THESIS

THE IMPACT OF CBD AND CBG ON GUT AND MENTAL HEALTH

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ABSTRACT

THE IMPACT OF CBD AND CBG ON GUT AND MENTAL HEALTH

Cannabinoid-laden products have fostered a growing industry with marketing for their supplementation centered on perceived therapeutic benefit. With biochemical research reporting a wide array of mechanisms of action displayed by cannabinoids this thesis narrows on the current state of therapeutic potential of cannabidiol (CBD) and cannabigerol (CBG) on cognitive health and gut health, forming what is known as the gut-brain axis (GBA). A compiling of the current state of the literature provides data that shows how these compounds show they may be of benefit. Like many other compounds in the nutritional supplement industry, insight is provided on how the touted benefits claimed for CBD and CBG are not fully supported with empirical evidence and are currently lacking a thorough investigation to determine their therapeutic role.

To address gaps in this area of the field I provide a detailed report on a study I performed to expand the foundation of how CBD and CBG affect health through interaction with the GBA. This was conducted with examination of their impact on gut microbial composition, metabolism of cannabinoid and endocannabinoid compounds, and how they impact anxiety in mice. This report provides new evidence that CBD and CBG alter the microbiome in a similar fashion and their metabolism is likely not dictated on the initial composition of the gut microbiome. It was discovered the cannabinoids are absorbed differentially based on sex, and that male mice consuming CBD have elevated levels of anandamide, an endocannabinoid produced by the body that has been shown to play a role in gut health and mental health.

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CHAPTER 1 – LITERATURE REVIEW

Cannabinoid Overview

Cannabinoids are a diverse class of lipophilic molecules native to the *Cannabis sativa* plant. There have been over 120 unique cannabinoids identified to date[1]. Historically, cannabis has played a significant role in holistic medicine. Evidence of its therapeutic use dates back approximately 5,000 years and cultivation of the plant has been dated back to the end of the last ice age, over 10,000 years ago[2,3]. Modern scientific investigation into cannabinoids was overwhelmingly centered on tetrahydrocannabinol (THC), largely due to its psychoactive effects. Recently, researchers have begun shifting their focus to non-psychoactive cannabinoids and cannabinoid analogs discovered in *Cannabis* and both related and non-related plant species[4,5]. Progression in the legislative arena and interest in cannabis-derived therapeutics have accelerated scientific efforts for exploring clinical uses of this historical ethnobotanical class of compounds.

The Current State of Cannabinoid Research

Cannabinoids can be classified as "major" or "minor", according to their quantities in *Cannabis* plants. THC and CBD are classified as "major" cannabinoids, as are cannabidiolic acid (CBDA), cannabigerol (CBG), cannabigerolic acid (CBGA), tetrahydrocannabinolic acid (THCA), cannabinol (CBN), and cannabichromene (CBC)[6,7].Research on cannabinoids beyond THC and cannabidiol (CBD) has been limited, primarily due to challenges associated with their low natural abundance in the plant and high variability across different *Cannabis* strains, complicating the ability to extract quantities sufficient for research purposes. THC and CBD been the most intensively studied cannabinoids, with CBG recently gaining attention. Biotechnological advancements, including genetic engineering of the cannabinoid synthesis pathways has enabled viable yields of certain cannabinoids to be extracted for research and commercial use[8–11]. However, these approaches are still in the early stages and require further development to expand cannabinoid research capabilities.

Many cannabinoids have been studied primarily in extracts containing complex cannabinoid mixtures rather than as individual compounds. As a result, many of the bioactivities of cannabinoids have been attributed to an "entourage effect," which suggests that synergistic interactions between the numerous cannabinoids results in greater bioactivity. However, recent findings examining isolated cannabinoids in comparison to mixed formulations has challenged this notion. For example, CBN, often touted for its sleep-

enhancing properties, had reduced efficacy when administered jointly with CBD in a human trial[12]. Similar observations have been reported in other studies, indicating limited to no benefit from combined cannabinoid formulations. One study that used an inflammatory model in guinea pigs revealed that both CBD and CBG independently reduced inflammatory markers in the lungs, but that this anti-inflammatory effect was ameliorated when used in combination[13]. These scenarios highlight studies where cannabinoid isolates may be more effective in achieving specific therapeutic outcomes, prompting the need for additional studies detailing the effects of individual cannabinoids. Additionally, there are concerns regarding selective reporting and reluctance to transparently acknowledge data that contradicts claims of beneficial entourage effects, given the widespread commercial use of extracts[14]. Ultimately, therapeutic decisions, whether cannabinoid isolates or mixed formulations, should depend on clearly defined clinical outcomes. Presently, current knowledge gaps prevent transparent and definitive clinical recommendations regarding optimal dosing and formulations. Establishing comprehensive, empirically based foundations for the effects of individual cannabinoids is essential to guide future research and to enable personalized cannabinoid-based therapies.

One key knowledge gap is the lack of conclusive evidence regarding the biochemical and enzymatic interactions of individual cannabinoids. To date, nearly all cannabinoids, including those typically found in higher abundances, lack substantial data characterizing their specific enzymatic pathways and mechanisms of action. Despite this limitation, current literature generally indicates that cannabinoid consumption is safe and produces minimal side effects. Another knowledge gap includes how to deliver cannabinoids for intended therapeutic effects. Cannabinoid preparations can include inhaled, oral, and topical forms. Cannabinoids are poorly bioavailable, with limited absorption when consumed orally. However, recent studies comparing different oral formulations of CBD have revealed methods to enhance absorption and bioavailability[15–17]. Additionally, co-consumption of cannabinoids with dietary fats has shown to significantly improve absorption in humans[18]. Once absorbed, cannabinoids are rapidly metabolized by the liver with the newly formed metabolites mainly being stored in adipose tissue or excreted through feces and urine[19,20]. The following sections highlight the bioavailability and bioactivity of CBD and CBG, two of the major commercially available cannabinoids.

CBD

Background of CBD

CBD, being non-psychoactive and abundantly present relative to most other cannabinoids, has been a primary focus of cannabinoid research. It is naturally synthesized from CBGA, which is enzymatically converted to CBDA via cannabidiolic acid synthase[21] (Figure 1). This subsequently undergoes non-enzymatic

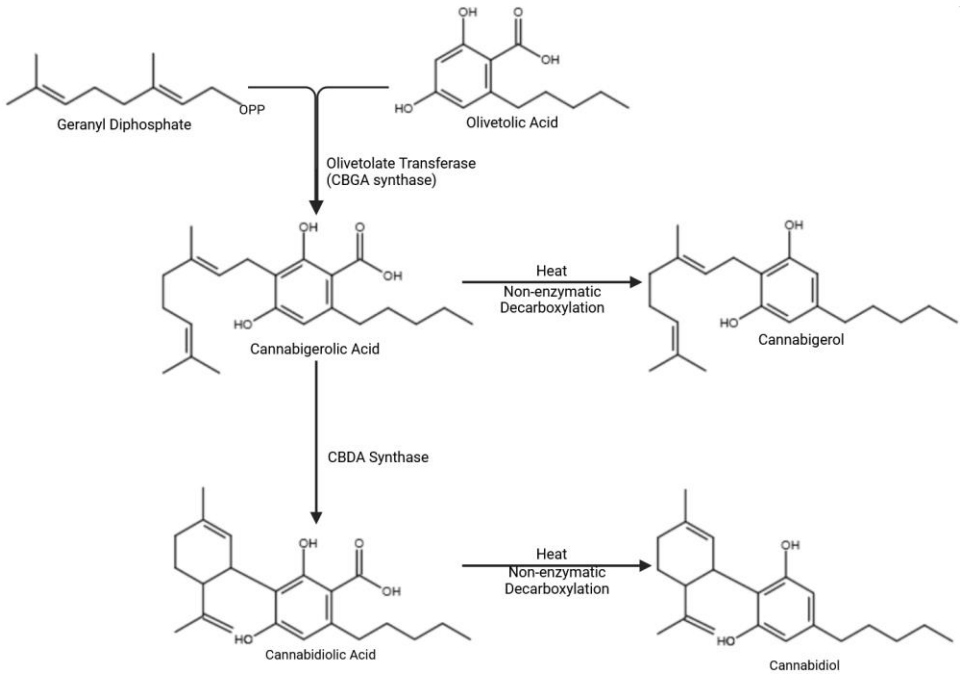

Fig. 1- Synthetic pathway of CBG and CBD in *C. sativa*.

decarboxylation to yield CBD. While primarily associated with *Cannabis sativa*, CBD has recently been reported in *Trema orientalis*, a shrub within the *Cannabaceae* family[22,23]. Although CBD remains the prominent subject of study, growing research interest has extended to its related structures CBDA and cannabidivarin (CBDV) for potential therapeutic use[24]. Currently, CBD has demonstrated broad potential for reducing chronic disease burden and improving gut and mental health through anti-inflammatory, anti-anxiety, and anti-nausea properties[19,25–30]. Mainstream use of CBD has largely been driven by its purported benefits in reducing anxiety and inflammation. In the sections that follow, we will discuss the current data examining the specific impacts of CBD on cognition and gut health in greater detail.

CBD and Cognition

CBD has received considerable attention for its potential therapeutic role in cognitive health. A significant milestone in cannabinoid research was the Food and Drug Administration's (FDA) approval of Epidiolex®, a CBD-based prescription medication for treating Dravet Syndrome and Lennox-Gastaut Syndrome, two rare forms of epilepsy[31]. Increasing evidence also suggests CBD may address cognitive impairments associated with Alzheimer's disease, Huntington's disease, Parkinson's disease, and psychosis[32]. One proposed mechanism thought to exert these benefits is by attenuating neuroinflammation. For instance, in an epileptic rat model, CBD treatment led to a dose-dependent reduction in pro-inflammatory cytokines IL-1β, IL-6, and TNF-α, while simultaneously increasing anti-inflammatory cytokines IL-10, IL-4, and TGF-1[33]. Further mechanistic insights into the potential neuro-protective benefits from CBD have been shown in cell studies, where CBD was shown to activate peroxisome proliferator-activated receptor gamma (PPAR-γ) pathways[34]. Another animal study using an inflammatory model to look at therapeutic mechanisms of action revealed modulation of the alpha-2 adenosine (A2A) receptor may help treat inflammation associated with multiple sclerosis[35]. Other inflammatory models in animals using lipopolysaccharide (LPS) have shown treatment with CBD also reduced depression-like symptoms[36]. Extending from this finding, CBD was also shown to prevent anxiety and behavioral impairments in mice with induced cerebral ischemia[37]. Along with PPAR-γ activation, these benefits were potentially mediated through serotonin 5-HT1α receptors, a receptor family widely studied for its role in anxiety and mood disorders. This mechanism was observed in a depressive mouse model where CBD prevented depressive-like behaviors through enhanced serotonin signaling[38].

One of the most common applications of CBD is for the therapeutic management of anxiety. Early human studies reported significant decreases in subjective anxiety and reduced experimentally induced anxiety during simulated public speaking[39,40]. Further evidence from a large clinical study suggested CBD could potentially improve sleep quality while simultaneously reducing anxiety symptoms in the majority of participants[41]. Notably, the anti-anxiety effects of CBD appear to follow a bell-shaped, dose-dependent response curve, emphasizing the importance of precise dosing[42,43]. Despite these encouraging results, definitive conclusions about CBD's anxiolytic effects remain elusive. A recent review in 2022 reported that approximately 70% of independent studies showed no significant impact of CBD on anxiety-related

behaviors[44]. The lack of significant outcomes was also reported by the same principal investigator in one of the first clinical trials examining the efficacy of CBD as a treatment for anxiety disorders[45]. This discrepancy underscores the critical need for standardized methodologies, including consistent dosing regimens, administration protocols, and outcome measures, to clarify CBD's true clinical potential. Given its clinical validation through FDA approval and ongoing therapeutic promise in mental health, CBD stands as the most extensively studied non-psychoactive cannabinoid. As research expands to encompass additional cannabinoids, utilizing CBD as a control will be essential to evaluate their relative therapeutic effects. Nevertheless, significant work remains in achieving a conclusive understanding of CBD's mechanisms of action and establishing clear causal relationships for its clinical efficacy as it related to cognition and anxiety.

CBD and the Gut

Gut health is a prominent issue, with rising diagnosis rates for inflammatory bowel disease (IBD) driving the search for new therapeutic strategies. Given inflammation's central role in these conditions, CBD, widely studied for its anti-inflammatory properties, has emerged as a potential therapeutic candidate. Cell culture studies utilizing transepithelial electrical resistance (TEER) assays to assess intestinal permeability have demonstrated protective effects of CBD on gut barrier integrity, particularly under inflammatory conditions[46,47]. Supporting evidence from animal studies further illustrates CBD's anti-inflammatory capacity in a murine colitis model, where treatment significantly reduced pro-inflammatory signaling through activation of PPAR-γ pathways and suppression of glial cell activation, facilitating tissue repair[48]. Preliminary human data also indicates potential protective effects, suggesting that CBD's anti-inflammatory actions might partially involve activation of cannabinoid receptor type 1 (CB1)[49]. However, despite promising findings from cell culture and animal studies, robust clinical evidence in humans remains limited, underscoring the urgent need for well-designed, rigorous clinical trials to conclusively determine CBD's therapeutic potential in managing gut inflammation.

An emerging area of research on CBD and gut health focuses on interactions between CBD and the gut microbiome. Although the direct effects of CBD alone on gut microbiota remain modest, one study found CBD treatment, administered alongside fish oil, increased beneficial gut taxa such as *Akkermansia muciniphila* and *Parabacteroides goldsteinii*, both commonly associated with gut health and probiotic benefits[50]. Conversely, the same treatment reduced the abundance of microbial families typically linked to negative health outcomes,

including *Marinifilaceae*, *Desulfovibrionaceae*, and *Ruminococcaceae*. However, caution should be applied when interpreting certain microbiome findings. For instance, a 2022 study using an epileptic rat model reported restoration of *Prevotellaceae_UCG-001* abundance in a high-dose CBD compared to epileptic controls, although it failed to reach statistical significance[33]. Additionally, the same study reported findings of high *Helicobacter* levels in the high-dose CBD group, although it was negatively correlated with pro-inflammatory markers. In an antibiotic-treated mouse model inoculated with *Clostridium sporogenes*, CBD treatment altered gut microbial composition, increasing taxa such as *Lachnospiraceae_NK4A136*, *Blautia*, *Desulfovibrio*, *Turicibacter*, and *GCA-900066575*, while decreasing *Muribaculaceae*, *Bacteroides*, *Alistipes*, and *Muribaculum*[51]. Notably, these changes represent shifts among both beneficial (e.g., short-chain fatty acid producers like *Blautia*) and potentially harmful (*Desulfovibrio*) bacteria, suggesting CBD modulates the microbiome's key taxa without fully disrupting its functional stability.

Beyond microbial composition, CBD treatment has demonstrated significant effects on microbiota-derived metabolites and host physiology. For example, one noteworthy metabolite significantly reduced by CBD treatment was indole-3-methyl acetate, a compound potentially linked to microbial tryptophan metabolism. Although the exact microbial origin of this compound remains unconfirmed, related indoles such as indole-3-acetic acid are established products of bacterial tryptophan metabolism and have been implicated in colon cancer models[52]. Another study using lipidomics and metabolomics revealed that CBD treatment significantly lowered inflammatory markers induced by high-fat, high-cholesterol diet, particularly those driven by LPS-mediated gut permeability[53]. Furthermore, CBD treatment was also shown to reduce circulating risk factors associated with cardiovascular disease, such as trimethylamine-N-oxide and phenylacetylglutamine, and improved lipid profiles by decreasing LDL and increasing HDL levels. In the context of human microbiome research, only one study has investigated the impact of CBD, which was done in cancer patients. This study reported restored abundance of *Alistipes* and increased microbial diversity compared to controls with cancer[54]. Importantly, current animal models typically do not utilize fecal microbiota transplants (FMT) from human donors, despite known metabolic and microbiome differences across species, especially regarding cannabinoid metabolism. Highlighting these interspecies differences, studies have reported variability in THC metabolism between mice and rats, and in cannabinoid pharmacokinetics across humans and domestic animals[55,56]. Such differences underline the critical need for

clinically relevant, translational study designs to accurately assess the potential therapeutic benefits of CBD in humans. Although the modulation of the gut microbiota by CBD requires further characterization, current findings suggest promising potential for its therapeutic application in gut-related disorders.

Although foundational research investigating the effects of CBD on gut health has advanced significantly through cell culture and animal studies, human studies examining CBD independently remain limited. The existing clinical literature on CBD's therapeutic efficacy for gut disorders is currently inconclusive. For instance, one clinical trial reported that lower doses of CBD (10 mg) did not effectively alleviate symptoms associated with Crohn's disease[57], whereas another study reported potential therapeutic benefit from a high-CBD extract in patients with colitis[58]. However, this observation of reduced inflammatory markers in colitis patients was partnered with elevated tolerance issues in the CBD treatment group. In another trial, patients with IBD did not have an improvement of symptoms compared to placebo after use of a CBD-infused chewing gum[59]. Collectively, these studies represent the extent of human trials specifically investigating CBD as a therapeutic treatment for colitis and IBD. This underscores a considerable gap between promising translational research findings and conclusive human clinical evidence. The performance of more human clinical trials is needed to fully determine CBD's therapeutic efficacy in human gut health.

CBG

Background of CBG

CBG has been referred to as the "mother of all cannabinoids," as it is the precursor cannabinoid from which all others are derived. Like CBD, CBG is primarily produced by Cannabis sativa plants but its derivatives and related analogues have been reported in *Helichrysum umbraculigerum*, a South African plant with historical roots in holistic medicine[60]. Unlike *T. orientalis*, the plant where CBD has also been detected, *H. umbraculigerum* belongs to the *Asteraceae* family and is unrelated to *Cannabis*. In *Cannabis*, CBGA synthesis occurs enzymatically via olivetolate transferase (also known as CBGA synthase) through the condensation of olivetolic acid with geranyl diphosphate (Figure 1)[1]. Typically, CBGA is rapidly converted into CBD, THC or CBC through enzymatic pathways, with smaller amounts alternatively undergoing non-enzymatic decarboxylation to yield CBG[21]. This rapid conversion results in low native levels of the compound in natural sources and has limited research into CBG. Fortunately, recent advancements in bioengineering have provided opportunities to produce high-CBG *Cannabis* strains as well as yeast strains that synthesize CBG, fostering

greater research opportunities[8–11]. As research into CBG expands, evidence suggests its potential therapeutic value across numerous applications, notably through antibacterial activity, neuroprotection, anti-inflammation, analgesia, mental health, cancer treatment, and metabolic health[61]. In the following sections we will specifically examine the current state of research regarding the effects of CBG on cognitive and gut health.

CBG and Cognition

Emerging studies are beginning to highlight the potential efficacy of CBG as a therapeutic for cognitive health. Preliminary cell-based research has provided a foundation for translational investigation. One study using cell lines to represent the blood brain barrier in an inflammatory model reported CBG having neuroprotective effects[62]. Another cell study modeling inflammation demonstrated that CBG reduced pro-inflammatory cytokines IL-1β, TNF-α and INF-γ[63]. Research utilizing an amoeba model, with follow-up studies using mouse embryonic fibroblasts, suggested that CBG may regulate mTORC1 signaling pathways implicated in multiple sclerosis (MS), a disease in which approximately half of patients experience some severity of cognitive impairment[64–66]. Another study using murine cells and a MS model in female mice revealed injections with CBG provided neuroprotective benefits through anti-inflammatory and anti-oxidative pathways[67]. Studies looking into CBG's impact on behavior has provided intriguing results on potential mood and anxiety applications. Observations from murine cell models suggest CBG is a potent agonist of A2A receptors and partial antagonist of 5HT1α receptors[68,69]. Clinically characterized antagonists of these receptors for treating anxiety are rare, suggesting CBG may have potential future applications. WAY100635, a piperazine drug that is typically used as a receptor inhibitor in experimental studies, is one of the few documented examples of this modulation being applied[70–72]. To date, no pharmaceutical interventions simultaneously antagonize 5HT1α receptors and agonize A2A receptors, underscoring the unique pharmacological profile of CBG.

Animal studies investigating these proposed mechanisms of CBG have yielded mixed results regarding its efficacy as a treatment for anxiety. Early rodent studies initially indicated promising anxiolytic effects of CBG, demonstrating reductions in anxiety-like behavior in rats[69] and in mice with the commonly used open field test[73]. These findings were supported by a more recent comparative study, in which a CBD formulation enriched with CBG exhibited anxiolytic properties while the formulation without CBD did not[74]. This anxiolytic effect in elevated plus maze tests was seen in both acute (after a single CBG-enriched

administration) and sub-chronic (after daily administration for 10 days) exposures. Additionally, CBG has also been shown to reduce blood pressure in animal models, possibly through agonistic A2A receptor signaling[75,76]. Of note, A2A activation can result in the inhibition of norepinephrine release from presynaptic neurons, an effect that could have therapeutic implications for the management of stress and anxiety[75]. In contrast, several animal studies using similar metrics and others like the light-dark test and acoustic startle tests in rodents found no significant anxiolytic production from CBG administration[77–80]. Given these conflicting outcomes derived from a limited number of studies with varying dosages and administration methods, further systematic investigation is necessary to conclusively establish the efficacy of CBG for mood and anxiety disorders.

Human studies investigating the therapeutic effectiveness of CBG for cognitive and mood-related disorders have only recently begun. A survey by Russo *et al*. indicated anxiety as the primary reason consumers use CBG[81]. Following this survey, a field trial led by Cuttler *et al.* suggested subjective anxiolytic improvements with CBG use[82]. This was seen from a 26.5% reduction in subjective anxiety ratings in the CBG group from baseline. However, the placebo group also experienced a similar anxiety reduction of 22.5%, including no significant differences between the groups on stress parameters. It is important to note that this trial was a self-reported field study, administering a single 20 mg dose of CBG and measuring outcomes in response to an induced online stress test up to 60 minutes post-consumption. Given evidence from both animal and human pharmacokinetic studies showing peak plasma concentrations of CBG (and CBD as a reference) occurring 2 to 5 hours following oral consumption[83–85], it is possible that the chosen 60-minute testing window missed the period of maximal effect. Adding to this, cannabinoid concentrations in brain tissue might occur even later post-consumption. The study also included participants who were already regular users of cannabinoid products, potentially introducing bias. To date, this is the only human trial investigating CBG intervention for mental health. Considering these factors, critical gaps remain in both translational and clinical research, emphasizing the need for further systematic studies designed to evaluate overall efficacy of CBG as a therapeutic approach.

CBG and the Gut

CBG's observed anti-inflammatory and antibacterial effects are suggestive of a potential therapeutic role in gut health. One area receiving attention is the treatment of colon cancer, supported by preliminary cell-

line studies. While one study found that CBG itself was less potent than other cannabinoids in reducing cancer cell viability, its acidic form (CBGA) significantly decreased reactive oxygen species (ROS) levels and viability in colon cancer cells[86]. Other studies using cancer cell lines have corroborated these findings, both with CBG independently and combined with other compounds, suggesting that inhibitory action on cancer growth is through modulation of transient receptor potential melastatin 8 (TRPM8) channels[87,88]. Related research involving mice in an induced colitis model alleviated the inflammatory state through reduced ROS and inflammatory cytokines[89]. Considering a rising prevalence of colon cancer diagnoses a clearer understanding of how CBG may be therapeutically utilized is needed. Additionally, growing rates of IBD incidences with diet emerging as a potential contributor, researchers have begun exploring CBG's potential to combat IBD's progression. In one rodent study, CBG treatment reduced inflammation with a high-fat, high sugar diet by lowering arachidonic acid levels, downregulating pro-inflammatory pathways and upregulating anti-inflammatory pathways[90]. While early findings are promising, more focused research is essential to determine the full scope and clinical relevance of CBG's role as a treatment for gut-related diseases.

Given CBG's noted antibacterial effects in other biological systems[91], there is growing interest in how it might modulate the gut microbiome. For example, a study investigating skin microbiota demonstrated CBG's potential to inhibit bacterial infections, including reducing viability and biofilm formation, thus fostering a protective environment against skin pathogens[92]. Similarly, preliminary investigations into oral health highlighted CBG's inhibitory effects against the dental pathogen *Streptococcus mutans* when administered in bacterial growth plates[93,94]. Another study utilizing cell-culture plates evaluated CBG combined with CBD on bacteria representative of the gut microbiota. This study revealed broad, clade-specific antibacterial activity, notably against Gram-positive taxa[95]. These studies collectively suggest that CBG possesses broad antibacterial activity with some targeting against pathogenic microbes, raising intriguing possibilities for similar effects in the gut microbiome. However, more translational studies in relevant animal models or human tissues are needed to determine the potential applicability of CBG as an antimicrobial.

Currently, no published study has specifically examined how CBG independently impacts gut microbiome composition. One study using a hemp extract high in both CBG and CBD in a mouse model of induced colitis reported reductions in IBD symptoms and reduced colonic damage[95]. This study also observed compositional shifts in the gut microbiome. Treatment with the hemp extract led to a slight, non-significant

rebound in alpha-diversity compared to the control group. This potentially highlights complex interactions in the gut influenced by the antibacterial properties of cannabinoids. Taxonomic analysis revealed shifts revealed high-CBG/CBD administration reduced abundance of groups within *Oscillospiraceae*, *Bacilli*, *Clostridia*, and *Lachnospiraceae* while increasing the abundance of taxa such as *Adlercreutzia*, *Romboutsia*, and an *Anaerovoracaceae* group. However, interpretation of these shifts remains complex, given the mixed associations of the altered taxa with health outcomes. Further analysis revealed beneficial alterations in microbial metabolic pathways, notably tryptophan metabolism, in the induced-colitis group receiving treatment. Tryptophan metabolism in the gut is largely driven by microbial activity, producing metabolites such as indole-3-acetic acid, which possesses gut-protective properties. Preservation of these beneficial metabolites by cannabinoid treatment underscores the importance of further evaluating interactions between cannabinoids and microbial metabolism in the gut. Given that this study is currently the sole investigation of CBG's effects on gut microbial composition, future research focused on specifically examining CBG in isolation is critically needed to further elucidate its therapeutic potential for gut health.

Summary and Research Objectives

Summary

CBD has significantly advanced research into the therapeutic potential of non-psychoactive cannabinoids. Historically, cannabinoid research beyond THC and CBD was hindered by limited cannabinoid yields following extraction from harvesting plants. However, recent innovations in biosynthetic chemistry and genetic engineering have provided researchers with access to a broader spectrum of the more than 120 known cannabinoids. As two of the major cannabinoids found in *Cannabis*, CBD and CBG have demonstrated promising therapeutic interactions relevant to cognitive and gut health. Notably, their unique receptor interactions compared to typical cognitive therapeutics offer novel and unique mechanisms of action for mental health interventions. Regarding gut health, preliminary evidence suggests that both CBD and CBG may confer beneficial effects without causing substantial disturbances to the overall gut microbiome, highlighting their potential in gut health applications.

Gaps in the Research

While animal studies have consistently demonstrated CBD's potential as an effective treatment for gut-related ailments such as colitis and IBD human trials remain limited and inconclusive. Similarly, CBD has

received considerable attention as a potential treatment for mental health conditions, notably anxiety. However, a thorough review of current data reveals substantial uncertainty regarding its efficacy and highlights the need for refined and definitive clinical trials. For CBG, early research indicates promising anti-inflammatory properties and potential as an anxiolytic, positioning it as a possible successor or complement to CBD. Despite this, therapeutic claims of CBG related to gut health remain largely untested, particularly regarding inflammation and alteration of the gut microbiome. Moreover, the literature addressing CBG's effectiveness for anxiety is limited, with the single human trial conducted thus far showing only modest effects with notable methodological limitations and potential participant bias.

To directly addresses several of these research gaps, we have designed a study in mice that have been colonized with human fecal gut bacteria. Specifically, germ free mice will receive one of two disparate microbiomes and be given orally administered CBD, CBG, or a placebo. We will assess bioavailability of CBD and CBG as it is impacted by differences in sex or microbiome composition and look at how chronic consumption alters the gut microbiota and intestinal barrier function. In addition, we will use cognition and anxiety tests to determine anxiolytic effects of these compounds. By investigating impacts on a human-associated gut microbiome mouse model, we will be able to more translationally determine how CBD and CBG independently alter gut microbial composition. Performing plasma metabolomics will allow us to observe how treatments and distinct microbial compositions alter both metabolism and absorption of cannabinoids. Performing barrier function tests using the transepithelial electrical resistance (TEER) assay we will be able to evaluate the anti-inflammatory effects of a CBD or CBG-conditioned gut environment on gut barrier function. Furthermore, by subjecting mice to standardized stress testing designed to induce anxiety, this study allows for direct comparisons between CBD and CBG on their anxiolytic potential. Cumulatively, combining both assessments allows for interpretation of how CBD and CBG impact gut and behavioral health through the gut-brain axis, providing insight on their potential as a combined therapeutic for gut and behavioral health.

Objectives

The first aim of this study was to analyze how CBD and CBG independently altered the microbial composition of two distinct microbiomes. The second was to evaluate how CBD and CBG independently impact enzymatic function of host gut tissues in mice as well as gut barrier integrity. The third was to see if

two distinct microbiomes would differentially metabolize the two cannabinoids. Our fourth and final objective was to examine how CBD and CBG influence anxiety-like behavior in mice.

Current literature presents inconclusive evidence regarding CBG's efficacy as a therapeutic for mental health conditions, as well as a lack of data on its independent effects on the gut microbiome. To address these gaps, we utilized a translational animal model employing FMT into germ-free C57BL male and female mice. Half of the received an FMT from one lean human donor while the other half received an FMT from one obese human donor. This increased the clinical relevance of our findings through microbiomes representative of distinct human populations. In addition to placebo controls, the direct comparison between CBD and CBG allowed assessment of their relative therapeutic strengths. By investigating CBD's effects, we validated and expanded existing limited research, while simultaneously providing the first comprehensive characterization of how CBG independently interacts with gut microbiome composition and function. Using a translational model with FMT from human donors allowed us to assess multiple connecting gaps in the literature, specifically by investigating:

- How different human-associated gut microbiomes metabolize cannabinoids differently.
- How distinct microbiomes affect – and are affect by – cannabinoid treatment, particularly regarding host gut enzyme function and barrier integrity.
- The functional implications of cannabinoid-induced microbiome alterations – and metabolism of two distinct cannabinoids – alter anxiety-like behavior, thereby providing insights into GBA mechanisms.

Collectively, these approaches provide critical translational insights into the therapeutic potential of CBD and CBG for treating both gut-related and cognitive disorders.

Acknowledgement

The synthesis figure was created using the Fisher Scientific Chemical Structure Search program[96].

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CHAPTER 2 – EFFECTS OF CANNABIDIOL (CBD) AND CANNABIGEROL (CBG) COMPARED TO PLACEBO ON MODULATION OF THE GUT MICROBIOTA, INTESTINAL BARRIER FUNCTION, AND ANXIETY IN MICE

[1]

Abstract

Cannabinoids like cannabidiol (CBD) and cannabigerol (CBG) have become increasingly available on the public markets in the United States. Commonly reported uses for their consumption include alleviating anxiety and reducing inflammation, which may impact gut health. However, there are limited data on how these compounds interact with the gut microbiota to influence intestinal barrier function and compound bioavailability. Therefore, we conducted a placebo-controlled study in male and female C57BL/6J gnotobiotic mice that received a fecal microbiota transplant (FMT) from one of two different human donors. Mice received 5mg of either CBD, CBG, or a placebo for 4 weeks. Gut microbiota profiles were assessed using 16s rRNA sequencing, plasma metabolites were measured by HPLC, and levels of anxiety were analyzed using an elevated plus, open field, and novel recognition test. test. We found that both CBD and CBG treatment resulted in higher alpha diversity in the gut microbiota compared to placebo and clustered independently of placebo samples in ordinations of Bray-Curtis distances. These patterns were observed regardless of starting microbiota and did not influence differences in circulating levels of CBD or CBG. Both CBD and CBG prevented increases in anxiety index (AI) scores in repeated bouts of an elevated plus maze (EPM) test, with CBD having significantly lower week 4 scores compared to placebo. We did not observe any changes in gut barrier resistance when fecal extracts from the mice were added to Caco-2 monolayers.

Introduction

Cannabis sativa is the source of more than 120 terpene compounds, called phytocannabinoids, that may have beneficial effects for human health. Archaeological records demonstrate a long history of ethnobotanical uses of *Cannabis* in the ancient world[1,2]. These uses of cannabis were similar to those reported

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today, and include treating pain, gastrointestinal distress, headaches, anxiety, and inflammation[3,4]. Among the phytocannabinoids produced by *Cannabis* plants, cannabidiol (CBD), a non-psychoactive cannabinoid, has been a primary focus of research pertaining to human health. Several human intervention studies have demonstrated the efficacy of CBD as an anti-anxiety supplement[5–9]. There is currently insufficient data in humans examining the therapeutic effectiveness of CBD on gastrointestinal (GI) conditions; however, there is a body of evidence in cell and animal models supporting it as a potential treatment combatting GI distress, intestinal barrier function and inflammation[10–14]. There is even less understanding of how CBD impacts the gut microbiome, with only a few studies in mice examining how CBD impacts gut microbial composition[15–17].

Another cannabinoid, cannabigerol (CBG) is gaining popularity in commercial products in the US but is much less studied than CBD. Cannabigerol is the parent metabolite for the over 120 other phytocannabinoids, earning the title "mother of all cannabinoids". To date, only one study has assessed the effects of CBG on gut health. In this study, a high-CBG hemp extract was tested in a mouse model for irritable bowel disease (IBD), where it was shown to reduce colonic damage and symptom severity[18]. Mechanistically, this may have been driven by modulation of the gut microbiome resulting in microbial profiles that were protective against gastrointestinal inflammation and distress. However, the extract was also high in CBD, limiting the ability to determine the independent effects of CBG. Several animal studies have also looked at the effects of CBG on anxiety, but the results are equivocal. Three studies in rodents reported that CBG did not produce anxiolytic effects[19–21], but two other rodent studies did show anxiolytic effects after CBG administration[22,23]. In human studies, participants taking a high CBG cannabis product noted considerable improvements in anxiety-related symptoms compared to taking conventional pharmaceuticals, and a follow-up placebo-controlled study demonstrated moderate evidence that CBG reduced anxiety but was overall inconclusive. Thus, there are considerable gaps in the current literature regarding how CBD and CBG impact gut health and anxiety.

One factor that may be important in determining the bioavailability and bioactivity of phytocannabinoids is the gut microbiome. The gut microbiome consists of trillions of organisms that live in the gastrointestinal tract and play a role in digestion, immunity, metabolic regulation, and pathogen protection. In addition, the gut microbiome has been associated with the mood regulation through various mechanisms including production of neurotransmitters, signaling along the gut-brain axis (GBA) via the vagus

nerve, regulation of stress hormone production, and production of metabolites like short chain fatty acids (SCFAs) that can cross the blood brain barrier[24]. Phytochemicals, such as phytocannabinoids, may interact with the gut microbiome in the colon, producing bi-directional effects that alter both the microbiota and phytocannabinoid structure and function. A better understanding of these interactions is needed to begin to unravel both the broader effects of phytocannabinoids on human health and the underlying reasons for variability in response to phytocannabinoids.

Here we use orally administered CBD and CBG in a human microbiota-associated mouse model to begin to unravel these effects. Specifically, we use two compositionally distinct human microbiomes- one from a lean individual and another from an obese individual- to determine whether individual differences in the microbiome impact circulating levels of these phytocannabinoids, and whether CBD and/or CBG alter microbiota composition. In addition, we use several assays to observe mouse behavior to determine whether CBD and CBG impact anxiety-like behaviors in the animals. Finally, we used fecal water extracts from the animals to determine whether the luminal contents of CBD or CBG-exposed animals produced effects on intestinal barrier function using trans-epithelial electrical resistance (TEER) in Caco-2 monolayers. Both CBD and CBG are touted to have many of the same health benefits, but there is a lack of empirical data to validate these claims. The completed studies use a translational human microbiota-associated mouse model to help address gaps in understanding how CBD and CBG impact the gut microbial community, intestinal environment, and anxiety-like behaviors.

Materials and Methods

Animal Experiment

Thirty-six germ-free (GF) male and female C57BL/6J (Jackson Laboratories) mice were bred at the CU Anschutz Medical Campus Gnotobiotic Core Facility. Mice were equally divided by sex and randomized to receive a fecal matter transplant (FMT) by oral gavage from either a lean (Microbiota 1; n=18) or obese (Microbiota 2, n=18) human donor. The donor material was previously analyzed and specifically selected because the two donors had distinct microbiota compositions, allowing is to determine whether the gut microbiome altered the metabolism and absorption of CBD and CBG. Our gavage protocol was performed as previously reported[25]. After a two-week stabilization period, mice were transported to the Live Animal Research (LAR) facility at Colorado State University, where they began a 10-day acclimation period. Mice were

individually housed and maintained with a 12/12 light cycle, at 73°F. Baseline weight and food intake were recorded. After acclimation, the 8-week-old mice were equally divided and randomized by sex and microbiota inoculum into one of three treatment groups: placebo, CBD, or CBG (Figure 1). Litter sizes divided the study

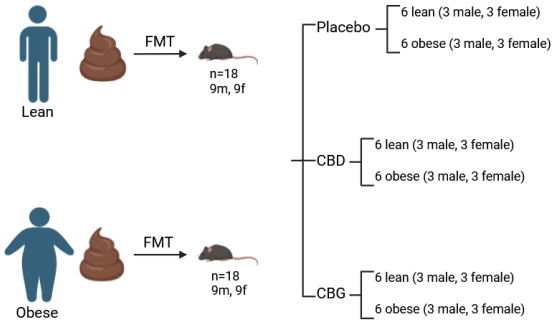


Fig. 1a

Fig. 1b

Figure 1- Experimental design of animal study. (Fig. 1a) Balanced, randomized inoculation of gnotobiotic mice from either a lean human donor or an obese human donor. Mice from both microbiome groups were then randomized in a balanced fashion to one of the three treatment groups, CBD, CBG or placebo. (Fig. 1b) Longitudinal design of the animal study. Baseline mouse body weight, food intake and cognitive testing was performed the week prior to commencement of treatment administration. Baseline feces was collected the days prior to treatment administration beginning. During administration, treatments were administered daily in the morning for 28 days. Cognitive testing was performed in week 4. Feces was collected at week 2 and week 4. Mouse body weight and food intake was recorded weekly.

into two cohorts, 16 mice in the first cohort and 20 mice in the second. Prior to treatment administration, baseline cognitive testing was completed. Mice began receiving oral administration of 100ul of a 5% concentration of either placebo of MCT oil, CBD, or CBG pipetted onto one-half of an oyster cracker. This was performed between 8am and 10am daily for 29 days. Weight and food intake were measured weekly and fecal pellets were collected at baseline (Week 0) and on Weeks 2 and 4 and cognitive tests were completed on Days 26-28, prior to termination. At Week 4, the mice were sacrificed after treatment administration. Blood was collected by cardiac puncture and tissues were weighed and collected for downstream analyses. All collected samples were stored at -80°C until analyzed.

Phytocannabinoid Administration

CBD and CBG (water-soluble nano-emulsions at 5% concentration), along with a placebo carrier emulsion (containing 5% medium chain triglyceride) were provided by Caliper Foods (Commerce City, CO). All three formulas were double purity tested and verified with a third-party state licensed lab in Colorado (SC

Labs, Denver, CO). Daily administration of 5uL of the intervention was pipetted in 100uL of liquid onto one-half of a Mini Baked Oyster Cracker (Kroger, USA; Avg daily intake of ½ cracker: 0.2g, 0.97kcal, 0.02g fat, 2.36mg sodium, 0.15g carbs, 0.01g protein, 0.01mg iron). Crackers were placed in an open area of the cage, free of obstruction or concealment and animals were observed to confirm consumption. Animals did not show any aversion to this mode of administration, and the cracker was typically consumed within 30 minutes of placement.

Behavior Testing

All behavioral tests were conducted at baseline, before treatment administration began, and at the end of the study. The schedule of cognitive testing was as follows: Day 1- Open field test (OFT) followed by novel object recognition (NOR) test familiarization; Day 2- NOR test; Day 3- Elevated plus maze (EPM) test. For day 1, one mouse completed OFT and NOR familiarization before moving on to the next mouse. A two-minute interval between completion of OFT and initiation of NOR familiarization was used to maintain standardized timing between tests for each mouse. After completion of the OFT, the mouse was removed from the apparatus. Any feces or urine was removed from the box and two identical objects were placed into the apparatus. Before and after each mouse the testing apparatus was cleaned with 70% alcohol to eliminate odor carry-over. Odors of the alcohol were allowed to dissipate before the next mouse was placed in the testing apparatus. This same schedule and process was completed for final cognitive testing on days 26-28 of the study.

All tests were video recorded with a camera placed approximately two meters above the apparatus, and videos uploaded to the ANY-maze Video Tracking software (Stoelting, USA), version 7.2 (64-bit, released Jan 1, 2023) for analysis. Immobility detection was set at the default 65% sensitivity, with immobility detection set at 3 seconds of no movement. On cognitive testing days mice were transferred to a procedure room where all tests were performed and allowed to acclimate to the room for two hours before testing commenced.

Open Field Test (OFT)

The apparatus used for the OFT was 50L x 50W x 38H cm in dimension with a 30 x 30 cm interior zone, white opaque color and a textured surface (fig. 2a). Mice were placed in the center of the apparatus and

Fig. 2a

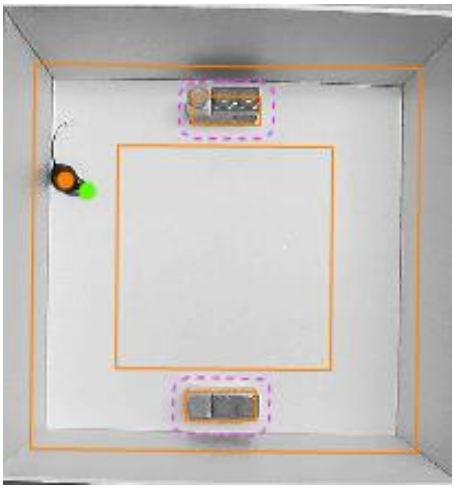


Fig. 2b

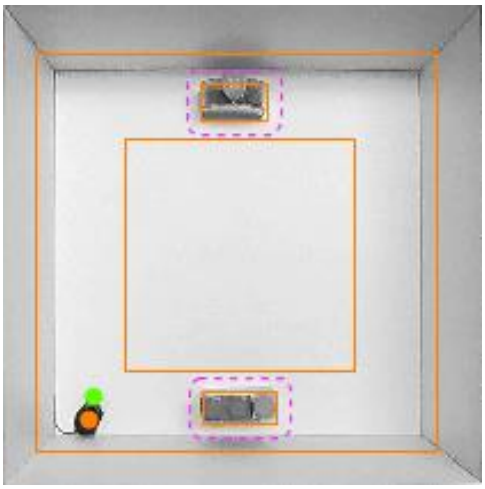


Fig. 2c

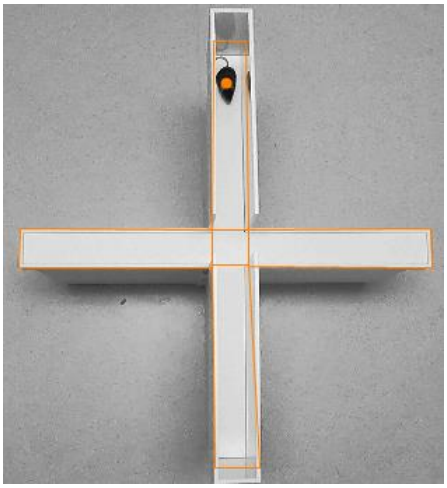


Fig. 2d

Figure 2- Apparatus configuration for cognitive testing, shown with zone configurations using the ANY-maze Video Tracking software. (fig. 2a) OFT with inner zone and outer zones. (fig. 2b) NOR test, familiarization session, with inner and outer zones and exploration zones around objects. (fig. 2c) NOR test, novel object session, with same zones as familiarization session, except for one object being replaced with a novel, unfamiliar object. (fig. 2d) EPM test with zones to delineate open arms, closed arms, and a center zone.

observed while freely exploring the box for five minutes. Anxiety-like behavior is determined by observing the amount of time spent on the perimeter of the box (exterior zone) versus the amount of time in the center of the box (interior zones) as well as the amount of time the mice is immobile. The common method for assessing the anxiety level of mice in the OFT is either taking the amount of time spent in the interior zone over the total time of the test or taking the distance traveled in the interior zone over the total distance traveled during the test[26]. We performed both methods of analysis.

Novel Object Recognition (NOR)

NOR testing was performed using the same apparatus as was used for OFT by adapting a previously published protocol[27]. Mice are first introduced to two identical objects in a familiarization phase (fig. 2b). 24-hours later, we conducted the novel object phase of the test, introducing one novel (unfamiliar) object with one of the familiar objects from the previous day (fig. 2c). In this phase, the task tests the ability of the mouse to recognize the familiar object and to spend most of the time exploring the novel object. A mouse that preferentially observes the novel object is determined to have recognized the familiar object, while a mouse that observes the novel object and the familiar object equally is determined to have inadequate recognition memory in discriminating the familiar object with the novel object test[28]. The duration of both the familiarization test and the novel object test is a maximum of 10 minutes or when the mouse completes at least 20 total seconds of exploration, whichever comes first. The exploration criteria of 20 seconds were set in accordance with Traschütz et al.[29] which mentions using 20 seconds of exploration for mice ages 3-6 months old. Scoring of exploration time occurred when mice touched or directed their head towards the object within 2cm. Mice that did not meet the exploration criteria before 10 minutes were determined to have failed to explore (FTE) and were not included in the final analysis. We used the discrimination ratio to measure the sensitivity and discrimination of exploring a familiar object versus a novel object. The recognition index ratio of time spent exploring the novel object over total object exploration time. The discrimination ratio is (time exploring novel object – time exploring familiar object) / total time of exploration. Scores range from +1 to –1, with a positive score indicating more time spent exploring the novel object and a negative score indicating more time spent exploring the familiar object.

Elevated Plus Maze (EPM)

EPM is a commonly used test to assess anxiety behavior in mice by using height and open arm environments to induce stress response. The apparatus is "+" shaped with two arms that are open, with no walls, and two arms that are closed, with walls (fig. 2d). The maze is elevated to a height of 39cm to simulate instinctual fear of heights in mice, and the open arms are to simulate natural aversion to open areas based on predation instincts. End-to-end length is 67cm, width of the arms in 5cm, and height of the walls of the closed arms is 15cm. We conducted EPM tests for 5 minutes. Level of anxiety assessed from the data is further

expressed with the anxiety index (AI), calculated by Cohen et el.[30]. The anxiety index is as follows: (AI) = 1 - (((time spent in the open arms/test duration) + (# of open arm entries/total number of entries))/2).

16s rRNA Processing and Sequencing

Mouse fecal DNA extraction was performed using the MPBio FastDNA SPIN Kit (Pittsburgh, PA), following manufacturer's protocol with additional wash prior to transferring to the Binding Matrix. Sequencing of the gut microbiome was performed using 16S rRNA sequencing of the hypervariable V4 region (515F-806R) fusion primer with 12bp Golay barcodes and Illumina sequencing adaptors. Samples were sequenced at the Colorado State University Next Generation Sequencing Core on an Illumina MiSeq using a v2 500-cycle kit with a 15% phiX spike.

The amplicon data was processed using QIIME2[31] (v2024.2) implementing DADA2[32] read filtering calibrated to improve the resolution of its dynamic error modeling. The number of reads used for the DADA2 learning algorithm was set to 15% of total frequency observed from demultiplexing (--p-n-reads-learn). Pooling method was set to "pseudo" for improved accuracy and low abundance ASV detection (--p-pooling-method pseudo). Taxonomic classification was performed using the SILVA[33] database (v138).

Metabolomics

Standards for phytocannabinoids, CBD and CBG, and endocannabinoids, N-arachidonoylethanolamine (anandamide; AEA), 2-arachidonoyl glycerol (2-AG), 1-arachidonoyl glycerol (1-AG), AEA-d4, and 2-AG-d5 were sourced from Cayman Chemicals (Ann Arbor, MI). Toluene, LC–MS grade reagents (acetonitrile, formic acid, and water) were purchased from Fisher Chemical (Waltham, MA). Plasma samples were subject to liquid-liquid extraction of endo- and phyto-cannabinoids following a previously published protocol[34]. In brief, 100uL plasma aliquots were extracted with 500uL of toluene containing the internal standards (CBD-d3, AEA-d4, and 2-AG-d5) followed with 20 mins of vortexing at 4°C. Five hundred microliters of water was added followed by another five min vortex step. Samples were kept at -80°C for 2 h to enable protein precipitation. Samples were equilibrated to 4°C and centrifuged for 20 mins at 2000x g followed by transfer of 300uL of the top organic layer, which was then dried at room temperature under nitrogen. Samples were resuspended into 50uL of 1:1 Acetonitrile: MeOH and vortexed for 10 mins and centrifuged for 5 mins prior to LC-MS/MS analysis. Blanks, standard curve, and pooled QCs were also made following the same protocol. Samples were analyzed using a PerkinElmer QSight 420 Triple Quad LC-MS/MS System and quantitation of

phytocannabinoids (CBD and CBG) and endocannabinoids (N-arachidonoylethanolamine [AEA], 1-arachidonoylglycerol [1-AG], and 2-arachidonoylglycerol [2-AG]) was calculated from standard curves of commercial standards.

Transepithelial Electrical Resistance (TEER)

To assess intestinal barrier integrity after exposure to phytocannabinoids we performed TEER analysis of Caco-2 monolayers exposed to cecal water extracts from the experimental mice. Cecal water extracts were prepared by reconstituting cecal contents in a 1:1 dilution with sterile water and pressed through a 0.2-micron filter. Caco-2 cells (3.3×10^3 cells/cm^2) were cultured on Transwell (cellQART®, Sabeu) inserts (0.3 cm^2) in 12-well plates containing DMEM. Cells were allowed to grow at 37°C at 5% CO_2 for 21 days to allow differentiation to confluent monolayers and media was changed every two days. The day before testing the monolayers received DMEM without FBS. For testing, TEER values were measured at baseline to confirm initial TEER of greater than 400 Ω cm^2. After baseline measurements, cecal water (15 uL, 1:10 diluted in DMEM without serum) from the mice were pipetted into the Transwells and tested in triplicate. TEER nodes were placed in 0.1 KCL fluid 24h prior to testing. Negative controls were used to normalize values from the residual resistance of the well inserts. TEER measurements were recorded at 2, 4, 8, and 24h using an EVOM2 Epithelial Voltohmmeter (World Precision Instruments).

Statistical Analysis

Analysis of anthropometrics, anxiety scores, and gut function was performed using ANOVA and mixed-effect models. These were validated with Shapiro-Wilks normality tests. Type II modeling for the ANOVA was performed with the *emmeans* R package for group comparisons and *post-hoc* analysis with Tukey-adjusted pairwise comparisons. A p-value of ≤ 0.05 was considered statistically significant and a p-value of < 0.10 was considered trending.

Downstream microbiome analyses were performed in R (v4.4.1), using packages *MicrobiomeAnalystR*[35] (v2.0). *Maaslin2* (v1.18.0)[36] was used to identify differentially abundant taxa. For alpha and beta diversity analysis, prevalence filtering was set to retain features with greater than four counts in at least 10% of all samples and total sum scaling was used to normalization read counts. Shannon Index was used for alpha diversity and was statistically analyzed using Kruskal-Wallis and adjusting for False Discovery Rate (FDR). Distance matrices for beta diversity analysis were constructed using Bray-Curtis dissimilarity

distances and visualized using Principal Coordinate Analysis (PCoA). PERMANOVA (Adonis test), PERMDISP, and pairwise PERMANOVAs were used to determine significance between groups. *Maaslin2* taxonomic analysis was performed using the compound Poisson linear model (CPLM). Significance was defined with a qval of <0.1 for significant results. Graphs and tables were constructed in R and visuals were constructed in BioRender.

Results

Anthropometrics

Neither treatment nor microbiome had a significant effect on body weight at termination, change in body weight, food intake, liver weight, spleen weight, cecum weight, mesenteric adipose tissue, or epididymal adipose tissue. When normalized by body weight, there was trending significance by microbiome on heart weight (p=0.053) (Figure 3). Mean heart weight for animals colonized by the microbiome of the lean

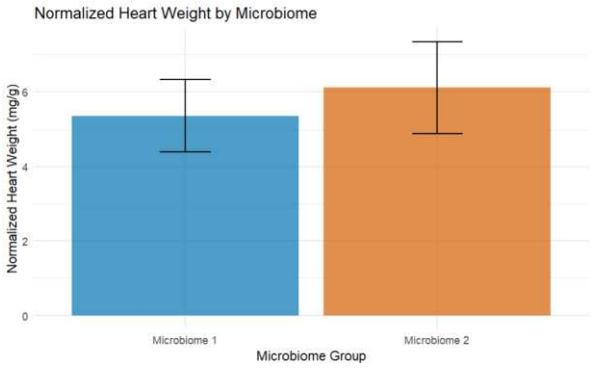


Fig. 3- Plot of mice heart weight (mg) normalized by mice body weight (g) visualized by microbiome group.

individual (microbiome 1) was 125.6 (±SD) mg, while it was 138.9 (± SD) mg in mice that were colonized with the microbiota of the obese individual (microbiome 2). We also observed a significant reduction in colon length between animals that received CBG or placebo (p=0.04), which was not observed when comparing CBD to placebo (p=0.13).

After normalization of subcutaneous adipose tissue (SAT) by mouse body weight we found a significant difference in SAT weight by treatment (p=0.002). Pairwise comparison showed a significant effect (fig. 4a) between CBD and CBG groups (p=0.01) as well as between CBD and placebo groups (p=0.003). Mean

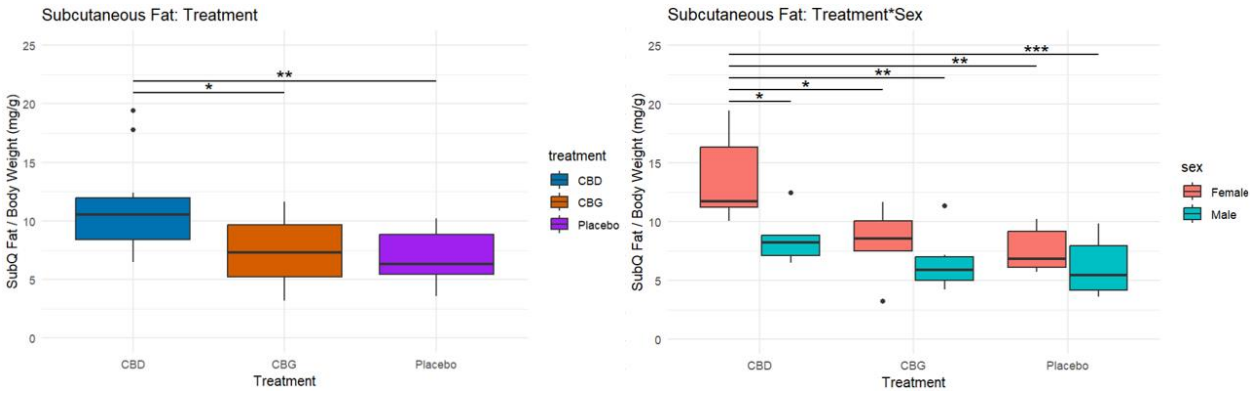


Fig. 4a Fig. 4b

Figure 4- Effect of treatment on SAT weight normalized by mouse body weight. (Fig. 4a) Effect of SAT weight by treatment group. (Fig. 4b) Effect of treatment on SAT weight by treatment*sex sub-groups. Significance is denounced * for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$.

SAT weight by treatment – CBD: 255.1 mg (sd=81.7), CBG: 168.5 mg (sd=70.7), and placebo: 156.5 mg (sd=53.7). Since there was also a significant effect by sex (p=0.007) an additional analysis was performed to look at the treatment*sex interaction. This revealed that females receiving CBD were driving the significance of both treatment and sex (fig. 4b). CBD female SAT weight was significantly higher than every other treatment*sex subgroup: CBD males (p=0.04), CBG females (p=0.03), CBG males (p=0.002), placebo females (p=0.009), and placebo males (p=0.0009). Tables 1a-c show anthropometrics of the mice by subgroupings.

Table 1a- Tissue metrics by treatment groups. Mean values reported with standard deviation in parentheses.

Tissue Weights: Treatment

Treatment	Epi (mg)	SAT (mg)	MAT (mg)	Liver (mg)	Heart (mg)	Colon (cm)	Cecum (mg)	Spleen (mg)
CBD	340.2 (153.1)	255.1 (81.7)	246.4 (103.7)	1187.6 (151.4)	129.7 (31)	5.4 (0.6)	652.8 (159)	70.9 (13.9)
CBG	280.7 (138.7)	168.5 (70.7)	299.5 (123)	1199.1 (183.5)	131.5 (39.6)	5.2 (0.6)	567.4 (117.2)	68.3 (10.2)
Placebo	273 (97.3)	156.5 (53.7)	273.3 (92.9)	1195.4 (173)	135.7 (20.4)	6 (0.9)	659.3 (85.5)	69.4 (7.4)

Table 1b- Tissue metrics by treatment*microbiome groups. Mean values reported with standard deviation in parentheses.

Tissue Weights: Treatment*Biome

Treatment	Biome	Epi (mg)	SAT (mg)	MAT (mg)	Liver (mg)	Heart (mg)	Colon (cm)	Cecum (mg)	Spleen (mg)
CBD	1	346.1 (162.7)	277.4 (89.7)	257.1 (148.6)	1189.3 (155.7)	125.4 (34.3)	5.2 (0.6)	663.7 (135.3)	76.4 (17.1)
CBD	2	334.2 (158.1)	232.8 (73.8)	235.6 (35.7)	1185.8 (161.7)	134 (29.8)	5.5 (0.6)	641.9 (192.3)	65.3 (7.8)
CBG	1	301.1 (203.4)	179.1 (97.4)	276.7 (160.3)	1222.4 (170.2)	121.6 (45.7)	5.2 (0.3)	588.5 (122.8)	70.2 (13.1)
CBG	2	263.8 (68)	157.9 (35)	322.3 (79.7)	1175.8 (209.3)	141.3 (33.6)	5.2 (0.9)	546.3 (118.7)	66.4 (7.1)
Placebo	1	262.8 (92.6)	133.3 (45.9)	279.4 (104.7)	1175 (175.3)	129.8 (16.1)	5.8 (0.8)	661.6 (91.2)	67.9 (7.6)
Placebo	2	283.2 (109.6)	179.6 (54.4)	267.2 (89.2)	1215.7 (184.8)	141.4 (23.9)	6.1 (1)	657 (88.1)	70.8 (7.5)

Table 1c- Tissue metrics by treatment*sex groups. Mean values reported with standard deviation in parentheses.

Tissue Weights: Treatment*Sex

Treatment	Sex	Epi (mg)	SAT (mg)	MAT (mg)	Liver (mg)	Heart (mg)	Colon (cm)	Cecum (mg)	Spleen (mg)
CBD	Female	300.4 (165.1)	282.6 (78.6)	259 (125.8)	1093.5 (80.5)	112.2 (18)	5.3 (0.6)	570.3 (123.5)	71.8 (10.6)
CBD	Male	379.9 (143.2)	227.6 (81.8)	233.8 (86.2)	1281.6 (150.7)	147.2 (32.5)	5.5 (0.5)	735.3 (154.9)	70 (17.6)
CBG	Female	195.6 (65.2)	171.4 (64.1)	316.4 (145.2)	1056.2 (136.3)	119 (39.9)	5.1 (0.9)	552.5 (123.9)	72.9 (11.9)
CBG	Male	351.7 (147.6)	165.6 (82.8)	282.5 (107.2)	1342 (80.5)	143.9 (38.6)	5.3 (0.3)	582.3 (119.8)	63.7 (6.1)
Placebo	Female	202.4 (54.3)	158.6 (41.2)	258.5 (106.6)	1050.8 (71.7)	134.6 (27)	5.8 (0.8)	605.9 (84.2)	74.3 (5.1)
Placebo	Male	343.6 (77)	154.3 (68.2)	288.1 (84.4)	1340 (102.7)	136.7 (13.5)	6.2 (0.9)	712.7 (46.6)	64.5 (5.9)

Anxiety and Cognition

There were no significant treatment effects on anxiety-like behavior in the OF test nor cognition using the discrimination ratio for the NOR test. For the OF test, total distance traveled was significantly different by microbiome grouping (p=0.028) at the Week 4 measurement and this was not observed at baseline (p=0.5). Microbiome 1 had a mean distance of 19.1 m while microbiome 2 had a mean distance of 16.2 m (Figure 5).

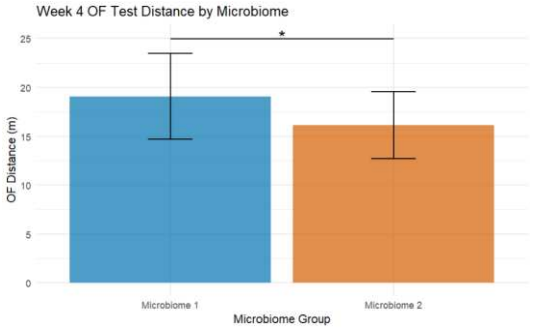


Fig. 5- OF distance (m) traveled in week 4 testing by microbiome group.

Out of 36 mice only 22 met exploration criteria at both baseline and week 4 testing. Due to experimenter-introduced variability in the first cohort of mice that were tested in the EPM test, we only proceeded with statistical analysis for anxiety scores in the second cohort tested. As a result, only 20 mice were included in the analysis (12 females and 8 males, 10 lean and 10 obese-associated microbiomes). By treatment allocation, 5 mice were in the placebo group, 6 in the CBD group, and 9 in the CBG group.

Using only cohort 2, we were not powered to look at treatment interactions with sex or microbiome for anxiety scores. The mean and standard deviation for the final AI scores of each group are as follows: placebo= 0.81 (sd=0.03); CBG=0.72 (sd=0.09); CBD= 0.67 (sd=0.1). At Week 4, there was a significant main effect for treatment (Figure 6; p=0.015), which was driven by a significant difference in AI scores between

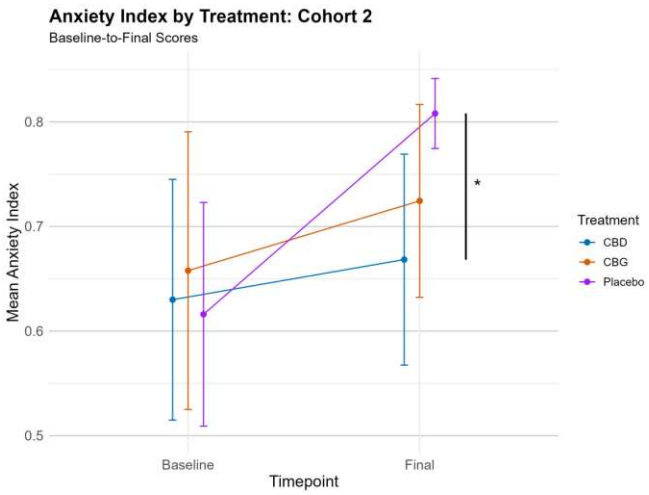


Fig. 6- Mean anxiety scores by treatment over time. * Denotes significance between CBD final AI and placebo final AI.

CBD and placebo (p=0.01). In addition, we ran a mixed-effects model to assess changes in AI over time in CBD and CBG treated mice. With repeated EPM testing, rodents show increased anxiety[37,38]; therefore, our objective was to determine if CBD and CBG prevented significantly increased anxiety from baseline compared to the placebo group. The placebo group did demonstrate significantly increased AI scores over time (p=0.005), and the CBD (p=0.92) and CBG (p=0.36) exposed animals did not (fig. 6). The CBD group had a 7.1% increase in AI, the CBG group had a 12.8% increase, and placebo group AI increased by 33.9%.

Gut Microbiota

Demultiplexing of 16S sequencing data resulted in a total of 8.97 million reads, with an average of 84,600 reads per sample. After denoising, the total reads were 7.12 million. Forward reads were truncated at 183bp, which resulted in poor merging of forward and reverse reads, so only forward reads were used for microbiota analysis. A total of 101 fecal samples were used for analysis (24 baseline, 32 week 2 and 35 week 4). Comparison of the baseline fecal samples showed that the animals clustered according to microbial inoculum group when looking at both the Phylum (p=0.01) and genus (p=0.001) (Figure 7b, d) levels but did not cluster by treatment or sex. At baseline, animals receiving Microbiota 1 from the lean individual showed a statistical trend for higher alpha-diversity at the phylum (p=0.07) and genus levels (p=0.09) (Figure 7a, c). There were no differences by treatment allocation or sex on parameters of alpha-diversity at baseline.

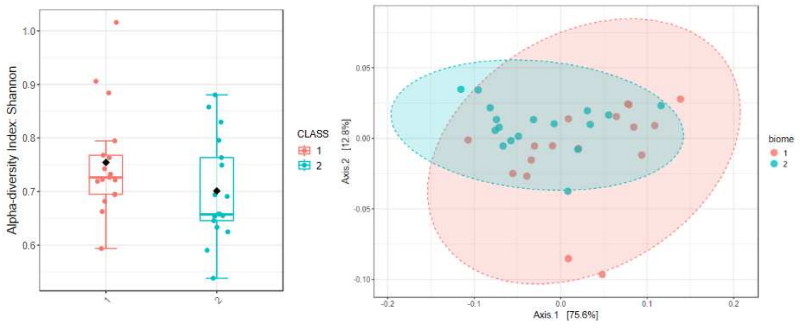


Fig. 7a Fig. 7b

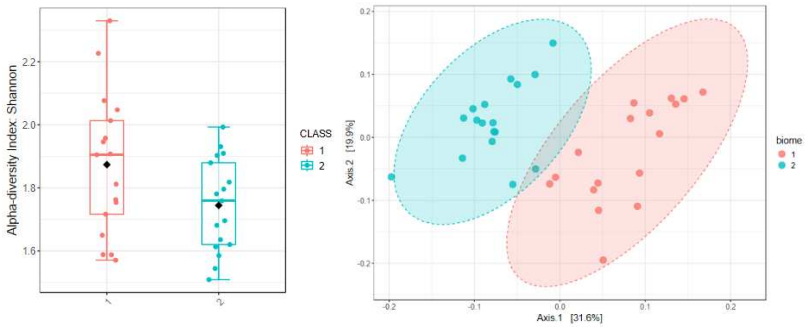


Fig. 7c Fig. 7d

Fig. 7- Alpha diversity (Shannon index) and beta diversity (Bray-Curtis dissimilarity) between microbiomes 1 and 2 at baseline. (fig. 7a) Alpha diversity at the phylum level. (fig. 7b) Beta diversity at the phylum level. (fig. 7c) Alpha diversity at the genus level. (fig. 7d) Beta diversity at the genus level.

No microbiota differences by treatment group were observed at Week 2. However, by Week 4, animals receiving the CBD (p=0.008) and CBG (p=0.02) treatments had significantly higher alpha diversity at the phylum level, compared to placebo (fig. 8a). At the genus level, only CBD had higher alpha diversity than

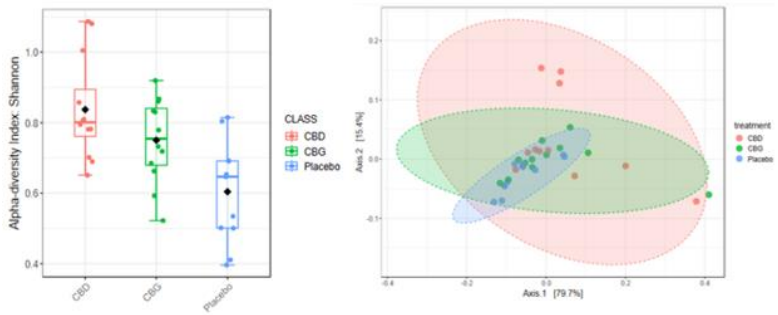


Fig. 8a Fig. 8b

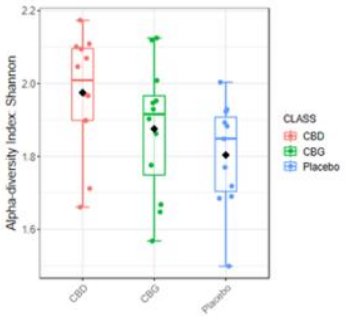


Fig. 8c

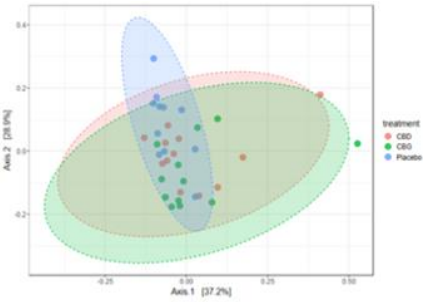


Fig. 8d

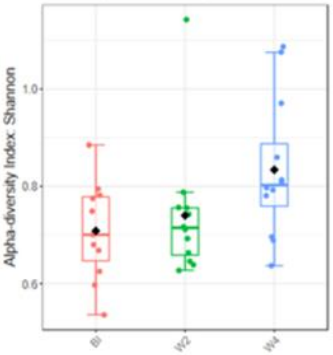


Fig. 8e

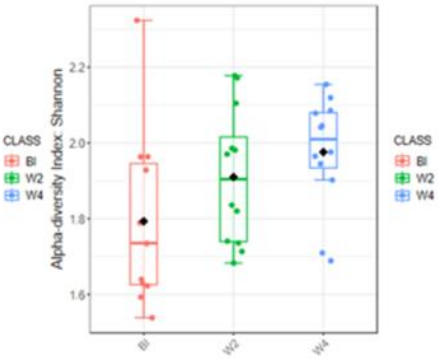


Fig. 8f

Fig. 8- Alpha diversity and beta diversity changes by treatment groups. (Fig. 8a) Week-4 phylum level alpha diversity by treatment group. (Fig. 8b) Week-4 phylum level beta diversity by treatment group. (Fig. 8c) Week-4 genus level alpha diversity by treatment group. (Fig. 8d) Week-4 genus level beta diversity by treatment group. (Fig. 8e) Phylum level alpha diversity over time in the CBD group. (Fig. 8f) Genus level alpha diversity over time in the CBD group.

the placebo (p=0.04) (fig. 8c). Similarly, PCoA of Bray-Curtis distances calculated at the phylum level showed significant clustering by treatment, with CBD distinct clustering separately from the placebo group (Pairwise PERMANOVA; p=0.006), while there was a trend for distinct clustering of the CBG group from placebo (Pairwise PERMANOVA; p=0.09) (fig. 7b). There was no significant difference in cluster dispersion (PERMDISP=0.37). At the genus level, both treatments were significantly different from placebo (Pairwise PERMANOVA; CBD, p= 0.017; CBG, p= 0.012; fig. 8d), while cluster dispersion did not differ significantly (PERMDISP=0.72). CBD was the only treatment to result increased Shannon's diversity over time with a trending increase in phyla-level diversity (p=0.06; Figure 8e) and significant increase in genus-level diversity between baseline and Week 4 (p=0.047; Figure 8f).

We next wanted to see if these changes we observed taking place were unique between the two microbiome groups. First, at the phylum level, we did not observe significant changes taking place in either

microbiome after 4 weeks of treatment. We only saw one trend at the phylum level between the CBD and placebo groups in microbiome 1 (p=0.10, PERMDISP=0.74, fig. 9b). We noticed significant changes taking

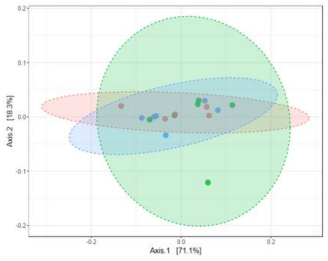


Fig. 9a

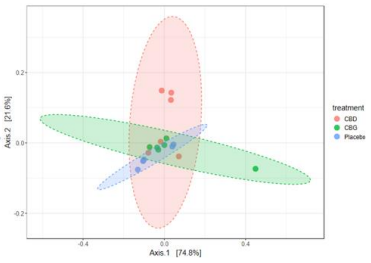


Fig. 9b

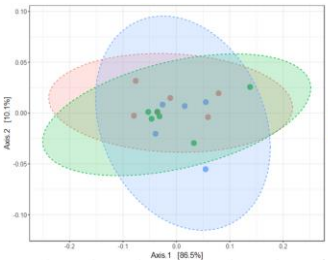


Fig. 9c

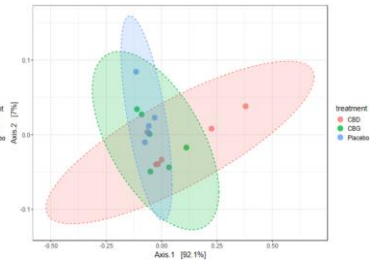


Fig. 9d

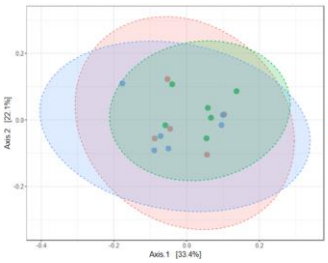


Fig. 9e

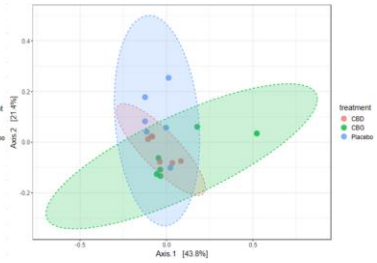


Fig. 9f

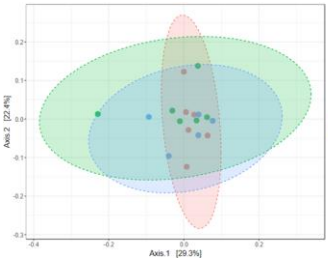


Fig. 9g

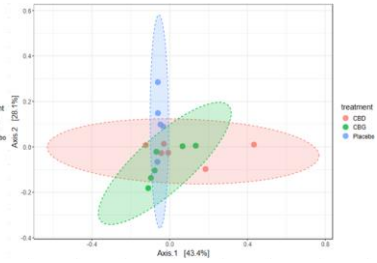


Fig. 9h

Fig. 9- Phylum level and genus level beta diversity from baseline to week 4 by treatment, shown by each microbiome group. (Fig. 9a) Phylum baseline beta diversity of microbiome 1. (Fig. 9b) Phylum week 4 beta diversity of microbiome 1. (Fig. 9c) Phylum baseline beta diversity of microbiome 2. (Fig. 9d) Phylum week 4 beta diversity of microbiome 2. (Fig. 9e) Genus baseline beta diversity of microbiome 1. (Fig. 9f) Genus week 4 beta diversity of microbiome 1. (Fig. 9g) Genus baseline beta diversity of microbiome 2. (Fig. 9h) Genus week 4 beta diversity of microbiome 2.

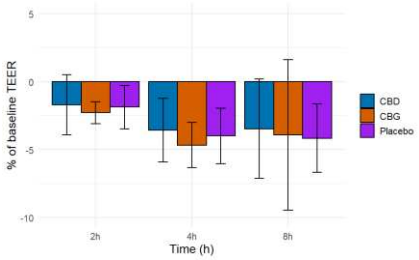
place at the genus level. At week 4, both CBD and CBG trended away from placebo (p=0.07 for both, fig. 9f) in microbiome 1. In microbiome 2, we saw at 4 weeks CBD trended from placebo (p=0.10) and CBG missed significance threshold compared to placebo (p=0.054, fig. 9h). However, we did not see the two cannabinoid treatments become dissimilar from each other, indicating they may be altering the microbiomes in a similar manner.

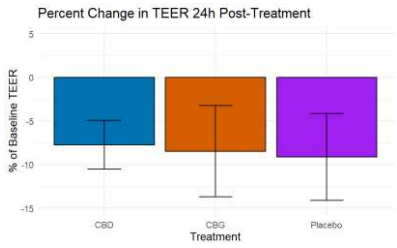
Changes in Differential Abundance with Cannabinoids

Both CBD and CBG treatment revealed shared and unique taxonomic shifts after 4 weeks of treatment. A qval of <0.1 was used to identify differentially abundant taxa. Compared to placebo, both CBD and CBG had increased abundance of *Oscillospiraceae* family (qval=0.0017 and 0.0018, respectively). CBD also showed an increased abundance of *Lachnospiraceae FCS020* group (qval=0.03). Comparing CBG to CBD only one taxon came up as significant, an uncultured bacterium in the *Roseburia* genus, which was positively associated with CBD treatment compared to CBG treatment (qval=0.056).

Effects of Phytocannabinoids on Intestinal Barrier Integrity

Phytocannabinoids have been touted for their anti-inflammatory properties. Therefore, to explore whether chronic consumption of these compounds impacted gut barrier function, we used cecal water extracts prepared from experimental mice to determine how CBD, CBG, or placebo exposure influenced the intestinal environment by measuring gut barrier integrity in Caco-2 cell monolayers. For initial analysis of the TEER assay, we first removed significant outliers determined by IQR calculations. This removed 1 sample at 2-hours post-treatment. We found no significant differences in triplicate TEER measurements by treatment, microbiome, or sex from baseline to 2-hours, 4-hours, or 8-hours post treatment (Figure 10a). For baseline and 24-hour post treatment recordings we had 6 wells for each sample. When initially ran, we did not see significance by treatment (p=0.15) or sex (p=0.14) 24-hours post-treatment with cecal extracts. However, we observed a trending effect by microbiome (p=0.056). After filtering out 3 outliers, all in the CBG group, we reran the ANOVA test. Treatment and sex were still not significant. After filtering there was a significant difference in TEER values by microbiome after 24-hours of cecal extract exposure (p=0.002). Data for the filtered 24-hour post-treatment changes can be seen in Figure 10b, c.





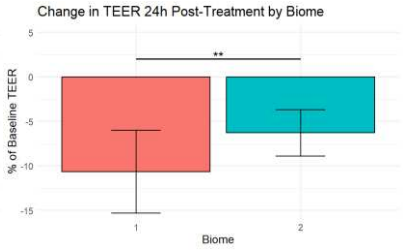
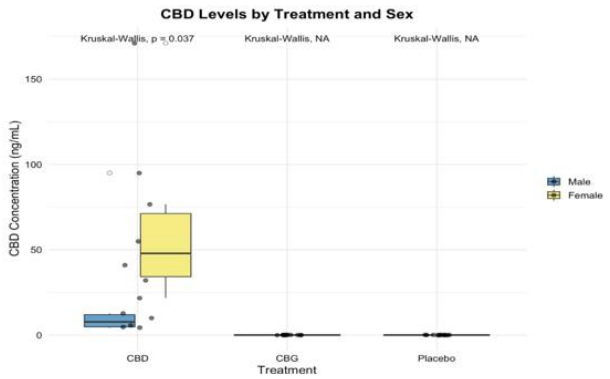


Fig. 10a Fig. 10b Fig. 10c

Fig. 10- Percent changes in TEER value compared to baseline means from exposure to cecal extracts. (Fig. 10a) Percent changes in triplicate TEER value by treatment compared to baseline means at 2-hours, 4-hours, and 8-hours post-treatment. (Fig. 10b) Percent changes in TEER value by treatment from baseline to 24-hours post-treatment with 6 wells per sample. (Fig. 10c) Percent change in TEER value by microbiome group from baseline to 24-hours post-treatment with 6 wells per sample. Significance is denounced * for $p < 0.05$ and ** for $p < 0.01$.

Plasma Metabolomics

We measured plasma levels of both endogenous cannabinoids (1-AG, 2-AG, AEA), as well as the parent phytocannabinoids that were administered as experimental treatments. CBD and CBG levels were only above the detection threshold in animals receiving those treatments. Microbiome composition did not affect circulating CBD or CBG concentrations. However, there were sex-based differences in CBD (p=0.037) (fig. 11a)



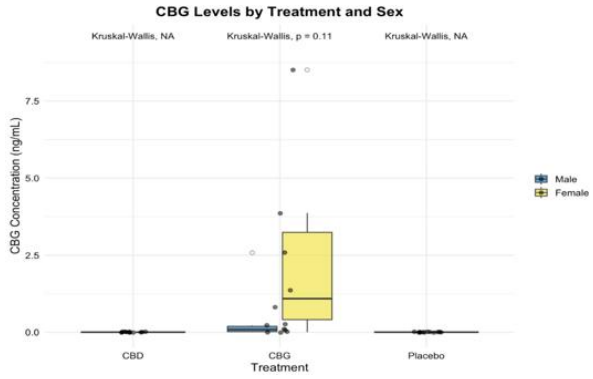
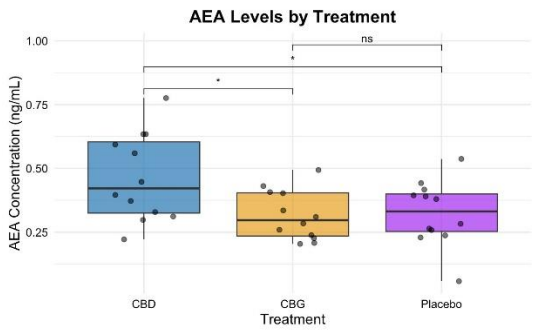


Fig. 11a Fig. 11b

Fig. 11- CBD and CBG concentrations in the plasma by treatment*sex from oral consumption. (Fig. 11a) Absorption of CBD into the plasma between male and female mice. (Fig. 11b) Absorption of CBD into the plasma between male and female mice.

concentrations, but not on CBG concentrations (p=0.11) (fig. 11b). Females had higher levels of the cannabinoids than their male counterparts (fig. 11). In the CBD group, mean plasma concentration for females was 66.2 ng/mL and for males 22 ng/mL. In the CBG group, females averaged 2.5 ng/mL while males averaged 0.5 ng/mL.

For endogenous cannabinoids, there were no significant differences in 1-AG or 2-AG by sex, microbiome, or treatment group. When it came to AEA, a significance by treatment was observed. The CBD group had significantly higher plasma AEA concentrations than both the CBG group (p=0.018) and the placebo group (p=0.033) (Figure 12a). Looking at this further, it was also observed that sex played a role, with



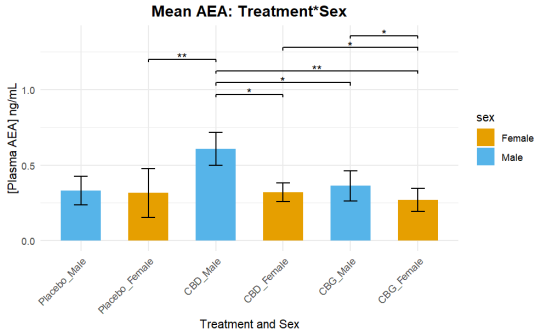


Fig. 12a Fig. 12b

Fig. 12- Concentrations of AEA in the plasma. (Fig. 12a) Concentration of AEA between treatment groups. (Fig. 12b) Concentration of AEA between treatment groups and sex. Significance is denounced * for $p < 0.05$ and ** for $p < 0.01$.

males in the CBD group having significantly higher AEA concentrations than the CBD females (males= 0.61 ng/mL, females= 0.32 ng/mL; p=0.025). Males in the CBG group had significantly higher in AEA concentrations than the CBG females (males= 0.36 ng/mL, females=0.27 ng/mL; p=0.03). CBD males had significantly higher levels of AEA than CBG males (p=0.05), CBG females (females= 0.27 ng/mL; p=0.001), and placebo females (females= 0.32 ng/mL; p=0.002).

Conclusion/Discussion

The overarching goal of this study was to examine the bi-directional interactions between two phytocannabinoids, CBD and CBG, and the gut microbiome. We colonized mice with two distinct microbiomes to determine whether microbiome differences altered metabolism of these phytocannabinoids such that the circulating levels of parent compounds were altered. While we were able to confirm that the different microbial inoculants led to distinct microbial profiles in the colonized animals, we saw no effect of microbiome on circulating phytocannabinoid levels. Gut microbes have been shown to extensively metabolize polyphenols and other phytochemicals, such as glucosinolates[39,40], but pathways for cannabinoid metabolism have not been widely studied. Beta-glucuronidases from microbes have been shown to deconjugate glucuronide metabolites of the psychoactive *Cannabis* metabolite, tetrahydrocannabinol (THC), releasing the

active form back into circulation[41]. Additionally, gut bacteria metabolize THC into 11-hydroxy-THC (11-OH-THC), and 11-nor-9-carboxy-THC (THC-COOH), which have varying bioactivity from the parent compound[41]. However, reports of specific metabolic reactions carried out by gut bacteria on CBG or CBD are lacking. One limitation of the current study is that we only measured parent compounds and therefore may have missed differences in microbiome-specific metabolites that were produced. Alternatively, enzymes that alter phytocannabinoids may be widespread and/or redundant in the microbiome resulting in similar levels of interaction with the phytocannabinoids despite differences in taxonomic composition of the microbiota.

Although there were no microbiota-associated differences in circulating phytocannabinoids, there were differences by sex. Female mice had higher plasma concentrations of CBD and were trending toward higher plasma CBG concentrations compared to males after consumption of the water-soluble CBD formulation. Similar data have been reported in two human studies between males and females consuming CBD. A randomized, double-blind, cross-over trial of 8 men and 8 women that orally consumed 25 mg of two different CBD formulations revealed women consuming a MCT-CBD formulation had significantly higher AUC of CBD metabolites at 8-hours ($p=0.02$) and 24-hours ($p=0.049$) post-consumption than their male counterparts[42]. While not significant ($p=0.11$) women in this group also had a 2.2-fold higher peak concentration than males. In comparison, consumption of self-emulsifying drug delivery system (SEDDS)-CBD formulation displayed similar bioavailability between males and females. In another randomized cross-over clinical study of 6 men and 6 women orally consuming 750 mg of CBD twice daily for three days and one dose on a fourth day reported women had significantly higher peak concentration ($p=0.015$) and significantly higher AUC ($p=0.028$) of the CBD metabolite 7-COOH-CBD than their male counterparts[43]. Our findings revealed similar trends as previously reported with concentrations of CBD in plasma higher in females than in males. Our findings also provide insight that a similar trend may be taking place with concentrations of CBG in plasma between males and females.

While there was no clear role for the microbiome in cannabinoid bioavailability, there were effects of cannabinoid consumption on the gut microbial composition. Increased alpha-diversity is thought to be an indicator of better health outcomes[44]. While this is not always the case, increased diversity does contribute to ensuring that there is redundancy in the functional capacity of the gut microbiome and provides stability and resilience. Therefore, the observed increase in Shannon's diversity with CBD and CBG intake may be indicative

of beneficial effects on health. Currently, other studies that have directly looked at the effects of CBD on composition of the gut microbiome in rodent models have not shown significant differences in alpha diversity compared to control groups[15–17]. Some differences in study designs likely contribute to our first reported finding of CBD resulting in higher alpha diversity than placebo. One using the same mice as our study, C57BL/6J were orally administered a lower quantity, 30 mg/kg, of CBD for four weeks[16] another using the same mouse model gave 50 mg/kg of CBD by oral gavage[15]. A study performed in rats had oral gavage administration of either 20 mg/kg or 100 mg/kg of CBD, but only for seven days[17]. Finally, our study used human-associated microbiota to colonize the mice making results potentially more translational to humans. Thus, the differences in microbiota, dose, and duration could account for why other studies have not reported increased Shannon's diversity after phytocannabinoid exposure.

There were several specific taxa whose relative abundances were changed in response to CBD and/or CBG consumption. Both CBD and CBG intake were associated with bacterial genera that have reported correlations with mental health. *Roseburia*, while higher in the CBD group compared to CBG group, was negatively associated with both phytocannabinoids. This genus has been positively associated with mental health and serotonergic signaling[45–47], and negatively associated with IBS severity[48]. *L. dorea*, negatively associated with CBD consumption in our study, has been shown to have mixed associations with mental health[49–51]. CBD consumption also had positive association with *L. FCS020* group. Investigation into this microbe in the context of mental health is limited. The current state of the literature is mixed, but some suggest *L. FCS020* group is positively correlated with depression and worsening mental health[52,53]. These results are interesting given that despite CBD- associated microbial profiles that would suggest worse outcomes in the anxiety and cognition tests, we saw attenuation of anxiety-like responses in animals that consumed CBD. As we reported, consumption of CBG resulted in a prevention of significantly increased anxiety-like behavior in repeated bouts of the EPM test and a decrease in *Paraprevotella*. This decrease in *Paraprevotella* is notable as it has been reported that elderly adults[54] and in athletes[55] displayed increased anxiety-like symptoms were associated with elevated levels of *Paraprevotella* in the gut. Despite changes in the gut microbiota, we did not see any changes in gut environment (i.e. cecal water extracts) that impacted gut barrier function as measured in our ex vivo cell culture model. Further studies should explore how a cannabinoid-altered microbiota may impact intestinal function *in vivo*.

Finally, we examined the impact of cannabinoid treatment on anxiety and cognition in the mice. As previously mentioned, repeated testing with the EPM increases anxiety-like behavior in rodents. We were able to demonstrate that oral administration of either CBD or CBG attenuates the induced anxiogenic response in anxiety-like behavior with repeated testing using the EPM. Week 4 AI scores showed the CBD group had a significantly lower AI score compared to the placebo group (fig. 5). While this shows therapeutic potential of CBD and CBG through an anxiolytic mechanism, deeper interpretation of these results needs to be considered. Treit et al. displayed through various forms of repeated testing that open arm avoidance is learned after the first bout of testing[37]. One form of this testing with diazepam showed that the anxiolytic effects of the drug did not carry over when the mice were in a non-drugged state. This presents the case that further research on CBD and CBG should be conducted to see if their anxiolytic potential is only displayed in a "drugged" state or if they aid in a neurological rewiring/relearning of stress response that carries over in a non-drugged state. There is also consideration of the increased open-arm avoidance (increased anxiety-like behavior) is less of an anxiety response and more of a learned avoidance from exposure to the environment[38]. This could be due to both reduced novelty of the environment and learned "danger" from rodent aversion to heights and open areas that expose them to predation. Future investigation may be worthy to symbiotically address the mechanisms of these factors. It is also noteworthy that our dosing of CBD and CBG was exceptionally high compared to current recommendations and clinical dosing. Even so, we report that ~250 mg/kg of CBD or CBG does not have a detrimental effect on cognitive function in the context of recognition memory nor anxiety. No abnormal behavior was observed during cognitive testing or during animal handling over the duration of the study. Using only cohort 2 for anxiety analysis in the EPM test we lost power to look at microbiome or sex interactions with the changes in anxiety scores we saw from the treatments.

We are the first to report gut microbiome changes associated with CBG consumption. Using a translational FMT model in germ-free mice, we were able to characterize these changes with human-associated microbes. This showed that both CBD and CBG may be altering the microbiome in a similar manner compared to a placebo. We are the first to characterize and compare the independent effects of both CBD and CBG on the microbiome outside of disease models. We reported that a microbiome associated with CBG consumption did not have harmful effects on intestinal permeability in a TEER assay. We are also the first to compare the anxiolytic potential of both CBD and CBG in isolation, showing both cannabinoids may have

similar therapeutic potential. We are the first to observe CBD consumption altering AEA levels in plasma in a sex-dependent manner, with male mice consuming CBD having significantly higher plasma AEA than their female mice counterparts. Another physiological response that we are the first to report is female mice consuming CBD accumulate a greater SAT weight than both their male counterparts and other treatment*sex subgroups. These findings were the result of mice receiving a supraphysiological dose. This dose was intentionally used to answer our main question about the bioavailability of these cannabinoids being influenced by distinct microbiomes. While this presented a translational limitation for a human equivalent dose, this supraphysiological dose did not result in detrimental or adverse effects during our analysis. Our small sample size may have prevented us from being able to detect more distinct differences from consumption between CBD and CBG on health. Experimenter-introduced variability in the first cohort prevented us from being able to look at treatment*microbiome or treatment*sex interactions in anxiety scores from our induced-anxiety testing with the EPM. This also prevented us from having a comprehensive analysis of the GBA by correlating anxiety scores with these interactions to observations we found in the plasma and in the microbiome. Overall, our findings add to the framework for future research investigating cannabinoids and the GBA to build upon.

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Contributions

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