease colon lengths trended down as expected in DSS-induced colitis.

Trial 3 males (disease versus control) were also subjected to TST during night hours of experimental day 9 only. Results were analyzed using 2 and 4 minute latency periods. In Figure 3.42 trial 3 male day 9 nighttime TST results using 2 minute latency are depicted. A significant increase in average immobility was noted for disease trial 3 males as compared to control trial 3 males, much like in trial 3 females. Figure 3.43 depicts trial 3 male day 9 nighttime TST results using 4 minute latency. No significance was uncovered upon analysis, but it is to be noted that trial 3 diseased males displayed a higher average immobility time as compared to controls.

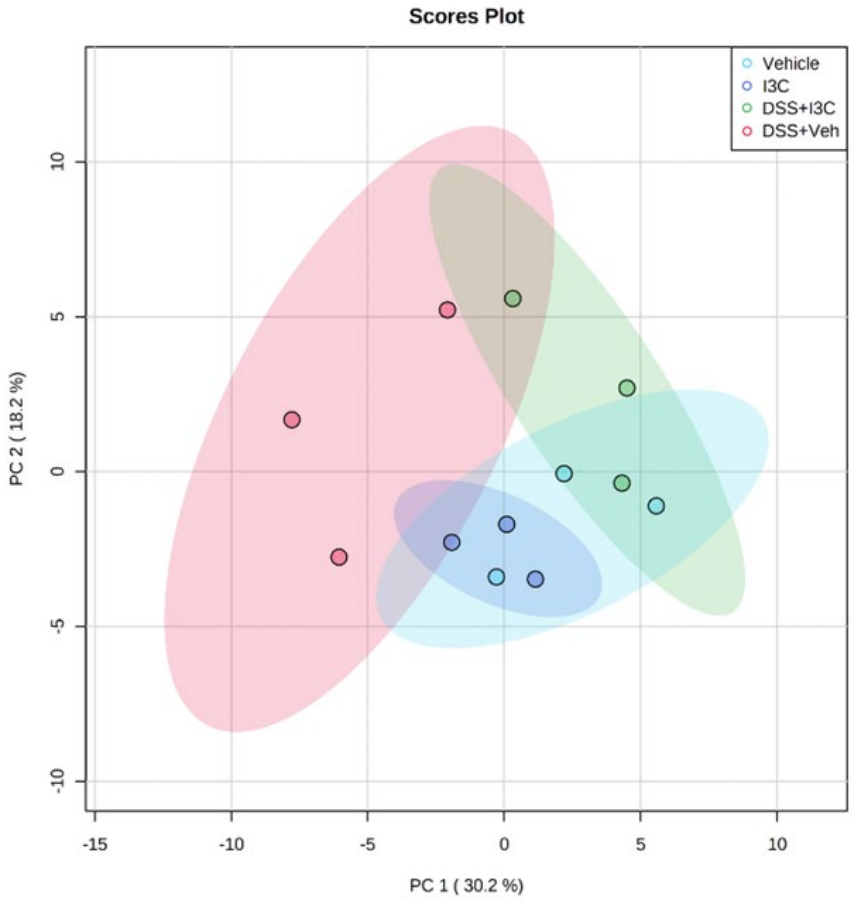

Figure 3.1: *I3C Fecal Metabolome is More Tightly Clustered with Controls*. Untargeted fecal metabolomics PCA plot showing samples in vehicle (light blue), I3C (dark blue), DSS + vehicle (red), and DSS + I3C (green) (n=3 per group). DSS+I3C group displayed a metabolomics profile more similar to that of controls versus DSS+Vehicle group.

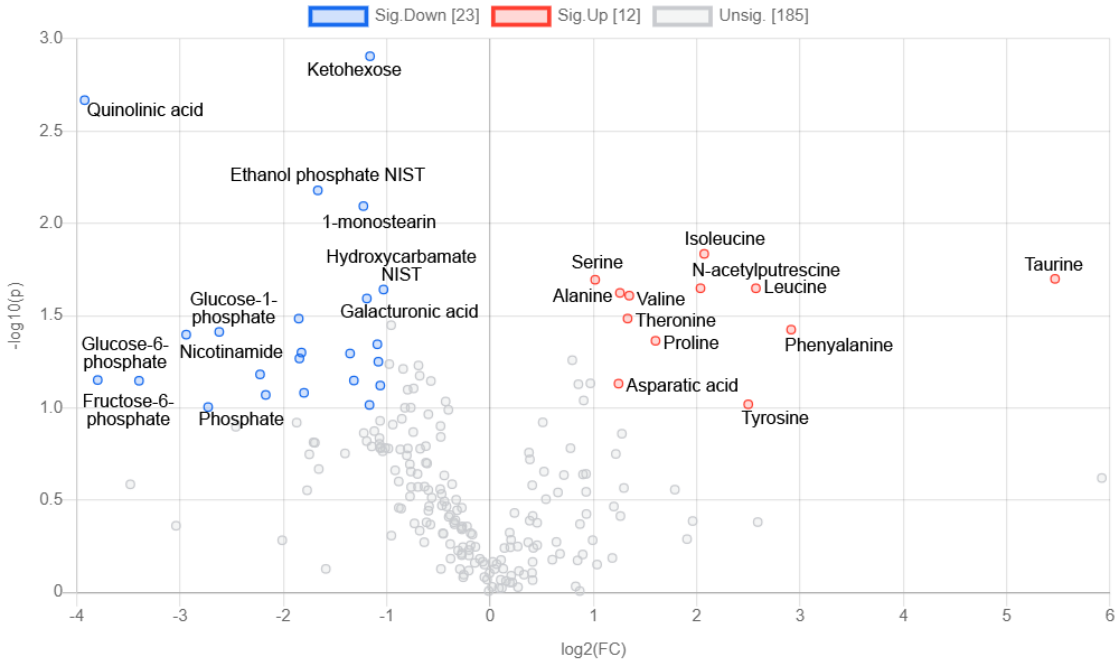

Figure 3.2: *I3C Alters Metabolome in DSS Models.* Untargeted fecal metabolomics volcano plot with significantly up (red) and downregulated metabolites between DSS + I3C versus DSS + Vehicle groups. Data showed neurotoxic QA to be significantly downregulated in DSS induced colitis after treatment with AhR ligand I3C.

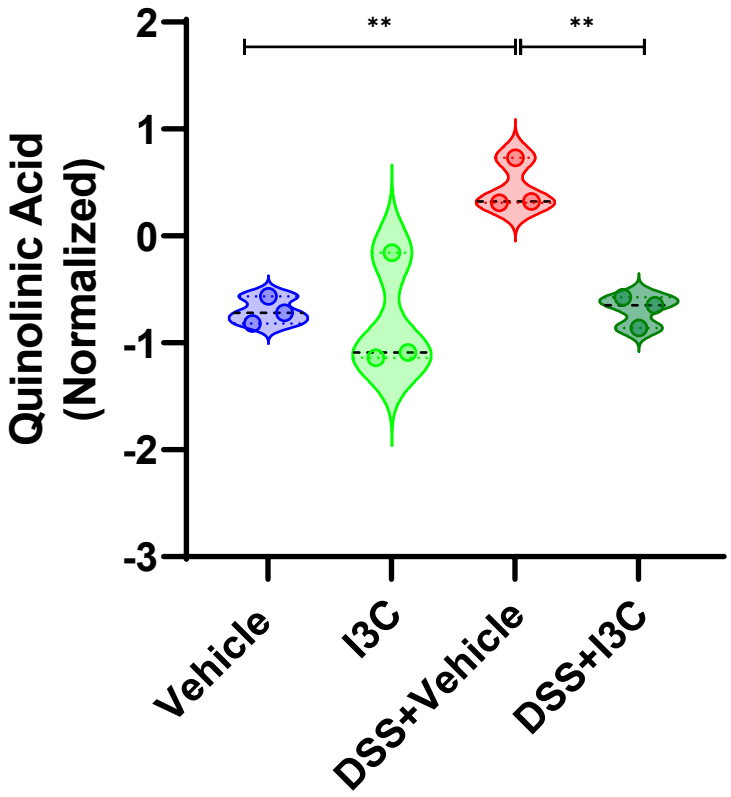

Figure 3.3: *I3C Prevents Increased Levels of QA.* Untargeted fecal metabolomics showing QA is reduced to control levels after I3C treatment in DSS induced colitis (**=0.01 for P value).

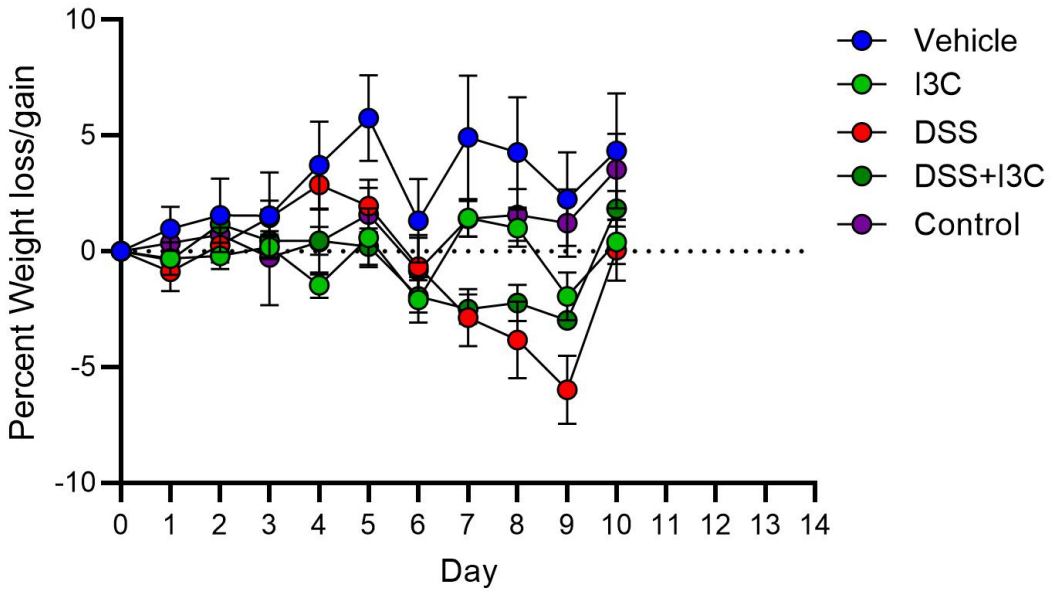

Figure 3.4: *Percent Weight Loss/Gain Trial 1 Females.* Percent weight loss/gain in trial 1 females (n=5 per group). Circles denote group percent weight loss/gain over entire group. No significant differences were found.

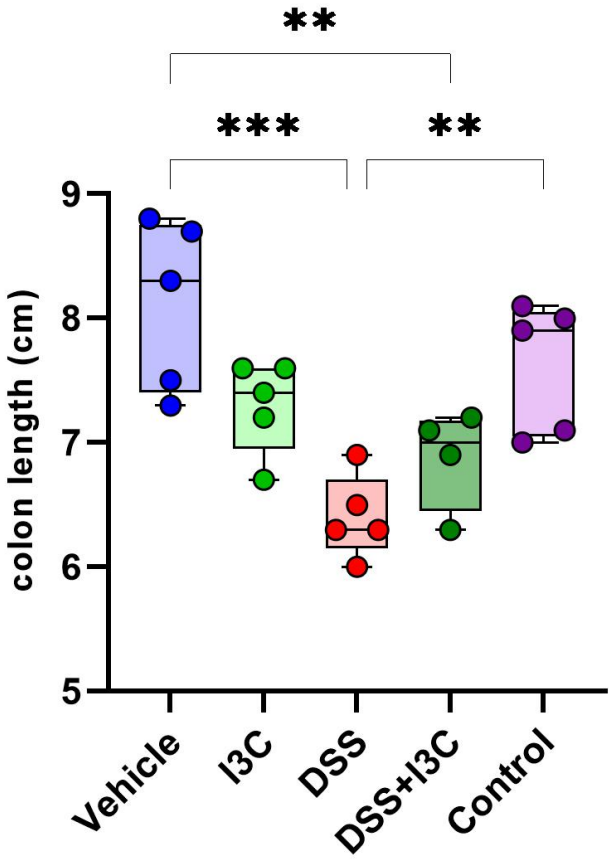

Figure 3.5: *Colon Length (cm) Trial 1 Females.* Colon length measured in centimeters for trial 1 females where **=0.01 and ***=0.001 for P value. Each circle represents a single colon length.

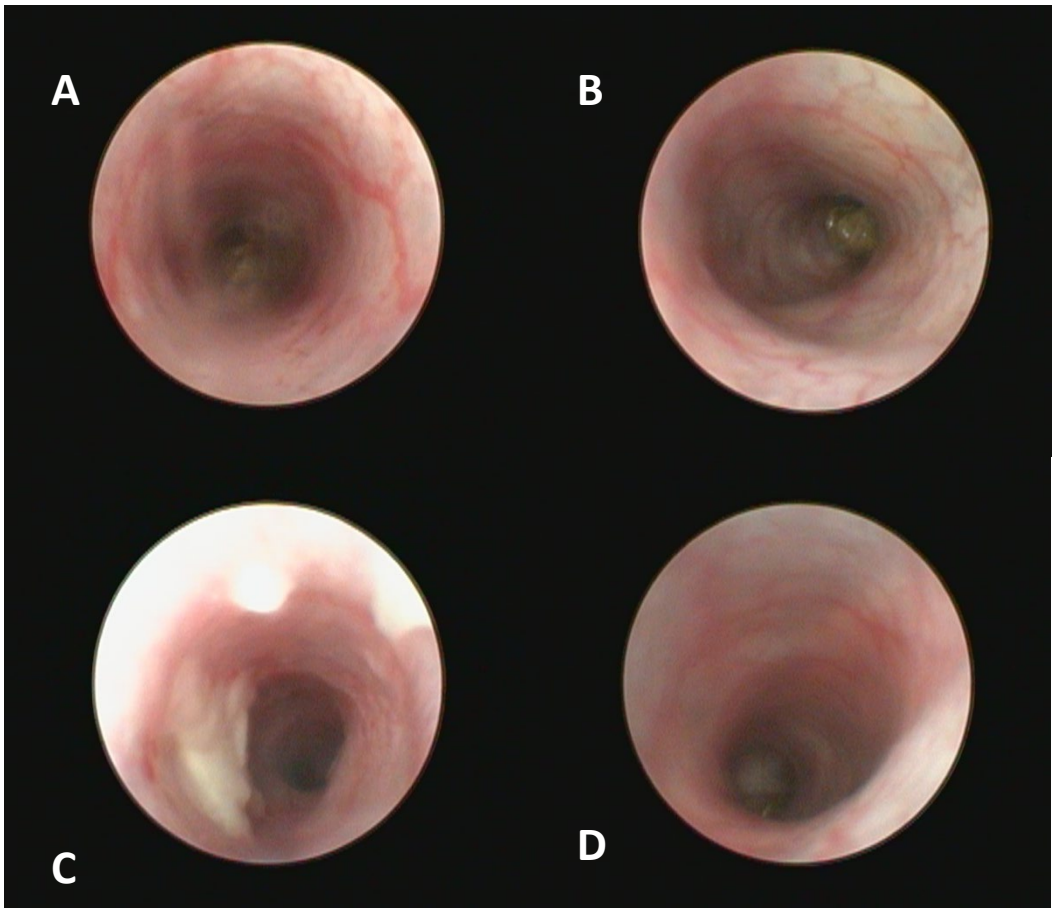

Figure 3.6: *Colonoscopy Images of Trial 1 Females.* Representative colonoscopy images of trial 1 females taken on experimental day 8. Image A corresponds to H2O+Vehicle, image B H2O+I3C, image C DSS+Vehicle, image D DSS+I3C. Image C, DSS+Vehicle, displays significant levels of tissue sloughing, decreased wall transparency, and spontaneous colonic bleeding. DSS+I3C, image D, shows significant reduction in symptoms displayed in image C.

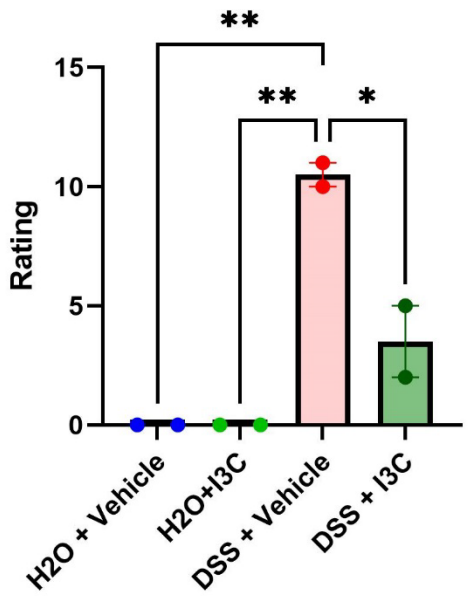

Figure 3.7: *Colonoscopy Scores of Trial 1 Females.* Scores of colonoscopy images from trial 1 females taken on experimental day 8 (*=0.05 and **=0.01 for P value).

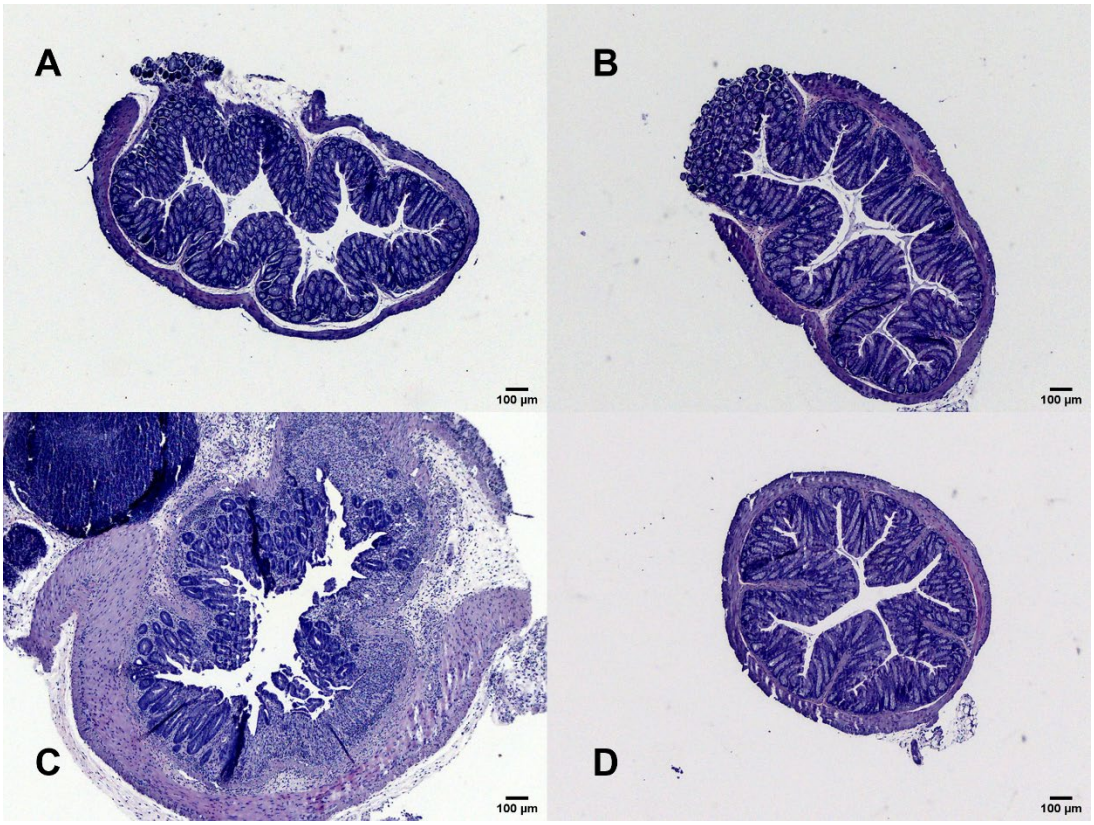

Figure 3.8: *Distal H&E-Stained Light Microscope Images from Trial 1 Females.* Motic Swift Line M3603C light microscope images of representative H&E-stained distal colon samples at 4x magnification from trial 1 females. Image A corresponds to H2O+Vehicle, image B H2O+I3C, image C DSS+Vehicle, image D DSS+I3C. Image C, DSS+Vehicle displays significant levels of epithelial cell loss, goblet cell damage, colonic crypt loss, and inflammation. Image D displays reduction in these symptoms after treatment with I3C.

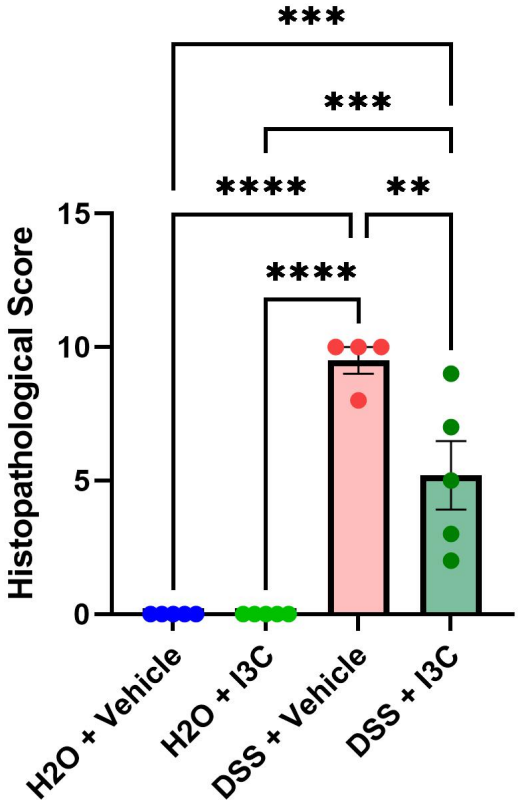

Figure 3.9: *Histopathological Scores from Trial 1 Females.* Histopathological scores of distal H&E-stain colon samples examined on Motic Swift Line M3603C light microscope at (**=0.01, ***=0.001, ****=0.0001 for P value).

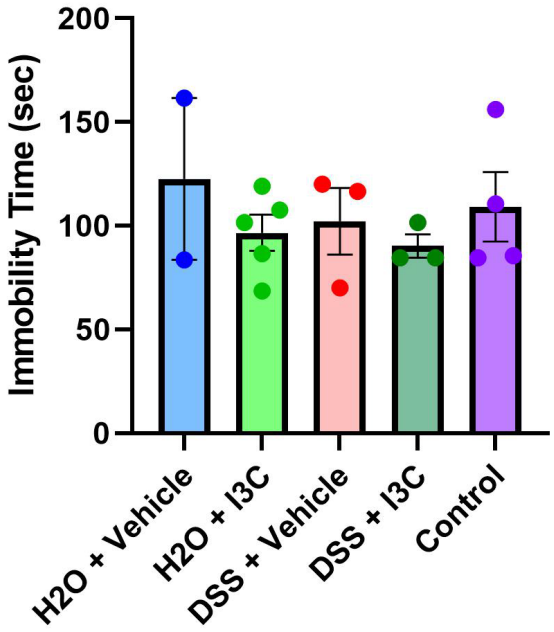

Figure 3.10: *Day 0 Daytime TST Trial 1 Females 2 Minute Latency.* Immobility times in TST performed during daytime hours on experimental day 0 for trial 1 females using 2 minute latency period (n=5 per group, missing data represents mice who tail-climbed or failed video playback). No significant differences were found.

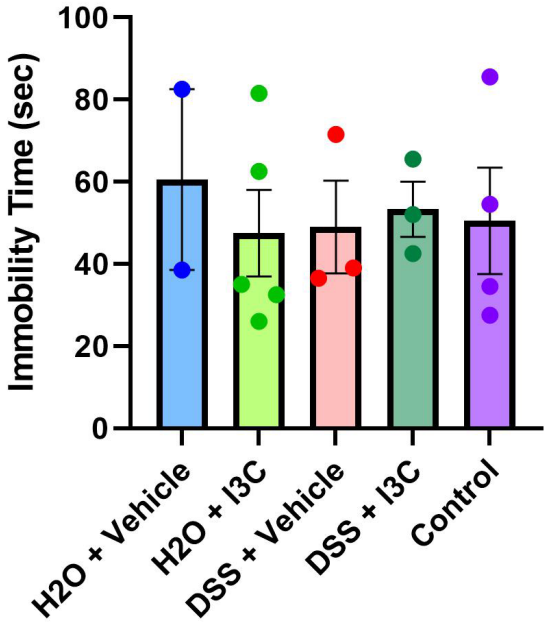

Figure 3.11: *Day 0 Daytime TST Trial 1 Females 4 Minute Latency.* Immobility times in TST performed during daytime hours on experimental day 0 for trial 1 females using 4 minute latency period (n=5 per group, missing data represents mice who tail-climbed or failed video playback). No significant differences were found.

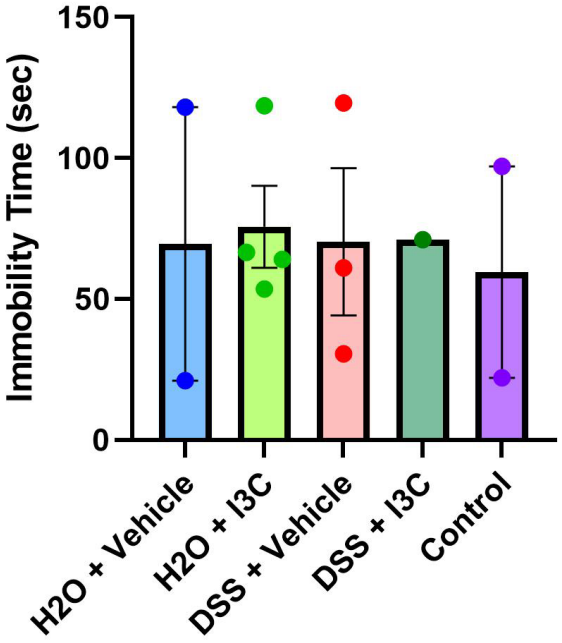

Figure 3.12: *Day 6 Daytime TST Trial 1 Females 2 Minute Latency.* Immobility times in TST performed during daytime hours on experimental day 6 for trial 1 females using 2 minute latency period (n=5 per group, missing data represents mice who tail-climbed or failed video playback). No significant differences were found.

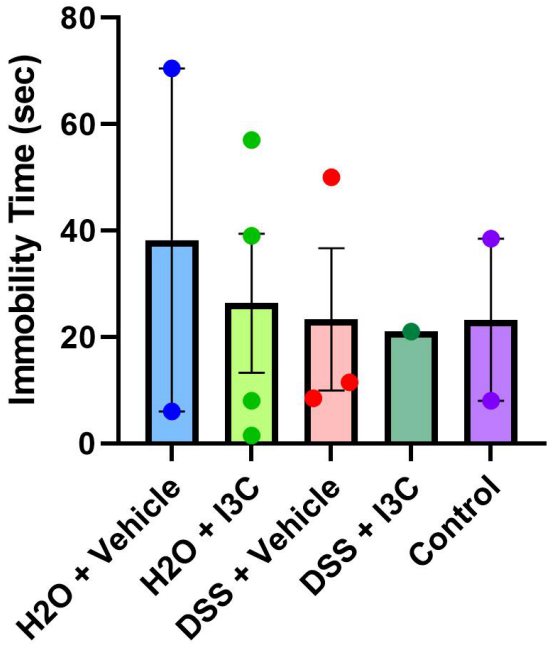

Figure 3.13: *Day 6 Daytime TST Trial 1 Females 4 Minute Latency.* Immobility times in TST performed during daytime hours on experimental day 6 for trial 1 females using 4 minute latency period (n=5 per group, missing data represents mice who tail-climbed or failed video playback). No significant differences were found.

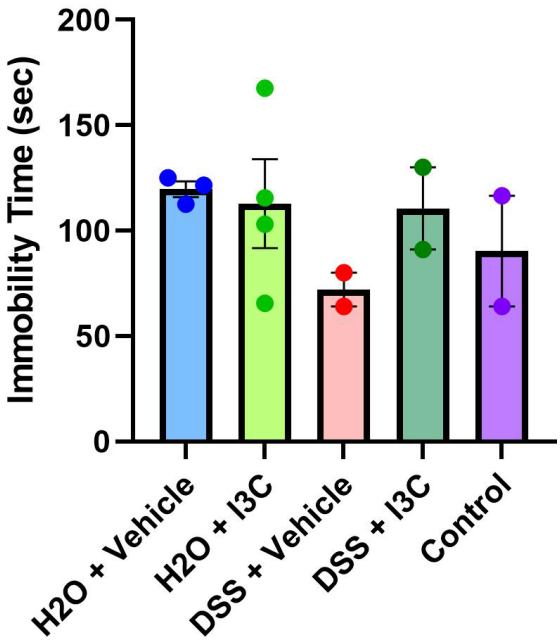

Figure 3.14: *Day 9 Daytime TST Trial 1 Females 2 Minute Latency.* Immobility times in TST performed during daytime hours on experimental day 9 for trial 1 females using 2 minute latency period (n=5 per group, missing data represents mice who tail-climbed or failed video playback). No significant differences were found.

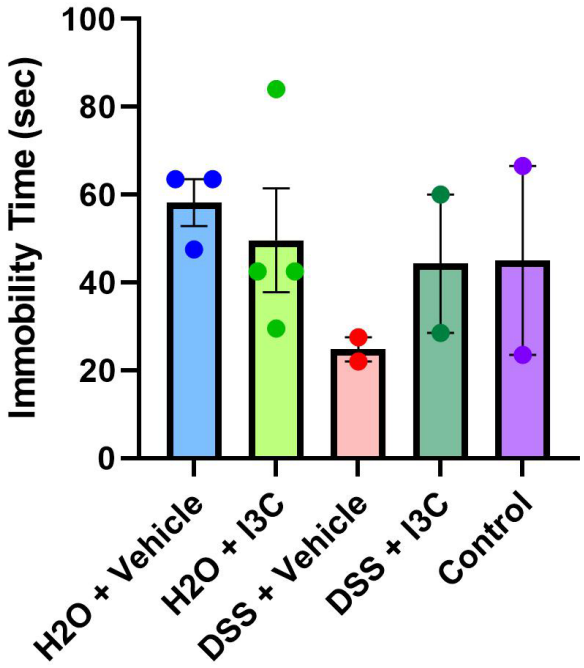

Figure 3.15: *Day 9 Daytime TST Trial 1 Females 4 Minute Latency.* Immobility times in TST performed during daytime hours on experimental day 9 for trial 1 females using 4 minute latency period (n=5 per group, missing data represents mice who tail-climbed or failed video playback). No significant differences were found.

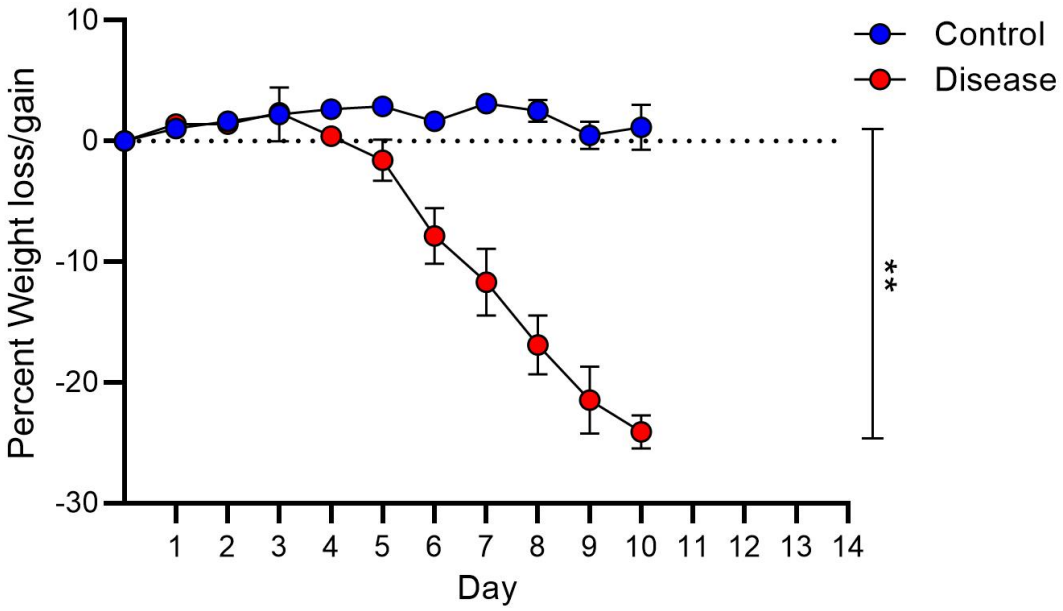

Figure 3.16: *Percent Weight Loss/Gain of Trial 1 Males.* Percent weight loss/gain in trial 1 males where **=0.01 for P value (n=4 for disease, n=3 for control).

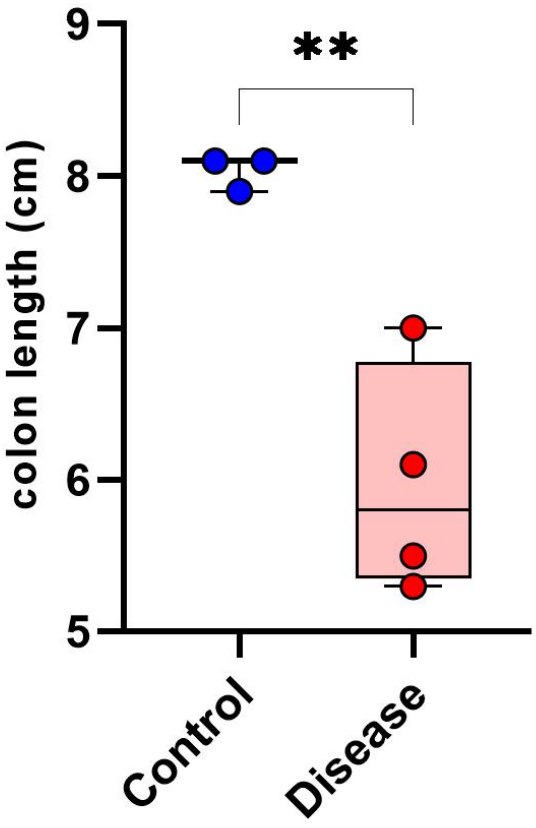

Figure 3.17: *Colon Length (cm) Trial 1 Males.* Colon length measured in centimeters for trial 1 males where **=0.01 for P value (n=4 for disease, n=3 for control).

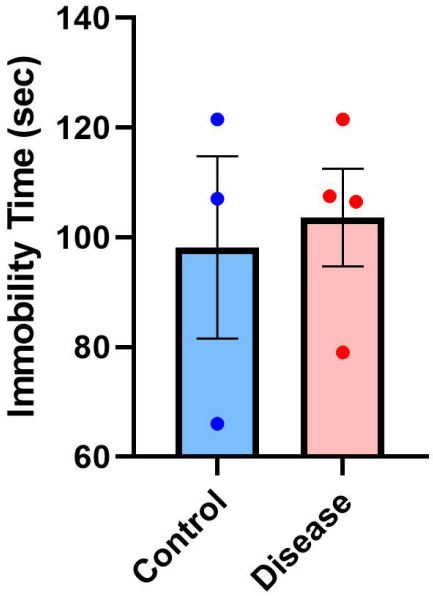

Figure 3.18: *Day 0 Daytime TST Trial 1 Males 2 Minute Latency.* Immobility times in TST performed during daytime hours on experimental day 0 for trial 1 males using 2 minute latency period. No significant differences were found.

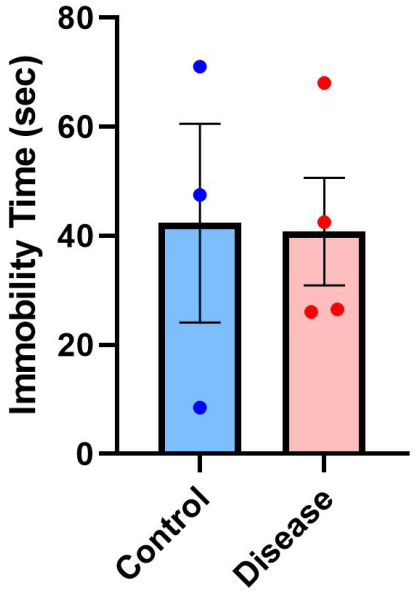

Figure 3.19: *Day 0 Daytime TST Trial 1 Males 4 Minute Latency Immobility.* times in TST performed during daytime hours on experimental day 0 for trial 1 males using 4 minute latency period. No significant differences were found.

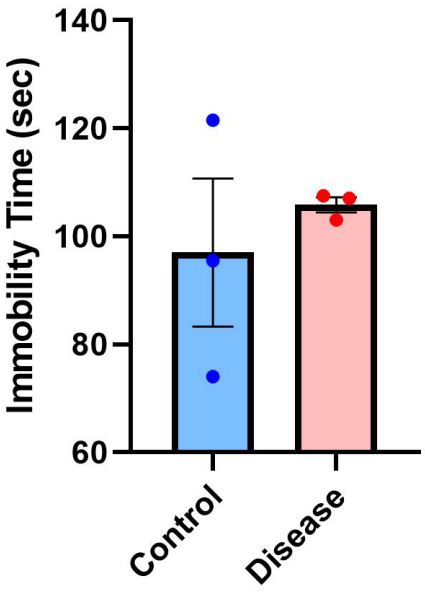

Figure 3.20: *Day 6 Daytime TST Trial 1 Males 2 Minute Latency.* Immobility times in TST performed during daytime hours on experimental day 6 for trial 1 males using 2 minute latency period. No significant differences were found.

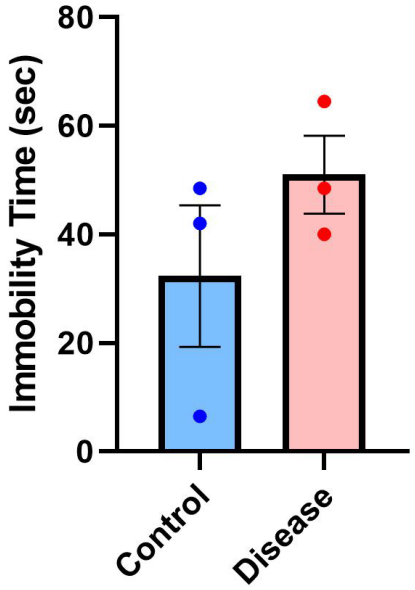

Figure 3.21: D*ay 6 Daytime TST Trial 1 Males 4 Minute Latency.* Immobility times in TST performed during daytime hours on experimental day 6 for trial 1 males using 4 minute latency period. No significant differences were found.

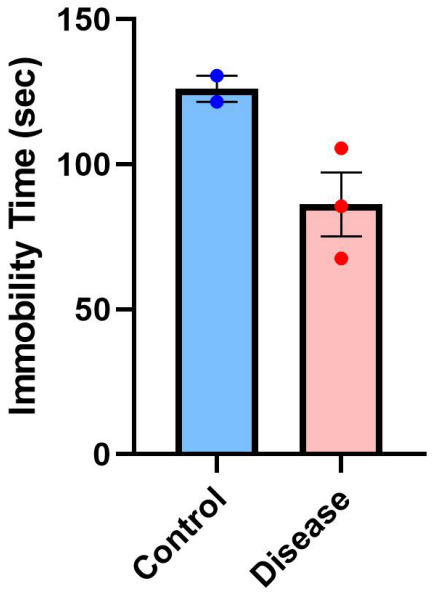

Figure 3.22: *Day 9 Daytime TST Trial 1 Males 2 Minute Latency.* Immobility times in TST performed during daytime hours on experimental day 9 for trial 1 males using 2 minute latency period. No significant differences were found.

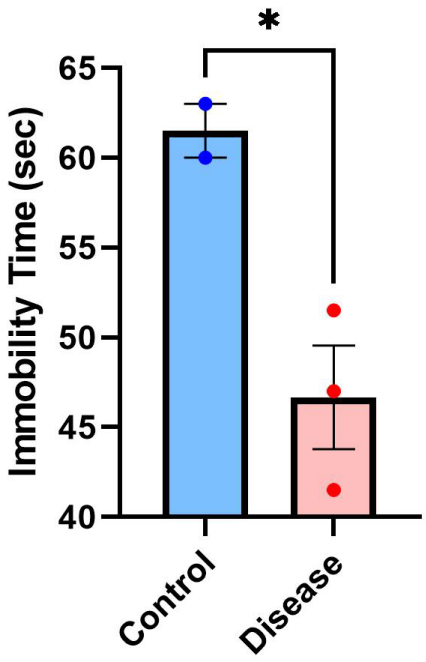

Figure 3.23: *Day 9 Daytime TST Trial 1 Males 4 Minute Latency.* Immobility times in TST performed during daytime hours on experimental day 9 for trial 1 males using 4 minute latency period. A significant difference was found in average immobility in the diseased group (*=0.05 for P value).

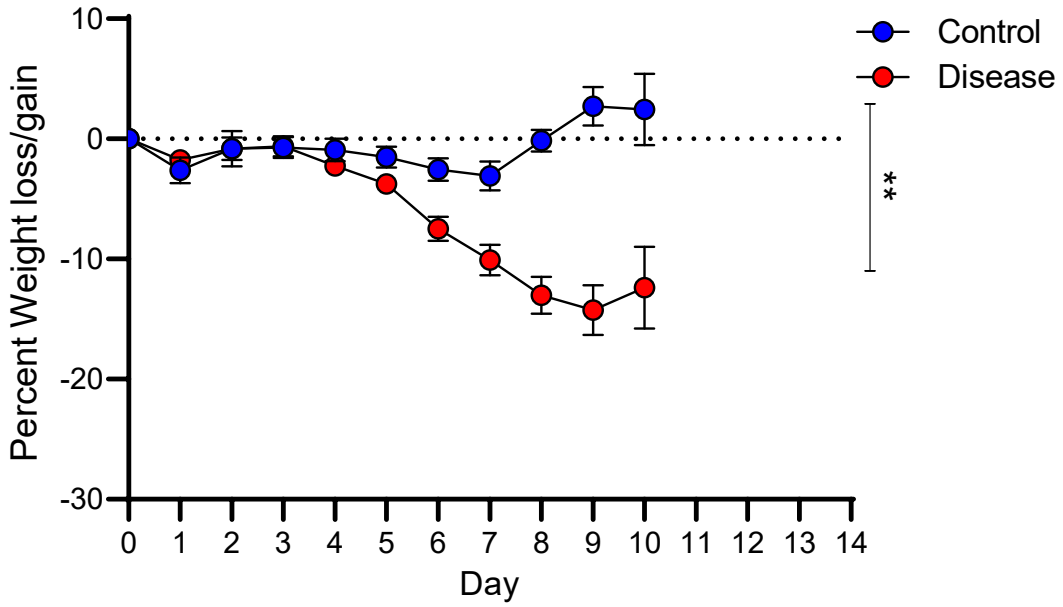

Figure 3.24: *Percent Weight Loss/Gain Trial 2 Females.* Percent weight loss/gain in trial 2 females where **=0.01 for P value (n=5 per group).

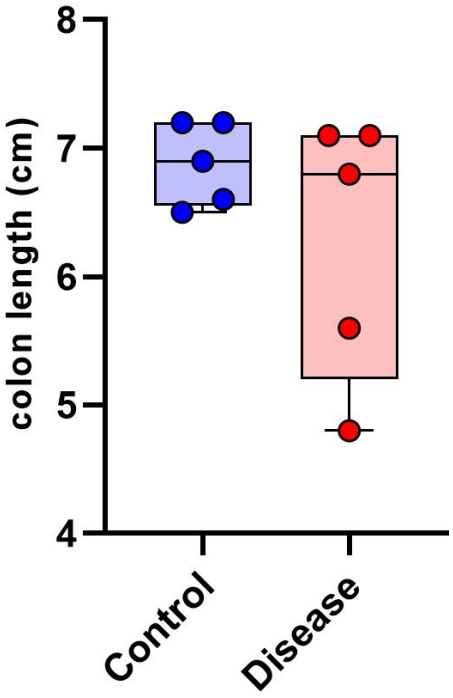

Figure 3.25: *Colon Length (cm) Trial 2 Males.* Colon length measured in centimeters for trial 2 females (n=5 per group). No significant differences were found.

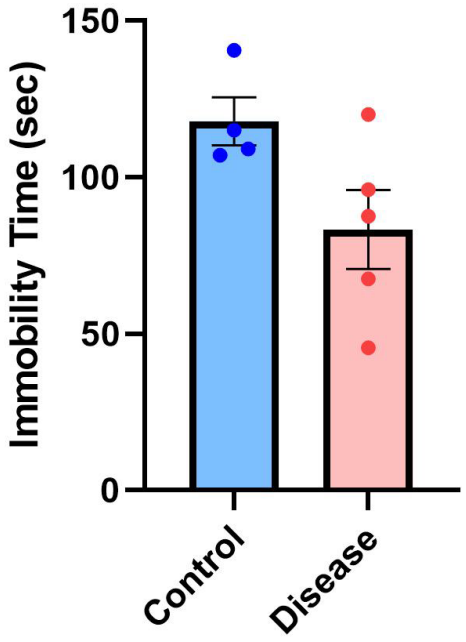

Figure 3.26: *Day 9 Daytime TST Trial 2 Females 2 Minute Latency.* Immobility times in TST performed during daytime hours on experimental day 9 for trial 2 females using 2 minute latency period. No significant differences were found.

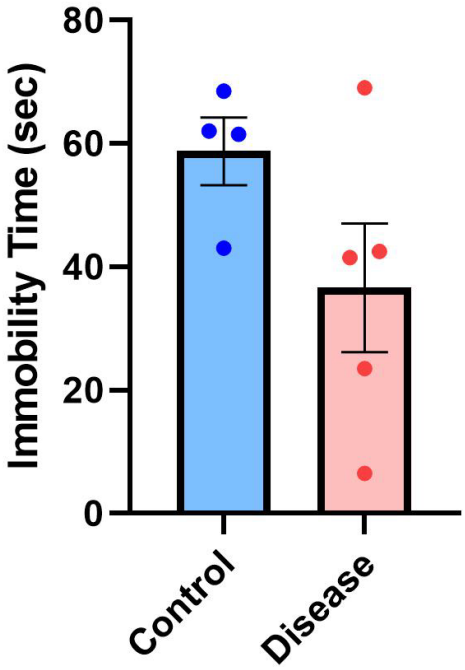

Figure 3.27: *Day 9 Daytime TST Trial 2 Females 4 Minute Latency.* Immobility times in TST performed during daytime hours on experimental day 9 for trial 2 females using 4 minute latency period. No significant differences were found.

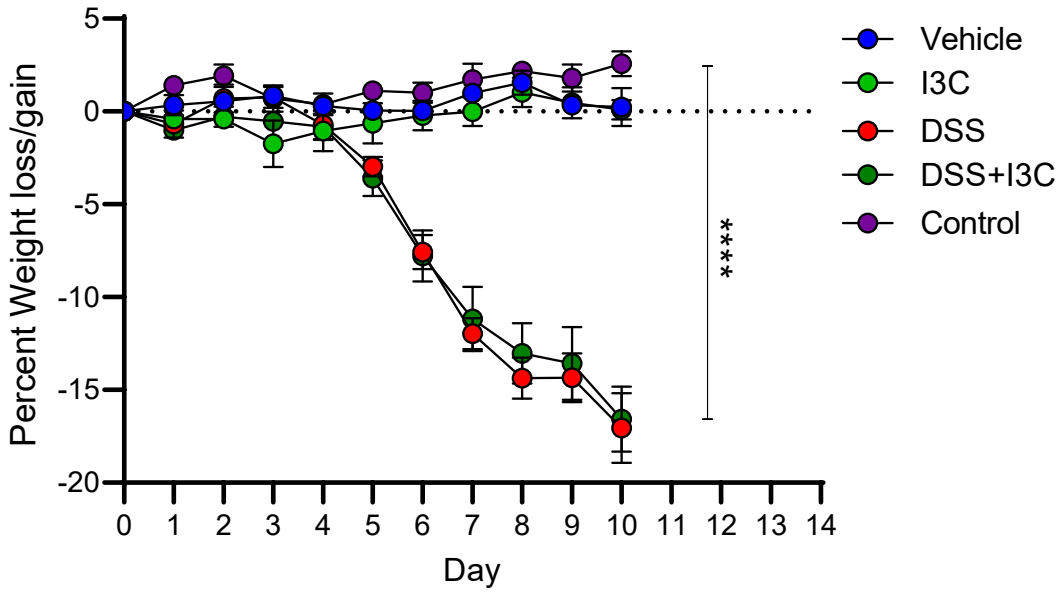

Figure 3.28: *Percent Weight Loss/Gain Trial 2 Males.* Percent weight loss/gain in trial 2 males where *=0.05, **=0.01, ***=0.001, ****=0.0001 for P value.

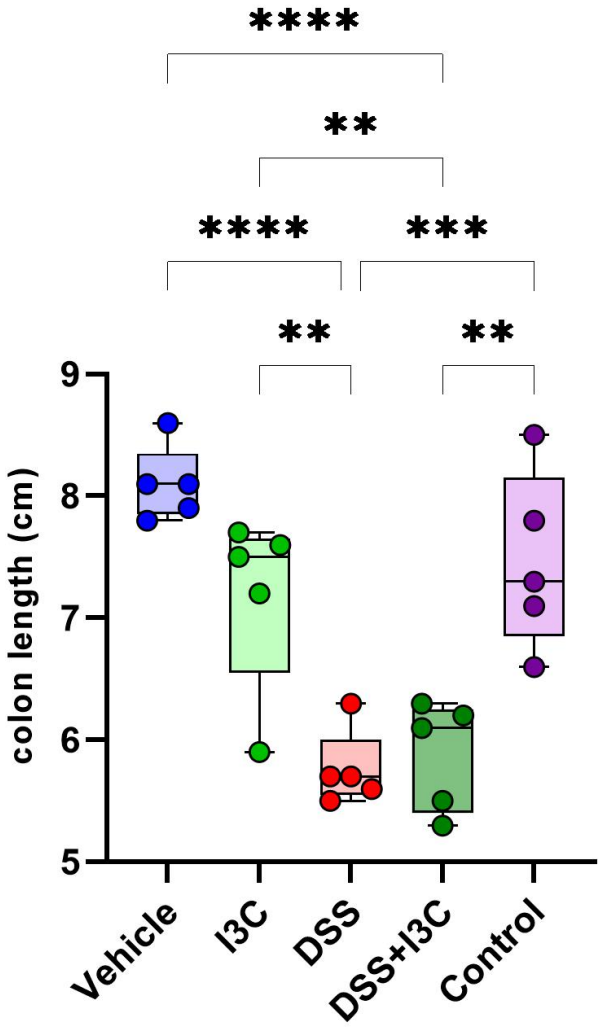

Figure 3.29: *Colon Length (cm) Trial 2 Males.* Colon length measured in centimeters for trial 2 males where **=0.01, ***=0.001, ****=0.0001 for P value.

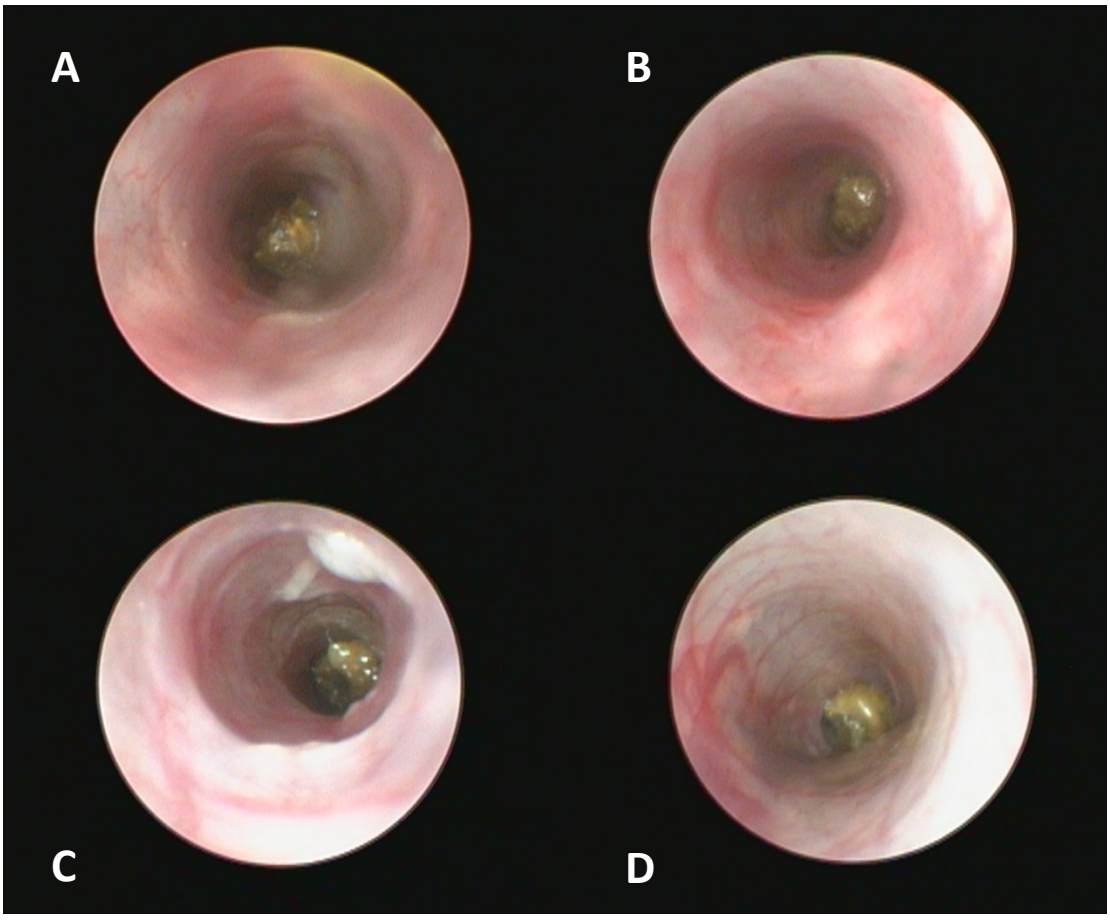

Figure 3.30: *Colonoscopy Images of Trial 2 Males.* Representative colonoscopy images of trial 2 males taken on experimental day 8. Image A represents H2O+Vehicle, image B H2O+I3C, image C DSS+Vehicle, and image D DSS+I3C. Image C, DSS+Vehicle depicts significant tissue sloughing and decreased colonic wall transparency. Image D, DSS+I3C shows nonsignificant decrease in colitis symptoms, as evidenced by blood lesion.

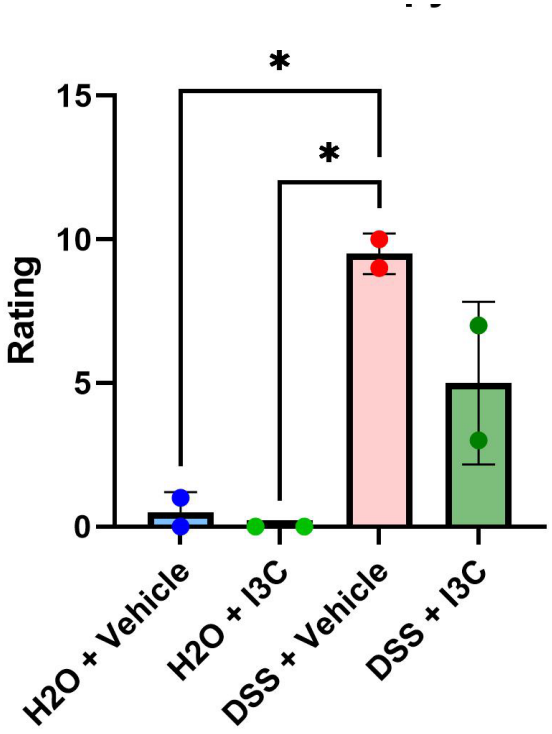

Figure 3.31: *Colonoscopy Scores of Trial 2 Males.* Scores of colonoscopy images from trial 2 males taken on experimental day 8 (*=0.05 for P value).

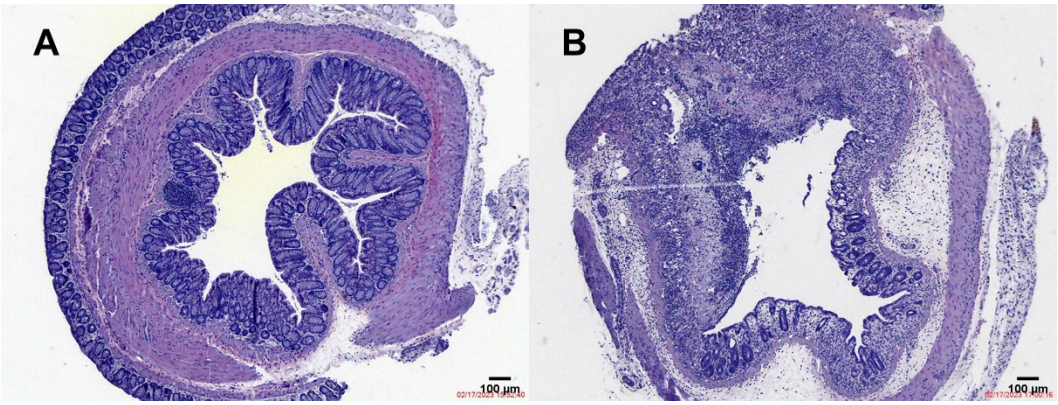

Figure 3.32: *Distal H&E-Stained Light Microscope Images from Trial 2 Males.* Light microscope images taken on Motic Swift Line M3603C of representative H&E-stained distal colon samples at 4x magnification from trial 2 males. Image A represents H2O+Vehicle; image B represents DSS+Vehicle. Note in image B significant epithelial cell loss, goblet cell damage, destruction of colonic crypts, and inflammation.

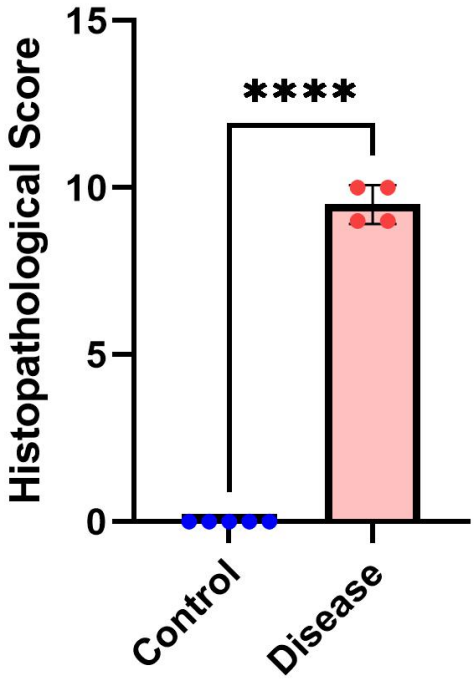

Figure 3.33: *Histopathological Score of Distal H&E-Stained Colon Samples from Trial 2 Males.* Histopathological scores of distal H&E-stain colon samples of trial 2 males H2O+Vehicle (control) and DSS+Vehicle (disease) examined on Motic Swift Line M3603C light microscope (****=0.0001 for P value).

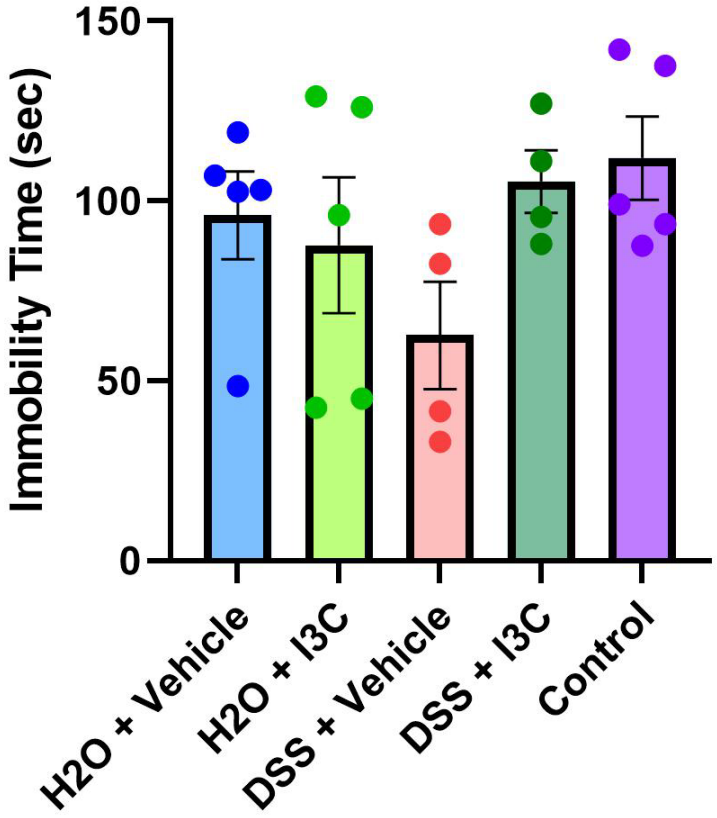

Figure 3.34: *Day 9 Daytime TST Trial 2 Males 2 Minute Latency.* Immobility times in TST performed during daytime hours on experimental day 9 for trial 2 males using 2 minute latency period. No significant differences were found.

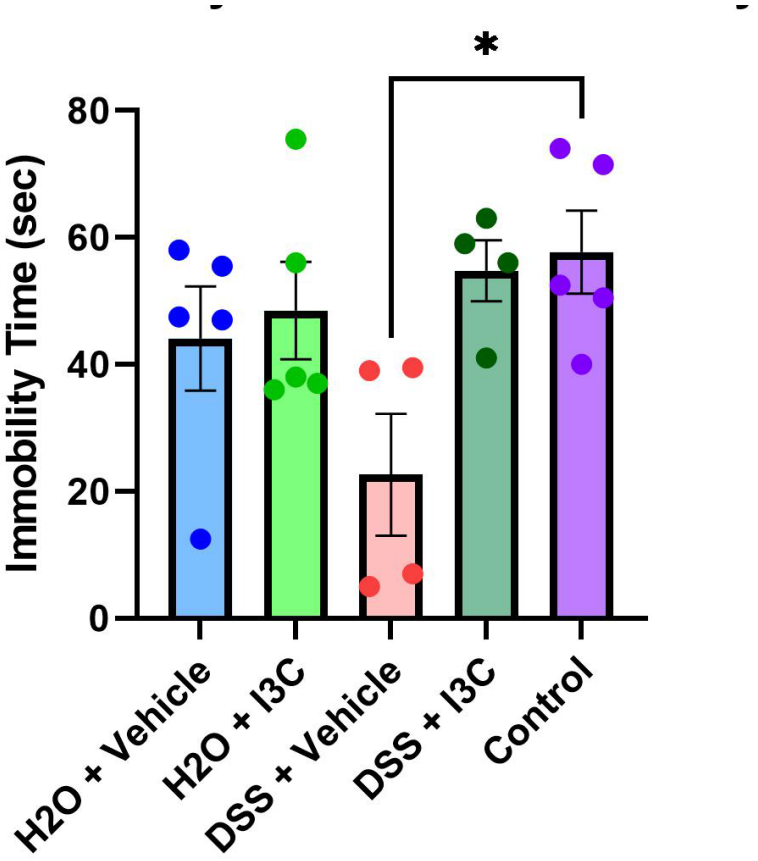

Figure 3.35: *Day 9 Daytime TST Trial 2 Males 4 Minute Latency.* Immobility times in TST performed during daytime hours on experimental day 9 for trial 2 males using 4 minute latency period (*=0.05 for P value).

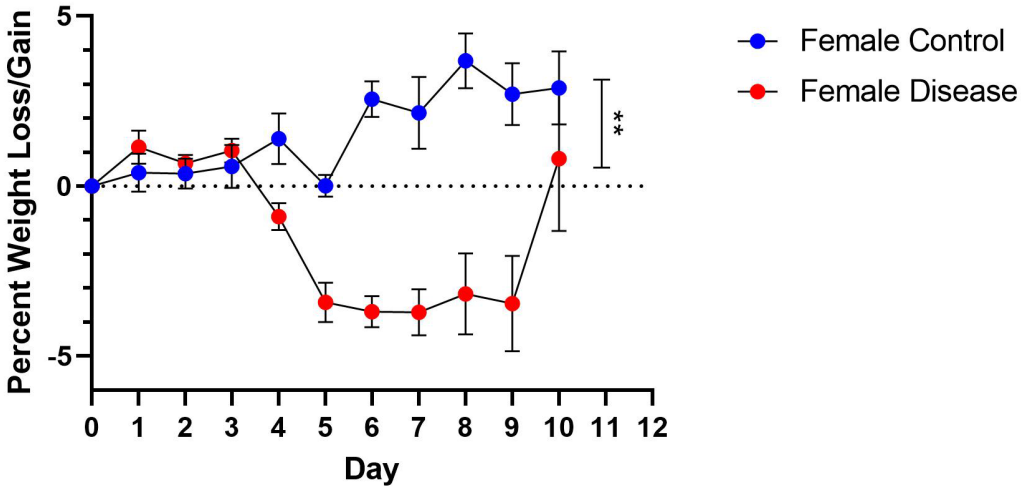

Figure 3.36: *Percent Weight Loss/Gain Trial 3 Females.* Percent weight loss/gain in trial 3 females where **=0.01 for P value.

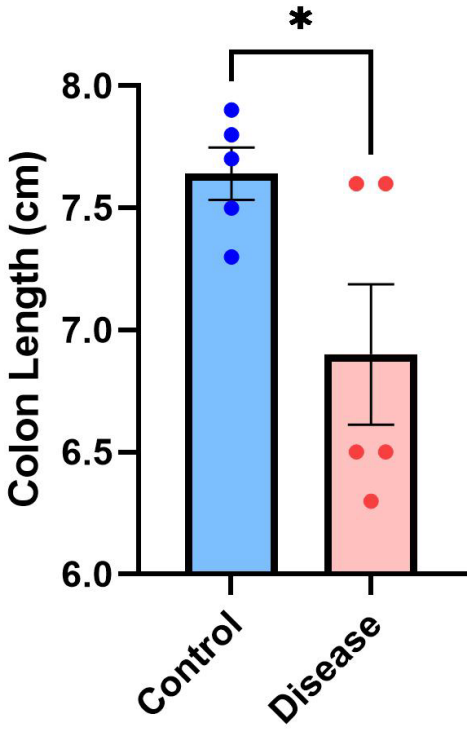

Figure 3.37: *Colon Length (cm) Trial 3 Females.* Colon length measured in centimeters for trial 3 females where *=0.05 for P value.

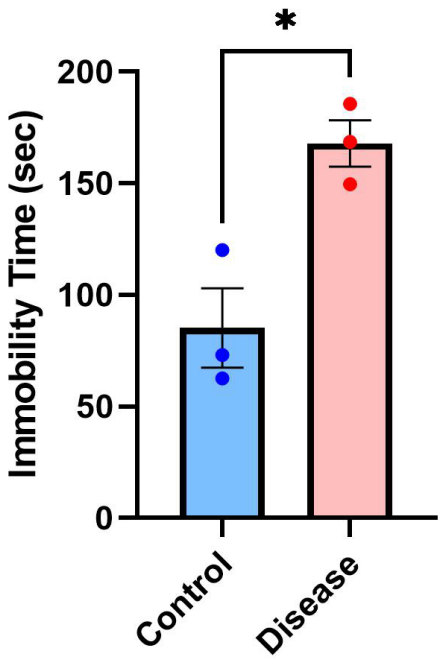

Figure 3.38: *Day 9 Night TST Trial 3 Females 2 Minute Latency.* Immobility times in TST performed during night hours on experimental day 9 for trial 3 females using 2 minute latency period (*=0.05 for P value).

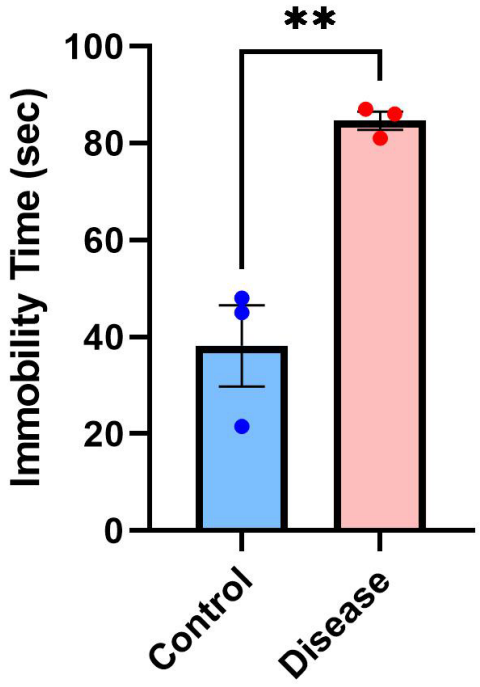

Figure 3.39: *Day 9 Night TST Trial 3 Females 4 Minute Latency.* Immobility times in TST performed during night hours on experimental day 9 for trial 3 females using 4 minute latency period (**=0.01 for P value).

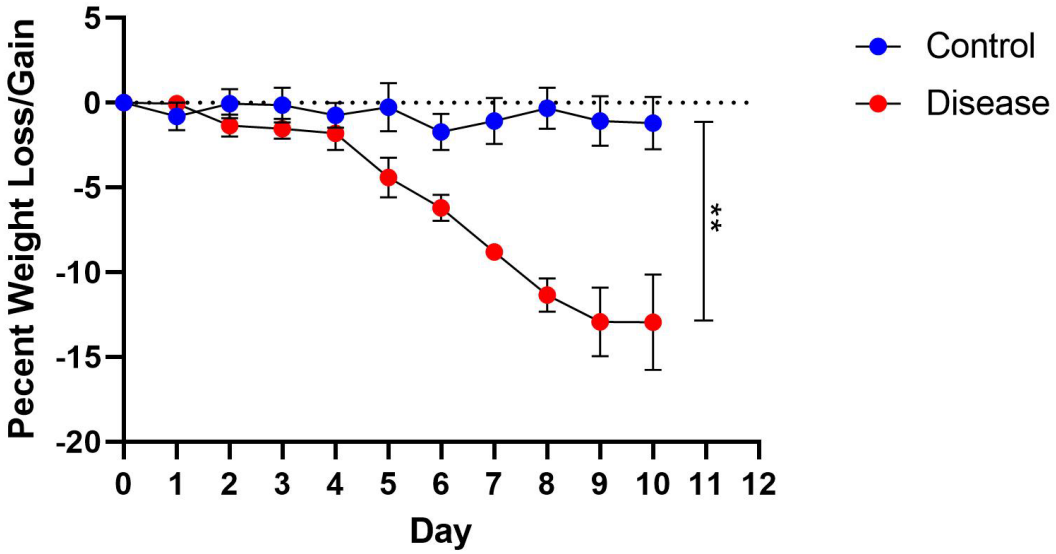

Figure 3.40: *Percent Weight Loss/Gain Trial 3 Males.* Percent weight loss/gain in trial 3 males where **=0.01 for P value.

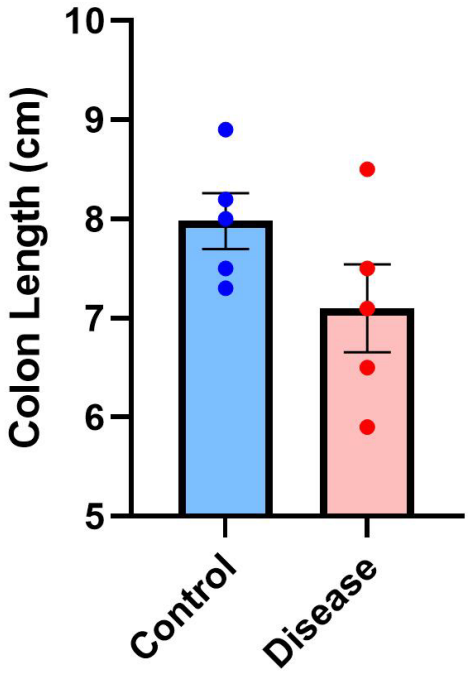

Figure 3.41: *Colon Length (cm) Trial 3 Males.* Colon length measured in centimeters for trial 3 males. No significant differences were found.

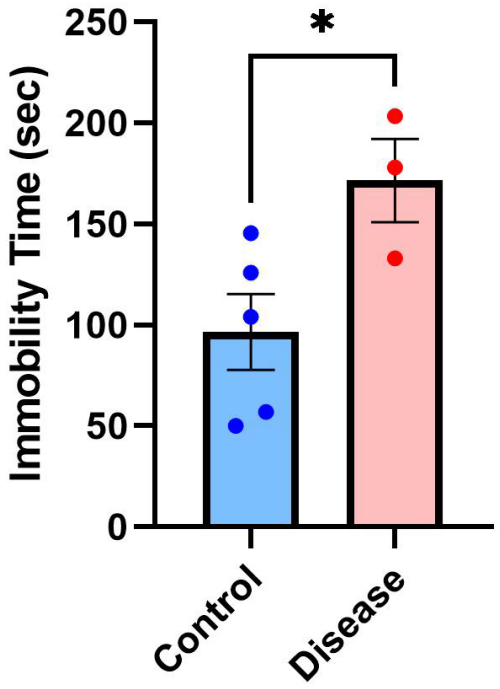

Figure 3.42: *Day 9 Night TST Trial 3 Males 2 Minute Latency.* Immobility times in TST performed during night hours on experimental day 9 for trial 3 males using 2 minute latency period (*=0.05 for P value).

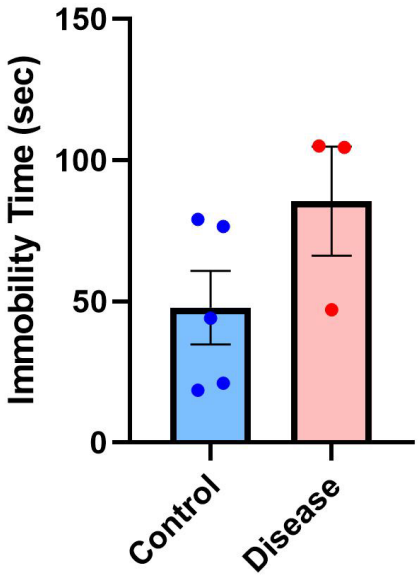

Figure 3.43: *Day 9 Night TST Trial 3 Males 4 Minute Latency.* Immobility times in TST performed during night hours on experimental day 9 for trial 3 males using 4 minute latency period. No significant differences were found.

CHAPTER 4

DISCUSSION

Current Experiment

In the current study, we investigated links between colitis and colitis-associated depression, particularly the potential role AhR might play in altering the gut metabolome to resolve both disease conditions. Results from untargeted metabolomics studies herein indicated the metabolomic profile of colitis-induced mice was different from that of healthy controls, which correlates with what is seen in human ulcerative colitis patients, particularly those diagnosed with comorbid behavioral disorders, such as depression and anxiety [108]. Most notably, QA was found to be significantly downregulated in the DSS model of colitis after treatment with I3C. This result indicated that neurotoxic QA could be involved in the mechanism in which I3C, and ultimately AhR, are known to ameliorate colitis and comorbid depression. Since QA is a known neurotoxin and recent studies have identified this metabolite as a possible contributor to both colitis and depression pathogenesis, the role of QA in both diseases should be explored further [81]. This result is in agreement with a study by Nikolaus et al. which found increased Trp metabolism lead to increased QA production in IBD patients; this same study also found an inverse association between levels of serum IL-22 and Trp in IBD patients compared to healthy controls, in line with previous findings from the Busbee lab [37, 85].

Clinical data from trial 1 males and females indicate successful disease induction with DSS as evidenced by colon length shortening, microscopic evaluation, and

colonoscopy images. I3C treatment in trial 1 females proved successful in ameliorating colitis symptoms as evidenced by colonoscopy and microscopic evaluation. Furthermore, investigation in trial 2 males and females also showed DSS to have successfully induced colitis symptoms. I3C did not significantly reduced colitis symptoms as evidenced by weight loss, colon length shortening, and colonoscopy images. In both trial 3 males and females DSS proved successful in disease induction as indicated by weight loss and colon length data. Summarily, clinical data from all trials indicates of successful induction of inflammation and ultimately colitis.

In expanding our immunology-based colitis and treatment studies into behavioral studies investigating comorbid depression states, trials using the TST were evolved over the course of our studies in order to best test our parameters and determine which experimental schema produced the most representative and replicative results. Trials were primarily evolved in terms of amount of TSTs and time of day of TST. In initial trials, unexpected results were obtained, particularly in regard to immobility times during the day cycle of tested experimental mice. In initial trials with daytime TST, disease mice had decreased mobilities on average. These results defied findings from other labs which have noted mice with inflammation to have increased average immobility in daytime TST when compared with controls [87]. Several ideas were postulated as to why these initial trials resulted in opposite trends in TST as in similar previous reports. Primarily, it is to be noted that this is a relatively new avenue of biomedical research and few labs have conducted experiments with similar parameters, thus pioneering data comes with hardships. Additionally, small sample size with no direct replicative trials is to be considered as a factor. In assessing clinical data, there is sufficient evidence that DSS did work to induce

colitis, and in most cases so did the treatment, at least in female mice. However, the question arises as to if this acute inflammation is persistent enough to lead to depressive-like symptoms, but results using other models of acute inflammation have measured TST increased immobility in murine models as compared to controls [89].

Another possible explanation lies in the normal behavior of mice. Since mice are inherently nocturnal, we postulate that perhaps time of day affected disease immobility in TST, though other labs have found significant increases in immobility of disease mice in daytime TST [87]. Time of day has been identified in previous studies as a critical biological variable for behavioral tests; however, many labs do not report time of day behavioral tests were conducted [109]. These findings highlight the importance of optimized and streamlined protocol of TST across the scientific community. Furthermore, it is of concern that many mice learned to beat the TST, thus reducing experimental power, and that multiple TSTs can induce an inherent level of stress on subjects which can skew results. Since stress has been shown to both increase intestinal permeability and BBB permeability, we sought to reduce stress by reducing the number of TSTs [58, 110].

Inasmuch, re-evaluating the manner in which the tests were conducted were explored in the later studies. In order to replicate previous reports showing increased immobility during TST, a marker of depressive-like behavior in mice, our later trials only included male and female disease and healthy controls. The difference in the later trials was that TST readings were taken during the night cycle, when mice are in their more active state. Results from these studies indicated colitis-induced depressive-like symptoms were observed in females and males in our disease groups, thus being more aligned with anticipated results based on past literature [111]. It is puzzling as to why TST data from

other labs have shown increased immobility is present in sick and inflamed mice during both day and night TST, but our data only reflects this trend during night TST. More investigation and additional trials are necessary to elucidate why.

Lastly, it was interesting to note that although the trend in diseased mice were indicative of less depressive-like state in diseased groups, I3C treatment did reverse this observation, restoring immobility times to control levels. This is in accordance with findings from other labs which have indicated I3C to alter depressive-like behavior, however in the opposite direction observed in previous studies [87]. However, results from these studies are of significance as I3C treatment appeared to reverse depressive-like behavior trends compared to colitis-induced mice. This is a new and exciting finding, and future studies will aim at furthering understanding how AhR activation, via treatment with compounds such as I3C, might better improve colitis disease outcome from both an inflammatory and behavioral perspective.

Limitations

This experiment only explored the relationship of IBD and depression in a model most fitting to that of UC. More experiments are necessary to explore this same relationship in the context of CD in order to best capture IBD as a whole. A limitation exists in using DSS as mode of colitis induction as well. Since DSS is administered through drinking water, this method is dependent on mice consuming sufficient water to induce disease. Further, this model also represented an acute model of colitis versus a chronic model. Since colitis in humans is inherently chronic inflammation with acute flareups, investigation into a chronic inflammatory model is warranted. It is to be noted that murine experiments, though widely used in biomedical research, are imperfect models of human disease.

Treatment injections of I3C for colitis are not guaranteed to work synonymously in human models as in mouse models. I3C is currently available as a dietary supplement and is safe for human consumption, but I3C as a treatment for colitis through injection must be studied in human clinical trials to further investigate. Other limitations with our animal models exist, for instance, the age of our experimental mice varied from 10 to14 weeks of age which could influence results. Dosage for I3C injection was aimed at 40mg/kg, having been calculated for 25-gram mice. Not all mice used in this experiment were precisely 25 grams, thus providing room for variation in results. In past experiments, the Busbee lab has weight matched groups to account for this variability. However, we did not weight match groups as groups were determined at arrival to the animal facility based on shipped cage mates. Experimental mice were grouped as arrived from the manufacturer to reduce stress of rehousing and conflicts between unfamiliar mice.

A limitation also exists in using only one behavioral test to indicate depressive-like symptoms in mice. In the near future, we will implement the sucrose preference test (SPT) which assesses anhedonia as another measure of depressive-like behavior in mice. Moreover, there exists limitations in using the C57BL/6 mouse model in TST. It has been noted in literature that this mouse strain often beats the TST by tail climbing, which did indeed occur multiple times throughout this experiment [112]. To overcome this obstacle, more trials are necessary for desired sample number. Publications from other labs have utilized ‘climbstoppers’ to prevent this behavior; if this problem persists we may include these in the future [113]. Furthermore, it has been noted that use of TST to conduct experiments concerning the investigation of colitis and depression is a relatively new avenue of research, thus our lab employed the TST in a variety of ways in attempt to

determine the most optimal experimental schema. Our lab began on initial trials with TST on day 0, day 6 and day 9 in the morning hours between 10am-1pm. With this schema a very large amount of mice learned to beat the TST by tail climbing. In concern that multiple TST may induce a level of inherent stress and learning not previously considered, our lab began TST only on day 9 the morning before sacrifice day. This resulted in less tail climbs but yielded similar results in high immobility across groups. Considering that mice are naturally nocturnal, the last experimental trial our lab employed TST on day 9 during dark cycle hours to mimic when mice would be most active. Studies from other labs have shown that behavioral testing during light versus dark hours often yield significantly different results, which our results certainly agreed with. Thus, more trials conducted under the dark cycle are critical and necessary [109, 114].

In addition to the aforementioned limitations, several issues with the TST apparatus were discovered, which included invalid power and energy output, lack of sensitivity to detect mouse movements, and inconsistent outputs amongst apparatus wells. Due to this, hand timing was the only viable option for proper data analysis. With hand timing, there exists the inherent possibility of human error in timing immobility in TST. To mitigate this error, two time points were taken from different lab members and then averaged together for final immobility times. An advantage of hand timing is the allowance of analyzing results with a variety of latency periods. Our lab analyzed TST data three different ways – 5 second latency period, 125 second latency, and 245 second latency period. To obtain more concrete results, an optimal latency period must be uncovered and will remain a limitation until such. Publications from other labs have used a variety of hanging durations and latency periods, it remains unclear which of these protocols is best suited for this

investigation [115-117]. Due to the apparatus problems which resulted in hand timing for immobility, timers were reliant on video feedback to time subjects. Unfortunately, data points were lost from failed playbacks. It was discovered throughout hand timing that if a mouse beat the test before all mice in that particular run were loaded, and the timer stopped that well, the video playback did not capture the next mouse video. Thus, for subsequent experiments, if a mouse tail climbed and beat the test, we did not 'stop' the mouse well until all mice were loaded for that run. Thankfully this error was mitigated by an early catch, but nonetheless – data points were lost. Of other limitations, this is the first time our lab has utilized TST thus, novice experience in this test is to be noted.

Future Directions

Our current study focused on a model best suited for the study of UC, in the near future the TNBS model will be employed in Balb/c mice to best capture IBD as a whole. This experimental schema represents an acute model of colitis. Thus, since colitis is inherently a chronic disease with acute flareups, investigation into chronic colitis inflammation is necessary and will be employed as a future direction. Furthermore, our lab has intentions of cytokine profiling – looking at both pro- and anti- inflammatory cytokines in circulation and in local tissues (colon and brain) in respect to behavioral adaptation in relation with IBD. Particularly, we intend to investigate IL-22, a chief cytokine involved in with AhR and I3C. Previous studies from our lab have shown the efficacy of I3C to be IL-22 dependent, thus leading to questions regarding how IL-22 could be mediating the I3C effect on other factors in association with both colitis and depression [37]. Of interest also are proinflammatory cytokines TNF-α, IL-1β and IL-6; additionally, anti-inflammatory cytokines such as IL-4 and IL-10 which have been shown to be altered in

both colitis and depression [87, 118]. Other members of the Busbee lab have utilized AhR knockouts: AV mice which have AhR knocked out in colonic epithelial cells and AR mice which have AhR knocked out in immune Rorc-expressing cells (e.g. IL-22 producing immune cells). Of potential interest to the future of this project is the employment of these knockout mice in order to better determine the role of AhR in colitis-associated depression.

We also aim to conduct FITC-Dextran permeability assays to investigate intestinal permeability to better validate our claims of "leaky gut". Retro-orbital injections of methylene blue or Evans blue will also be conducted for BBB permeability investigation. In order to quantify BBB leakage, we aim to conduct confocal imaging on brain sections using a combination of Evans blue and high molecular weight FITC-Dextran according to the protocol developed by Xu et al [119]. In order to better obtain data from brains, we also intend to solidify a perfusion protocol in order to best isolate substances from the brain and heighten our microscopy imaging capabilities. Moreover, we aim to incorporate other imaging methods in our brain analysis like light microscopy immunohistochemistry or visualization of the BBB tight gap junctions with transmission electron microscopy. Beyond, we aim to quantify BBB components and lymphocytes in the mouse brain through utilization of flow cytometry. Of specific quantification interests are brain microvascular endothelial cells and adherence of monocytes and T-cells, similar to a protocol developed by Williams et al. [120]. We also would like to investigate brain M1 microglia which are implicated in neuroinflammation in response to proinflammatory cytokines using flow cytometry similar to methods conducted by Srakočić et al. [121, 122].

To expand on the current project, our lab aims to solidify an optimal TST protocol in terms of 1) time of day to conduct TST (light vs dark cycle) and 2) optimal latency

period. Once TST protocol is optimized, more experimental trials will be necessary to accrue reliable data. In parallel, we hope to establish a baseline immobility for healthy control mice by increasing sample numbers. Of immediate requirement is investigation into night TST with mice treated with I3C to see if there are any differences observed in immobility trends in I3C treated animals as compared to daytime TST. It is also a goal to acquisition better use of our current TST apparatus in order to avoid hand timing and any potential bias which may come with such. In the future, we would be interested in expanding this project to include investigation on stress and anxiety in relation to IBD. Evidence suggests that there exists a relationship between stress, anxiety, and depression [123, 124]. Moreover, studies have shown that chronic stress promotes colitis by disturbing the gut microbiota and triggering immune system response [125, 126]. Studies have also linked high levels of anxiety to IBD; in systematic review and meta-analysis it was found that up to one third of IBD patients experienced anxiety symptoms [60]. Thus, beyond the TST, we hope to include more behavioral testing (forced swim test, sucrose preference, elevated plus maze, etc.) to best determine the relationship between IBD, depression, and other associated behavioral manifestations like stress and anxiety.

Additionally, our lab would be very interested in conducting gut microbiome analysis with colitis-induced depressed mice. The human gut is colonized by bacteria at birth and changes composition throughout host development. The average adult intestine contains a total of approximately 100 trillion microbes, equating to ten times the number of human cells in the body [127]. Thus, the gut-microbiome contains more than 5 million genes, outnumbering host genetic potential by two orders of magnitude [128-130]. The subsequent symbiotic relationship equates to the metabolic capacity of the liver; thus gut-

microbiota are effectively an additional organ [131]. Consequently, marked gut-microbiota alterations are associated with IBD presentation and changes in gut-microbiota equate to changes in metabolomic products and activities. In both UC and CD, gut-microbiomes display decreases in overall biodiversity as compared to healthy controls, along with phylum level increases in Proteobacteria and decreases in Firmicutes [132]. Many publications have shown significant differences in gut microbiota in depressed patients versus healthy controls [133, 134]. In line with other publications, it is a goal of the Busbee lab to analyze gut microbiota contents of colitis-induced depressed and healthy control mice using 16s rRNA sequencing to determine gut microbiota differences in said mice [135]. Additionally, fecal microbiota transplant (FMT) studies have introduced a promising avenue of biomedical research in both colitis and depression. FMT studies have shown that transference of fecal matter from both colitis patients and depressed human patients into GF mice result in altered colonic function and altered behavior [98, 133, 136, 137]. Thus, exploring this avenue is a promising future direction for the Busbee lab.

It is our primary goal to quantify Trp metabolites (also called kynurenines) –KYNA and chiefly QA from feces, blood, and brain. In the future, we will isolate and quantify these kynurenines in efforts to connect them to decreased/increased depression in experimental animals. Thus far our efforts to do so have remained fruitless as Trp metabolites lack methods for quantitative and simultaneous detections with desired sensitivity and specificity [138]. Yet another issue with isolating metabolites of Trp relates to their physical properties and structure. Many contain isomers that are impossible to distinguish from one another using traditional antibody-based techniques. Current methods for detection of our chief metabolite, QA, involve gas chromatography mass spectrometry

with derivatization to form a hydrophobic ester which we are in process of attempting [139]. If problems with quantification and detection persist, our lab intends to measure enzymes involved in the TRP-KYN pathway such as IDO, KATs and KMO to obtain data on the presence of downstream kynurenines. Particularly, KMO is the primary enzyme which shunts the breakdown of kynurenine towards production of neurotoxic QA versus production of neuroprotective KYNA. Thus future studies involving use of a KMO inhibitor are of particular interest [140].

The "monoamine hypothesis of depression" is of relevance to future directions of this project in regards to assessment of depression parameters. This hypothesis states that imperative brain functions depend on the presence and actions of various neurotransmitters at both the pre- and post-synaptic membranes of neurons in the brain [141]. This hypothesis essentially proposes that decreased availability of known mood regulators, like dopamine, norepinephrine, and serotonin, result in decreased neurotransmission and impaired cognition which may lead to depression. Studies have confirmed that decreased serotonin and dopamine are closely related to depression [142, 143]. Taking this into account, our lab intends to conduct enzyme-linked immunosorbent assays (ELISAs) using whole brain and sectioned brain supernatant in an attempt to detect and quantify amounts of both serotonin and dopamine in colitis induced depressed mice compared to healthy controls. Of note is serotonins relationship to inflammatory markers and the TRP-KYN pathway. Increased inflammatory markers lead to increased IDO which shunts available Trp to be degraded by the TRP-KYN pathway toward potentially neurotoxic QA and away from production of serotonin [144]. In consideration that serotonin also promotes gut motility, isolating this compound will be necessary [145].

Prospectively, we aim to examine the brain tissue from specific brain regions like the hippocampus and prefrontal cortex. Many studies have looked specifically at these regions due to their relationship to neuroplasticity which is said to be disrupted in depression. Neuroplasticity can be defined as the brain's capacity to rewire its connections and reshape its structure by strengthening or weakening synaptic transmission [146]. The "neurotrophic theory of depression" explores the potential for depression pathogenesis to be dependent on neurotrophic factors which control cell proliferation and differentiation in the CNS [147]. Of note, brain-derived neurotropic factor (BDNF) has been implicated in inflammation-derived depression. It is widely accepted that BDNF is integral to use-dependent plasticity mechanisms, such as long-term potentiation, learning and memory [89]. BDNF reduction in the hippocampus and prefrontal cortex have been linked to increased depression phenotypes [148]. Conversely, BDNF increase in the nucleus accumbens in the ventral tegmental area of the brain has been linked to increased depression phenotypes [149]. Levels of BDNF are associated with brain function. In general, studies have shown that reduced levels of BDNF are associated with synaptic plasticity and neuronal atrophy, while increased levels of BDNF are associated with neuronal survival and differentiation in the brain [150-152]. Thus, investigation into BDNF in the hippocampus and prefrontal cortex, and possibly the whole brain, is warranted. Our lab intends to conduct ELISAs in the very near future to examine this neurotrophic factor.

The hippocampus, specifically, is the most frequently studied brain region in depression research. Reports of volumetric changes in adult hippocampus have been noted in depressed patients which could result from a neurodegenerative reaction to increased glucocorticoid levels in depression [153, 154]. The hippocampus contains high levels of

glucocorticoid receptor (GR) and glutamate, both of which have been further linked to depression [155]. The hippocampus regulates the hypothalamus–pituitary–adrenal (HPA) axis, which makes it more susceptible to stress and depression [156]. Disturbances in the HPA axis activity are linked to a large array of physiological and mental health disorders [157]. The so called "Dysfunction in hypothalamus–pituitary–adrenal (HPA) axis hypothesis of depression" refers to the idea that dysregulation in the HPA axis, most notably through glucocorticoid sensitivity, is a major driving force of depression. Studies have shown that depressed patients often exhibit hyperactivity in the HPA axis resulting in increased glucocorticoids [158]. Glucocorticoid hormones, cortisol and corticosterone (CORT) are the effector hormones of the HPA axis. These hormones act in a negative feedback loop to control the amounts of CORT and cortisol. CORT levels vary by time of day and exponentially increases with environmental or physiological stress. Cortisol is known to contribute to neuronal survival, neurogenesis, and neuronal excitability; it is thought that high levels of cortisol may contribute to depressive symptoms by impairing these brain functions [159]. Cortisol binds GR and acts in part to regulate neurotrophic factors like BDNF to induce neuronal cell death and alter the hippocampal neurogenesis. It is thus possible that dysfunction in the GR could contribute to changes in the depressed brain [159, 160]. Of importance, studies have shown that there exists a relationship between HPA axis dysregulation and inflammation which may play a role in IBD and depression severity [53, 161]. One such study found that environmental stress induced gastrointestinal permeability was mediated by endogenous glucocorticoids in rats [110]. Furthermore, studies have shown that stress during pregnancy can cause maternal gut dysbiosis. This, in turn, can influence the development of the HPA in the offspring by

influencing gene expression in the brain, cortisol levels, and immune system functioning [162]. Considering such, investigation into dysregulation of the HPA axis in IBD is warranted.

Concluding Remarks

Though many theories to depression exist, these theories are likely not separate, but are parts of the whole picture that is depression. Thus, though we posit here that inflammation drives depression, that is not exclusive - many things may in fact drive depression. It is with careful review and investigation that we draw the conclusion that inflammation is a major contributing factor to both of these disorders. In this current study, much more investigation is necessary to cover the scope of this project. Nonetheless, our results show that colitis- induced mice had altered immobility times, with results depending on the testing time of day, when compared to healthy controls. These altered immobility times, which are a measure of depressive-like behavior in mice, were reversed upon treatment with I3C. These results suggest that colonic inflammation can lead to altered behavior and increased depressive-like symptoms through dysregulation of the gut metabolome but may be restored after treatment with an AhR ligand like I3C. Collectively, this suggests that activation of AhR can improve colitis-induced symptoms, which include colitis-associated depressive-like behavior.

Science remains to elucidate the exact mechanisms in which IBD and depression are linked. It is from current understanding that the process by which this occurs is likely through “leaky gut” allowing bacteria to migrate more freely into the bloodstream resulting in immune overactivation and stimulation of proinflammatory cytokine production. This subsequently increases expression of IDO, resulting in altered Trp metabolism (in part by

gut microbes) and altered AhR activation. This then signals through the GBA resulting in an imbalance in QA/KYNA production, impaired function of the BBB, and subsequent neuroinflammation with microglia activation and astrocyte atrophy (Figure 4.1). In future studies we aim to uncover and understand more of the relationship between IBD and depression.

Figure 4.1: Summary of Relationship Between Colitis and Depression

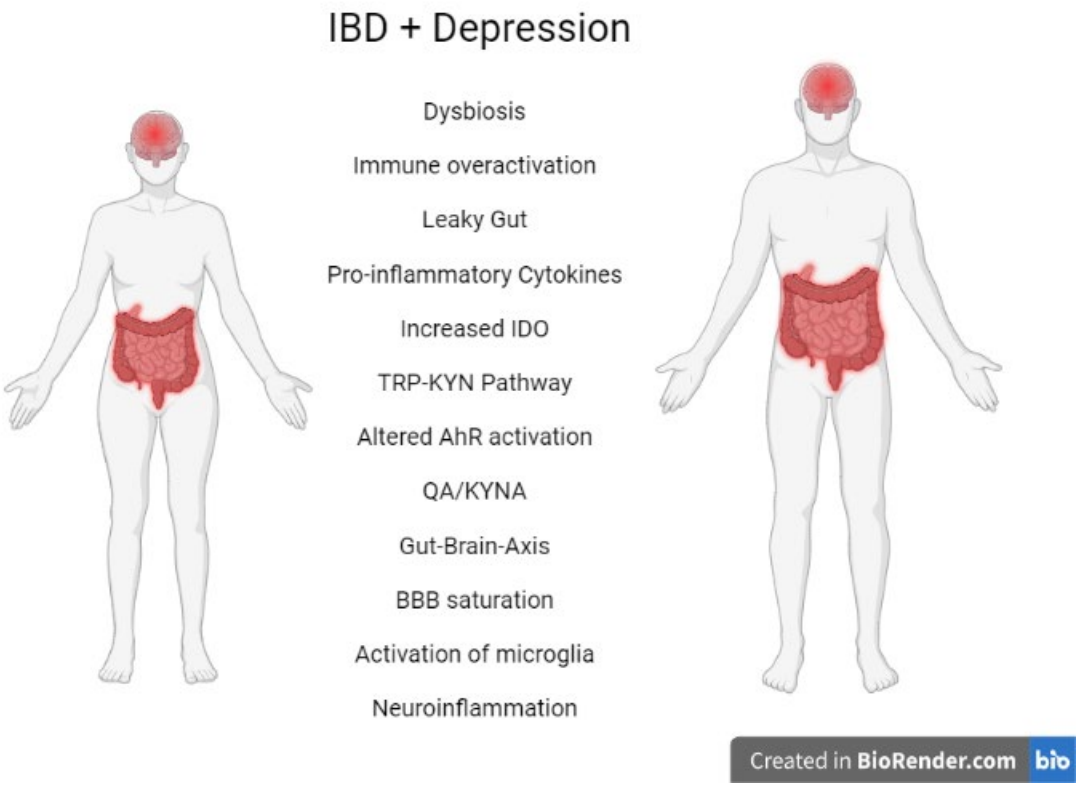

Figure 4.1: Details of the current understanding of the relationship between colitis and depression in a nonlinear list. Gut dysbiosis leads to immune overactivation and "leaky gut" allowing bacteria to migrate more freely into the bloodstream resulting in stimulation of proinflammatory cytokine production. This subsequently increases expression of IDO, resulting in altered Trp metabolism (in part by gut microbes) and altered AhR activation. This then signals through the GBA resulting in an imbalance in QA/KYNA production, impaired function of the BBB, and subsequent neuroinflammation with microglia activation and astrocyte atrophy

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