DISSERTATION

EXPLORING DIFFERENTIAL BIOLOGICAL AND PSYCHOLOGICAL EFFECTS OF REGULAR ALCOHOL AND CANNABIS USE IN HUMANS

Submitted by

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ABSTRACT

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Cannabis is the most commonly used substance among individuals who consume alcohol, but there is conflicting evidence regarding the effects of cannabis on alcohol use and specific health outcomes. Furthermore, physiological responses to long-term and regular use of these substances differ substantially. Research has established deleterious health effects of heavy and persistent alcohol use, including imbalance of the gut microbiome (termed dysbiosis) and systemic inflammation. Conversely, research shows cannabis to have consistent anti-inflammatory properties and support mucosal defense and repair of the intestinal wall. However, very little research investigates the effects of alcohol and cannabis co-use on these biological symptoms. Even further, the microbiota-gut-brain axis details the bidirectional relationship between the gut and psychological function. Current literature demonstrates an inarguable influence of gut health and systemic inflammation on psychiatric symptoms such as depression and anxiety. Even further, certain personality traits have been linked to behavioral tendencies such as substance use.

The present study aims to investigate physiological and psychological differences across individuals with different patterns of alcohol and cannabis use (individuals who exclusively and regularly use cannabis-only, alcohol-only, individuals who regularly use both alcohol and cannabis, and individuals who abstain from both substances). The study encompassed 4 multi-disciplinary aims spanning health outcomes impacted by substance use. The aims were to: (1) explore trait depression, anxiety and impulsivity as well as personality traits among the four groups, (2) examine differences in baseline circulating levels of endocannabinoids related to substance use patterns, (3) investigate group differences in gut microbiome composition and intestinal permeability, and (4) explore group differences in circulating inflammatory markers. These aims were tested through collection of fecal and blood samples,

as well as administration of several psychological assessments and substance use questionnaires to individuals between the ages of 21-58 who were screened into groups according to their reported use of alcohol and cannabis.

An observational cross-sectional design was used to collect data from the alcohol-only, cannabis-only and abstinence groups, while the alcohol and cannabis co-use data was supplied by a sister experimental study following identical eligibility criteria and near-identical data collection methods (barring some design constraints within the co-use group). The control group was expected to exhibit the healthiest profiles in all measures, followed by the exclusive cannabis use group. The exclusive alcohol use group was expected to exhibit the poorest health outcomes, with the co-use group falling between the exclusive alcohol and cannabis use groups.

Eligible participants completed a virtual consent and instructional session followed by 14 days of online daily diaries detailing substance use, exercise and mood, and then completed their participation with a laboratory session involving biological sample collection and several electronic surveys. Statistical evaluation of group differences included analyses of covariance as well as non-parametric Kruskal-Wallis tests to investigate aims 1, 2 and 4. Aim 3 analyzed data from an advanced biostatistics pipeline for microbiome analysis, comparing alpha and beta diversity metrics as well as relative abundance and total read counts of identified taxa.

Results revealed some persistent themes suggesting that cannabis use in the absence of alcohol predominantly exhibits protective or regulatory effects on immune and microbiome homeostasis, while alcohol consumption supports a more inflammatory and dysbiotic profile. While these data cannot speak to causality of observed relationships, the overall results support the notion that alcohol and cannabis use display differential influences on the gut microbiome and immune signaling in ways that may converge with mental health symptoms and personality traits. The current F31-supported study establishes a preliminary groundwork for more extensive and rigorous research on integrative psycho-physiological processes and how they are impacted by differing patterns of alcohol and cannabis use.

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DEDICATION

This work is dedicated to my dad, Phillip A. Archey.

Thank you for being my first and forever biggest fan.

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Chapter 1. General Introduction

Cannabis is the most commonly used drug among consumers of alcohol[1], but the existing evidence is conflicting regarding the effects of cannabis on alcohol use and specific health outcomes[2]. Additionally, physiological responses to long-term and regular use of these substances differ substantially. Heavy and persistent alcohol use has known deleterious effects on the gut, such as leaky gut and inflammatory bowel disease[3,4], while recent research suggests cannabis may exert systemic effects opposite to the effects of alcohol, such as reducing or preventing alcohol-induced liver, bowel and systemic inflammation[5–10]. These protective effects likely stem from endocannabinoid receptor signaling and the gut-brain axis.

The endocannabinoid system is comprised of cannabinoid receptors, enzymes and several other signaling molecules distributed throughout the central nervous system and periphery[11–13]. Cannabinoid receptors bind exogenous cannabinoids such as delta-9-tetrahydrocannabinol (THC, the psychoactive component of cannabis), as well as endogenous cannabinoids produced by the body. This binding begins a cascade of systemic events[12]. The endocannabinoid system has been linked to emotional control and cognitive processing[14–17], as well as gut-brain signaling[18]. Animal studies also suggest that chronic alcohol exposure and withdrawal may induce long-term up-regulation of prominent endogenous cannabinoids[19–21]. Cannabis appears to alter levels of endogenous cannabinoids as well, downregulating receptor expression and thus causing an increase in circulating endogenous cannabinoids[22–24].

Importantly, the endocannabinoid system is heavily distributed throughout the gut[11,25]. In particular, cannabinoid receptors are prevalent in the gut and have an essential regulatory role in the gut-brain axis[26–30], interacting with the gut microbiome and affecting signaling processes throughout the body[31–34]. Alcohol is shown to disrupt gut function by causing an imbalance of

bacterial flora (known as dysbiosis) in the gut and loosening tight junctions in the intestinal wall, thereby increasing permeability and promoting widespread inflammation[35–39]. On the contrary, emerging evidence suggests that exogenous cannabinoids like those in cannabis may be associated with decreased dysbiosis and improved intestinal barrier function[40,41].

Finally, psychological factors (namely anxiety and depression) may be related to the associations between cannabis, alcohol and gut function. Specifically, many cannabis users begin using regularly while experiencing depression[42–44], and self-reports of regular cannabis users often mention anxiety reduction as a motivator for use[45,46]. Similarly, depression is frequently found to be comorbid with alcohol use and dependence, while social anxiety disorder co-occurs with alcohol use disorders at high rates[47,48]. Further, recent studies suggest bidirectional links between gut microbiome health and depression and anxiety[49–51]. Given the evidence discussed, attempts to use alcohol or cannabis as relief for depression and anxiety symptoms could have vastly different long-term health outcomes. In fact, the combination of mental illness and alcohol use could have severe implications for gut health and consequently, worsening psychological symptoms over time.

These associations found separately throughout the literature point to a need for investigation of interactive relationships between gut health, substance use, and disordered mood regulation. Ultimately this knowledge could provide insight into unexplored treatment options for alcohol use disorder and perhaps for affective disorders such as major depressive disorder (MDD) or generalized anxiety disorder (GAD). In particular, cannabis may be a viable option for improving gut function in heavy alcohol users. In turn, this improvement could have positive implications for mental health outcomes in this population as well.

The present study aims to investigate physiological and psychological differences across individuals with different patterns of alcohol and cannabis use (individuals who exclusively and regularly use cannabis-only [CO], alcohol-only [AO], and individuals who regularly use both alcohol and cannabis [co-use], versus abstaining controls).

1.1 Alcohol & Cannabis Use and Mental Health: General Impacts and Current Treatment

Cannabis and alcohol are frequently used together[1], but current data regarding the impact of each substance on the other as well as health outcomes is limited. Regular alcohol use causes systemic inflammation and disrupts the health of the gut microbiome[3,4,52–54]. However, the effects of acute and long-term cannabis exposure are less understood. Notably, recent research suggests that cannabis may exert some systemic effects which are opposite to the effects of alcohol, such as being protective against alcohol-induced inflammation[5–9]. These findings are consequential as excessive alcohol use accounts for more than 85,000 deaths in the United States[55] and costs the US economy an estimated $249 billion each year[56]. In the same vein, the prevalence of individuals struggling with depression and anxiety continues to increase, and these mental health conditions cost the U.S. an estimated $247 billion each year[57]. Alcohol and cannabis use disorders are often comorbid with psychiatric disorders (i.e. depression and anxiety) and suicidal ideation[47,48,58], yet treatment for these combined issues is lacking. Similarly, cannabis users often claim to use cannabis to reduce feelings of anxiety or treat depressive episodes[59–61]. Given the social and economic impacts of alcohol, substance use and psychiatric disorders alongside the established associations between substance use and mental health concerns, the potential for harm reduction effects associated with cannabis should be considered and explored.

Despite extensive preclinical and clinical research revealing risk factors surrounding the etiology and maintenance of alcohol use disorder, currently available pharmacological and psychosocial treatment options are generally considered to be only moderately effective[62,63]. Because alcohol use disorder is multi-faceted, involving interactive neurobiological and psychological components[64,65], effective treatments must consider the underlying molecular mechanisms of the disorder as a whole. This deeper level of understanding was prioritized in the recent 2017-2021 NIAAA Strategic Plan[66], wherein uncovering novel pathways for treating alcohol-related diseases were a primary goal, with particular focus on alcohol's effect on the interactions between the gut, liver and brain, including disruption of gut microbiome and intestinal permeability. Preclinical research investigating the endocannabinoid system (ECS) provides evidence that compounds found in the cannabis plant – chemicals such as delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD) – can bind to cannabinoid receptors in the central nervous system and initiate positive effects in individuals who drink heavily, such as decreasing alcohol consumption[67] and inflammatory effects of alcohol-related disease[10]. Evidence demonstrates that processes are regulated by endocannabinoid receptor signaling in the gut[25,68], and are negatively impacted by heavy alcohol consumption[69,70] potentially explaining this effect. Furthermore, studies suggest that chemicals from the cannabis plant could work collectively to acutely treat symptoms of mood and anxiety disorders[71].

More research is needed to explore the mechanisms of interaction between each of these systems. A deeper understanding could lead to consideration of the ECS as a source of intervention when treating the harmful effects of alcohol and alcohol use disorder, and could inform our understaning of cannabis as a possible harm reduction tool for people who drink heavily[72]. Moreover, this type of intervention could have a positive secondary impact on mental

health via the effect of endocannabinoid signaling in the gut. Studies show that psychological and behavioral factors such as anxiety, depression and substance use are influenced by gut health and endocannabinoid signaling[73–77]. Even with this evidence, treatment of depression and anxiety disorders has not seen significant advances in several years. Investigating the mechanistic components of these disorders through the ECS is a novel and potentially promising approach to finding more effective and accessible treatment options.

1.2 The Endocannabinoid System

The endocannabinoid system (ECS) is comprised of cannabinoid receptors (CB1 and CB2), enzymes and several other signaling molecules distributed throughout the central and peripheral nervous systems[11,12]. CB1 is the primary receptor of the central nervous system, whereas CB2 is more prominent in the periphery[13]. Wherever they are located, both CB1 and CB2 bind chemicals called cannabinoids that circulate throughout the body. Endogenous cannabinoids are produced naturally by the body, while exogenous cannabinoids are found in the cannabis plant and include THC, the primary intoxicating component in cannabis. There are several implications of endocannabinoid signaling and the impact it has on processes across biological systems. For instance, the ECS has been linked to emotional control and cognitive processing[16,17,77], which may explain to some extent why many cannabis users name emotional dysregulation (i.e. anxiety or depressive symptoms) as a motivation for use. Further, CB1 receptors are densely populated in the gut, showing clear associations between the ECS and gut-brain signaling[18]. Based on this evidence alone, it is very likely that the ECS is critically involved in many biological processes, with impacts which have yet to be fully fleshed out. Altogether, the culmination of current literature (discussed more in-depth in this document) can

lead us to hypothesize that interactions between neurotransmission, psychological processing and gut-brain signaling are perhaps mediated by the ECS.

1.3 The Microbiota-Gut-Brain Axis

The gut-brain axis (GBA) refers to the bidirectional communication between the gastrointestinal tract and central nervous system, connected in various ways including the immune system, enteric nervous system, and the vagus nerve[31]. The GBA explains the complex regulatory role each system has on the other in terms of maintaining homeostasis and proper functioning. Another means of connection between the gut and brain is the gut microbiome, which refers to the quantity and composition of microbes that live in the human gut. The human gut is home to a multitude of microorganisms that survive in a symbiotic relationship with the human host[31]. The diversity and abundance of these microorganisms have vast implications for the GBA and has thus been termed the microbiota-gut-brain axis (MGBA)[32,33].

It is worth noting that the immune system is a critical modulator of gut-brain communication[78,79]. An abundance of research demonstrates that the gut microbiome can considerably impact expression of inflammatory cytokines, affecting systemic and neurologic inflammation[78,79]. This impact may be directly related to alcohol use, which is known to have deleterious effects on the gut. Specifically, heavy drinking causes inflammation in the gut along with disruption and dysregulation of the normal balance of gut microbiota[4]. This disruption in bacterial composition of the gut is known as dysbiosis, which can be accompanied by compromised integrity of tight cellular junctions in the intestinal wall, increasing permeability and thereby allowing endotoxins to leak into the bloodstream (i.e. 'leaky gut')[3,4]. This leakage causes a myriad of issues, including severe emotion dysregulation[49,80] and systemic inflammation[3,70,81]. Additionally, the vagus nerve is another important gut-brain communication

pathway of communication which alcohol is thought to interfere with by inhibiting vagal control of inflammation and cardiovascular regulation[31,82]. This interference can lead to a cycle in which alcohol-induced gut issues send signals to the brain via the vagus nerve, potentially worsening cognitive performance and emotional dysregulation[83]. Further, chronic alcohol use can lead to lasting vagal nerve damage causing long-term impairment and inflammation[84].

Overall, chronic alcohol use can cause inflammatory disease of the liver, bowel and brain[3,4,37,39,85], while cannabis appears to have opposite effects[86–90], promoting healthy immune function. Specifically, emerging evidence suggests that exogenous cannabinoids may be associated with decreased dysbiosis (measured in part by increased microbial diversity) and improved intestinal barrier function[40,41,86,91,92]. A growing body of literature has connected cannabis to decreases in oxidative stress, inhibiting production of cytokines, and decreasing symptoms of inflammatory bowel and alcoholic liver diseases[88,92–96]. Further, some research suggests that co-users of cannabis and alcohol may be experiencing some harm-reduction effects of cannabis, specifically via the immune effects of cannabis opposite to those of alcohol[97–99]. Given the research surrounding alcohol, cannabis and the immune system, investigation into other systems within the MGBA is warranted to discover whether similar ameliorating effects of cannabis are seen when compared to alcohol.

1.4 Endocannabinoid System Interaction with the Microbiota-Gut-Brain Axis

Importantly, the endogenous cannabinoid system is heavily distributed throughout the MGBA. In particular, CB1 receptors densely populate the gut and brain, playing an essential role in gut-brain communication[28,100]. CB1 receptors interact with the gut microbiome and affect signaling processes throughout the body[31–33,101], regulating gut function and intestinal permeability[68,96]. The binding of these receptors by both endogenous and exogenous (e.g. those

found in cannabis) cannabinoids activate signaling cascades within the MGBA and can have an inhibitory effect on the production of inflammatory cytokines in the gut, liver and brain[29,88,95,102,103].

In turn, specific short-chain fatty acids that comprise the building blocks of endocannabinoids are heavily impacted by gut microbiome composition[104,105]. Disruption caused by alcohol then downregulates the production of endocannabinoids causing a compensatory upregulation of CB1 receptors[20,106]. Notably, animal models show that decreased endocannabinoid signaling in the hippocampus and amygdala may contribute to excess alcohol consumption, craving and dependence[21].

1.5 Psychological and Behavioral Interactions

Brain regions with higher CB1 concentration correlate with areas thought to be critical for emotion processing (e.g. the basal ganglia and hippocampus)[14,107–109]. Depressed individuals have also been found to have low levels of circulating endocannabinoids throughout the brain and body[110]. Additionally, regulation of synaptic transmission by CB1 receptors affects the subsequent release of neurotransmitters known to influence mood, such as serotonin and dopamine[111,112], and several studies have demonstrated adverse psychiatric effects as a result of global CB1 blockers, including severe anxiety and notable depressive symptoms in individuals with no history of depression[75,110,113,114]. Conversely, administration of THC in imaging studies shows that amygdala response decreases with negative emotional content, but increases with positive emotional content, with an overall shift in bias toward positive information and affect[75,115–118]. The culminating evidence suggests that the ECS likely plays a regulatory role in symptoms of anxiety and depression by way of the CB1 receptor. Moreover, in reference to

immune modulation, clinical depression and anxiety increase systemic inflammation[119–121], which accounts for some psychological impact on gut health.

Drawing further connections, recent studies support bidirectional links between gut microbiome health and stress response, depression, and anxiety[78,122], as well as alcohol use disorder[49,82]. Gut microbiota are now known to be important to the synthesis and secretion of serotonin by neuronal cells in the gastrointestinal tract[123], which may be a significant contributor to the development and maintenance of depression[124,125]. Additionally, reduced endocannabinoid signaling in the hippocampus has recently been associated with changes in the gut microbiome[126]. These findings exhibit a possibility of reverse effects of mood dysregulation on gut health. In response to stress, the composition of microbiota is altered within the gut, decreasing the prevalence of certain precursors to endocannabinoids. This results in reduced endocannabinoid production, leading to heightened occurrence of depression and other mood disorders[126].

1.6 Inflammatory response

Inflammation is a fundamental process of the immune system designed to protect the body from injury and infection. It is managed through a complex signaling cascade involving cytokines, which are small proteins that regulate immune activity. Pro-inflammatory cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α), direct immune cells to sites of injury, while anti-inflammatory cytokines, such as interleukin-10 (IL-10), counterbalance this response to restore homeostasis[127]. When too many pro-inflammatory cytokines are released and accompanied by too few anti-inflammatory cytokines, the imbalance can lead to chronic conditions such as autoimmune disorders and inflammatory bowel disease (IBD)[128]. A rapid

release of pro-inflammatory cytokines that is not balanced (known as a cytokine storm) results in an exaggerated immune response that can lead to tissue damage and systemic dysfunction[128].

One pathway by which inflammation can become a chronic and widespread issue is through disturbances in composition of the gut microbiome[129]. When severe disruption occurs, the result is gut dysbiosis, wherein healthy microbes become surmounted by opportunistic or pathogenic bacteria, and microbial diversity severely decreases[129]. Over time, dysbiosis can lead to compromised cellular tight junctions of the epithelial wall and increased intestinal permeability, commonly referred to as "leaky gut"[130]. The breakdown of tight junctions allows endotoxic bacterial products such as lipopolysaccharides to enter into the bloodstream, thereby activating a systemic immune response[127]. This cascade results in the release of additional inflammatory mediators, including C-reactive protein (CRP), which is indicative of liver inflammation, as well as pro-inflammatory cytokines such as IL-6 and TNF-α[37,130,131]. Changes in circulating levels of pro- and anti-inflammatory cytokines, as well as increased levels of other inflammatory biomarkers such as CRP can be measured in blood or plasma, providing a window into general immune system activity.

Prolonged alcohol use has been consistently associated with dysbiosis**.** Thus, alcohol-related gut dysbiosis provides a direct mechanistic link between substance use, systemic inflammation, and downstream health outcomes. Cannabis, in contrast, has been shown to exhibit anti-inflammatory effects, although these are nuanced and depend on the specific cannabinoid and its interaction with the endocannabinoid system. Δ9-tetrahydrocannabinol (THC) can activate CB1 receptors to promote IL-10 secretion, thereby suppressing pro-inflammatory cytokines and reducing tissue damage[102]. Cannabidiol (CBD), specifically in high-CBD extracts, has also been shown to reduce pro-inflammatory cytokines such as TNF-a and IL-

1b while increasing IL-10, providing potentially protective effects in dysregulated immune signaling, including viral and autoimmune diseases[88]. These findings suggest that cannabis use may mitigate systemic inflammation through direct immunomodulatory effects.

Importantly, inflammation has been strongly implicated in mental health outcomes. Elevated levels of pro-inflammatory cytokines and markers such as CRP have been consistently seen in individuals with depression and anxiety[132]. Systemic inflammation can increase reactivity in the amygdala, known to be a key brain region for processing emotions including fear and anxiety[133]. Further, poor emotional regulation and negative attentional bias are associated with increased inflammatory responses[134–136]. Even more, chronic stress contributes to this cycle by dysregulating the endocrine and immune systems, leading to neuroinflammation and symptoms of anxiety and depression (figure 1.1)[129,137].

The multi-directional relationship between stress, inflammation, and mental health further highlights this connection. Psychological stress can activate immune pathways through sympathetic nervous system signaling, including adrenal activity and responsiveness, which increase cytokine secretion[133] . These immune changes in turn exacerbate psychiatric symptoms, forming a reinforcing feedback loop that links gut health, systemic inflammation, and emotional well-being (figure 1.1)[138–140]. In the same way, common issues like depression, anxiety and stress can cause inflammation through multiple pathways which can be either acute or become chronic and impact overall health and well-being[133,138,141]. Studies find that individuals with depression often present with increased levels of inflammatory markers including CRP, IL-6 and TNF-a[132].

Altogether, these findings suggest that alcohol use promotes systemic inflammation through gut-mediated pathways, while cannabis may be protective with its anti-inflammatory properties. At the same time, inflammation itself plays a critical role in mental health outcomes,

establishing a multidirectional model in which substance use, immune function, and mental health are deeply intertwined and significantly impacted by substance use.

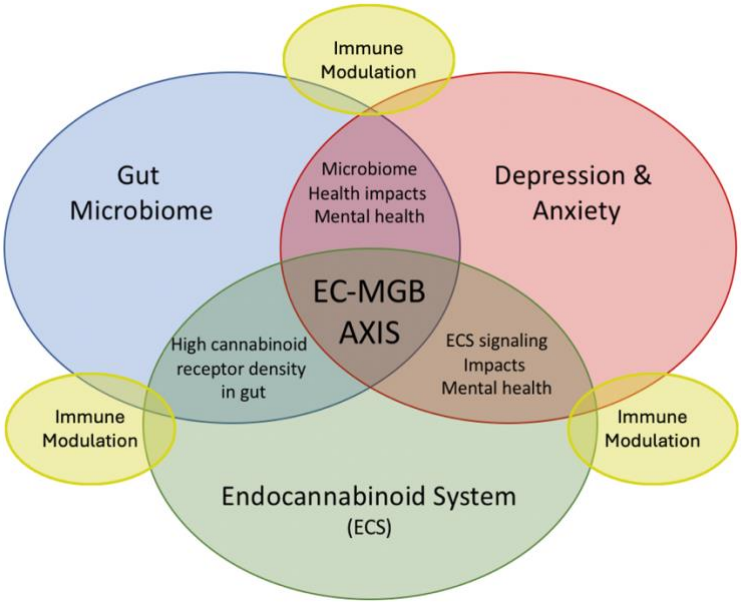

Figure 1.1 Interactions between the endocannabinoid system and microbiota-gut-brain axis with considerations of immune signaling

1.7 Exploring an ECS-MGB Axis

The associations discussed above, between the ECS, MGBA and psychological/behavioral variables and the impact of alcohol and cannabis use have been tested separately but, to our knowledge, these relations have not been explored holistically by any research to date. The nuanced and likely bidirectional relationships between these variables point to a need for investigation of interactive relationships between gut health, endocannabinoid signaling, substance use, and disordered mood regulation (as seen in depression and anxiety) as a whole. Distinct intersections have been supported by multiple studies but have yet to be merged into an integrative model, particularly with the goal of providing insight into novel treatment options for these disorders. We propose an "ECS-MGBA" model (as seen in Figure 1.2), which captures the bidirectional relationships between the gut microbiome, ECS and

psychology/behavior, also considering the interplay of immune system activation. Because it seems we cannot affect one aspect of the "ECS-MGB" system without affecting another, and because substances such as alcohol and cannabis appear to have important effects within each component of this model, the ECS-MGBA should be considered and explored further within the context of cannabis and alcohol consumption.

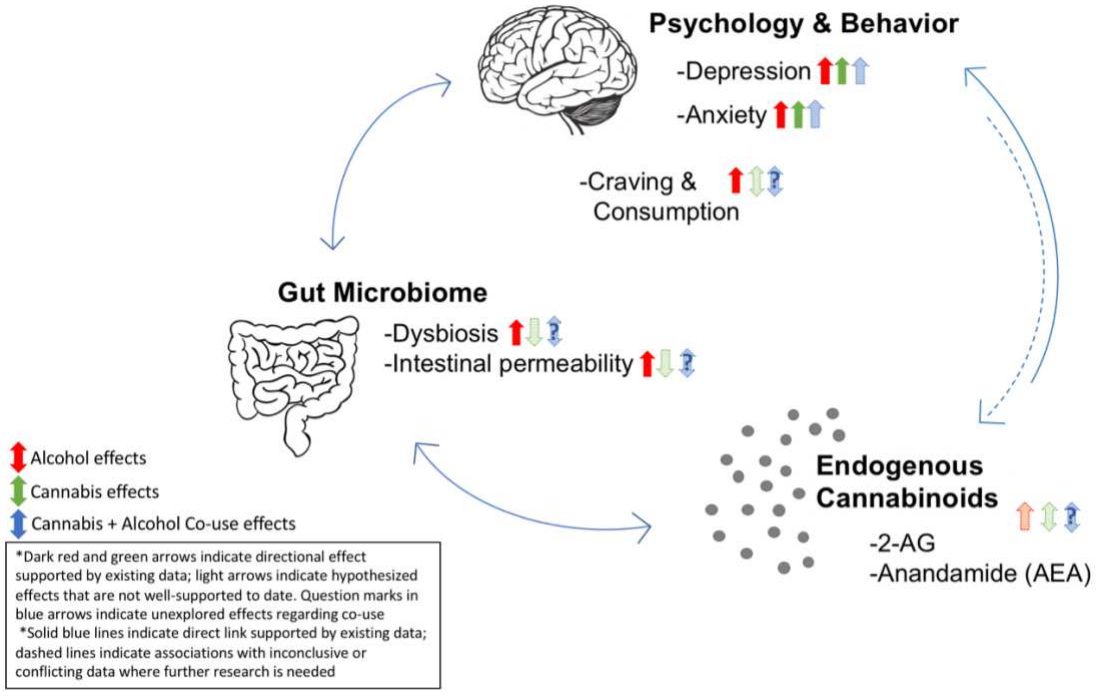

Figure 1.2 Diagram of proposed ECS-MGBA interactions and evidence-based effects of cannabis and alcohol use.

This research takes a novel approach to answering questions that combine multiple facets of psychology and biology. There is little if any existing literature that considers the interplay of the endocannabinoid system, gut microbiome, substance use and emotion dysregulation altogether. By considering these systems as a conglomerate within the proposed ECS-MGBA, we may achieve a greater understanding of the interactions taking place. Furthermore, a better understanding of these interactions could pave the way for more effective and even more attainable treatment options for individuals with substance or alcohol use disorders.

1.8 Study Objective & Specific Aims

The present study aims to investigate physiological and psychological differences across individuals with different patterns of alcohol and cannabis use – individuals who exclusively and regularly use cannabis-only [CO], alcohol-only [AO], individuals who regularly use both alcohol and cannabis [co-use], and individuals who abstain from alcohol and cannabis use [control].

Aim 1. Explore trait depression, anxiety and impulsivity among the four groups.

Hypothesis 1. The highest levels of depression and anxiety are predicted to be seen in the AO group followed by the co-use group, while the control and CO groups are predicted to show the lowest levels overall.

Aim 2. Examine baseline circulating levels of endocannabinoids and differences in relation to substance use patterns.

Hypothesis 2. Endocannabinoid levels should be elevated with higher substance use; we expect the highest circulating levels of endocannabinoids in the co-use group, followed by the AO and CO groups, and finally the control group.

Aim 3. Investigate the effects of regular cannabis and alcohol consumption on gut microbiome composition and intestinal permeability across the four groups.

Hypothesis 3a. 16S gene sequencing is expected to reveal the lowest microbial diversity and lowest abundance of healthy bacteria (e.g. Bifidobacteria[142]) and the highest levels of bacteria associated with inflammation (e.g. Proteobacteria[130,143]) in the AO group, followed by the co-use and CO groups, while the control group will show the highest levels of diversity and healthy bacteria with diminished levels of inflammatory bacteria.

Hypothesis 3b. The AO group is predicted to show the highest levels of intestinal permeability biomarkers of the four groups, followed by the co-use group, with the control and CO groups showing the lowest levels.

Aim 4: Explore inflammatory biomarkers (e.g. C-reactive protein and cytokines) in plasma for differences between substance use groups.

Hypothesis 4. The AO group will reflect levels of pro- and anti-inflammatory cytokines that are indicative of higher levels of systemic inflammation, with the control group and CO group display cytokine levels that reflect lower levels of systemic inflammation. The co-use group is expected to display an inflammatory profile that lies between the AO and CO groups.

Chapter 2. General Methods

2.1 Participant Eligibility

Inclusion and exclusion criteria were designed to match those of Dr. Hollis Karoly's K23 (K23AA028238) study protocol as data were shared to provide a comparison cannabis and alcohol co-use group. Most criteria were in place in consideration of participant safety or impact to biological variables of interest. Criteria for groups of heavy drinkers and regular cannabis users were set to ensure consistency and detection of group differences in biological and self-report measures during analysis. Recruitment involved the use of posted and mailed flyers, email broadcasts, word of mouth, and online advertisements via social media including Facebook, Reddit and Craigslist.

To be considered for participation, recruited individuals must have been between the ages of 21 and 60 years and able to provide informed consent. Individuals were considered ineligible and excluded from participation if they reported any of the following: daily tobacco use; seeking treatment for AUD or SUD; illicit drug use in the past 60 days; major medical conditions or illnesses contraindicating alcohol or cannabis use; immune disease; current use of psychotropic, steroid or immune medication (except anti-depressants); pregnancy (or trying or become pregnant) or breastfeeding; a history of or current psychotic disorder, bipolar disorder or major depressive disorder with suicidal ideation.

Interested individuals contacted the research team via email or accessed a link provided on recruitment materials and completed a detailed eligibility screening questionnaire. Upon review, trained research assistants contacted potential participants to follow up and confirm eligibility with a screening questionnaire. Eligible individuals still interested in participating

were then scheduled for their virtual consent session to initiate enrollment into the study. Individuals who did not meet eligibility criteria were contacted and thanked for their interest.

2.2 Group Classification

Participants were recruited and screened into one of four groups based on their reported substance use patterns. Each group required specific parameters surrounding substance use to be met. Group membership was determined upon screening and admittance into the study. Cannabis group membership required individuals to report use of cannabis at least three times per week for the past three months. Cannabis group members reported drinking no more than one standard alcoholic drink per week in the past three months. Alcohol group membership followed the NIAAA criteria for heavy and/or binge drinking, requiring individuals to report drinking at least five drinks per occasion for males or four drinks per occasion for females on at least five days per month for the past three months. Alcohol group members reported using cannabis no more than once per month in the past three months. Co-use group members met criteria for both cannabis and alcohol groups combined, reporting regular and heavy use of both cannabis and alcohol. Finally, individuals who reported abstinence from both cannabis and alcohol comprised a control group for comparison. Control group members reported using cannabis no more than once per month and drinking no more than one standard alcoholic drink per week for the past twelve months.

2.3 Study Design & Procedures

Session procedures and compensation for the K23 study's co-use group varied due to differing aims and protocol, however baseline data collection, sample storage and processing methods remained largely consistent, with the exception that the K23 co-use group was asked to abstain from alcohol and cannabis for 24 hours prior to their lab session due to safety concerns

expressed by the Institutional Review Board. This requirement was not imposed upon the CO, AO or control groups. Minor differences in handling of fecal samples between the co-use sample and the other samples were realized some time into data collection. These differences primarily involved the timing from biological sample collection to processing and storage. Due to the length of time of the mobile lab session as well as the variable driving time associated with travel to participant's homes, it was typically several hours between blood collection and processing times during which samples were stored in a small refrigerator. Fecal swab samples also remained at room temperature through the duration of the session and travel time. Samples for the other 3 groups were generally processed and stored within 30 minutes of the laboratory session.

Participation was initiated by a 20-minute virtual meeting via Zoom wherein the study procedures and expectations were explained and participants provided informed consent. Upon completion of the virtual session, an initial demographic and mental health questionnaire was sent to the participant via email, and an in-person laboratory session was scheduled for two weeks later. Within this 14-day period, participants completed online daily diary entries documenting diet, substance use, exercise and mood. Figure 2.1 provides a visual representation of the study timeline and procedures.

Daily Diary Data Collection

Participants were asked to keep a detailed daily diary of their cannabis use and alcohol consumption, mood and exercise for 14 days between the virtual and laboratory sessions. They completed online entries once each day in the evening, responding to prompts in reference to the past 24 hours. The survey asked questions which included the following: *1) Did you use cannabis? 2) How much did you use in grams? (pictures are provided to aid in estimation of*

amount), 3) What cannabis did you use? (enter strain name and THC potency, as indicated on all dispensary packaging), 4) When did you use cannabis? (input: "start time", "end time"), 5) Did you drink alcohol? 6) How many drinks? (standard drink equivalents for beer, wine and liquor will be provided for reference), 7) When did you drink? (input: "start time", "end time"), 8) How much did you crave alcohol? (scale of 0-10) and 9) Did you consume alcohol and cannabis together (e.g., did you drink alcohol while feeling the effects of cannabis or vice versa)? These questions allow for objective measurement of temporal patterns of alcohol and cannabis use. Also included in the daily diary survey was a Visual Analogue Mood Scale[144] and report of total minutes of aerobic and anaerobic exercise performed that day.

Amount and frequency of substance use as reported in the daily diary were used to confirm group assignment. In cases which reported substance use was largely inconsistent with group assignment, (i.e. an individual assigned to co-use or AO who reported no alcohol use), participants were re-assigned to the appropriate substance use group. (Note: small shifts in data were seen, but these re-assignments did not alter overall results.)

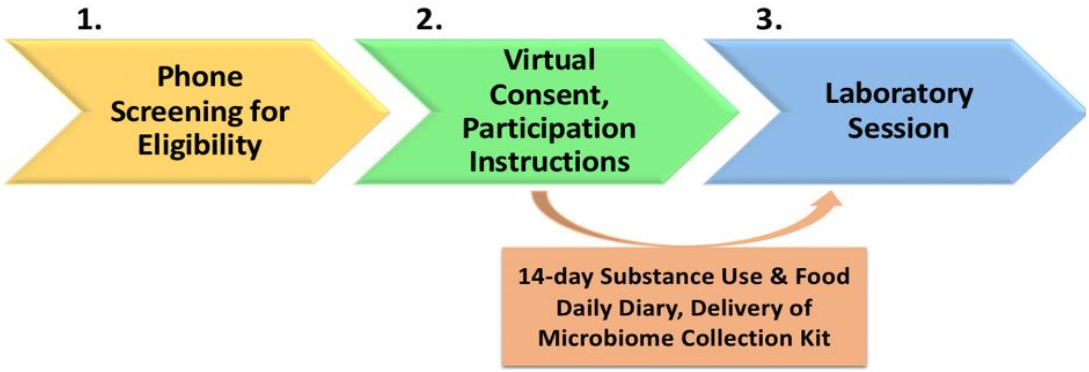

Figure 2.1. Study procedure timeline

Laboratory Session

Two weeks after the virtual baseline session, participants visited the CSU campus laboratory to conclude their study participation. Sessions were scheduled any time between 8am

and 8pm. At this session, participants delivered their fecal swab samples and researchers collected a 10 mL blood sample. To confirm group assignment and ensure absence of illicit substances and pregnancy, participants completed a urinalysis. A positive result for illicit substances or pregnancy resulted in ineligibility and dismissal from the study. The remainder of the laboratory session was used to complete several self-report assessments regarding demographics, a detailed diet inventory, mental health and substance use patterns, as well as a single cognitive task.

Compensation

To encourage compliance with daily data acquisition over the 14 days, participants were incentivized with $2 per daily entry completed, in addition to the $45 earned for completing the laboratory session. In total, participants could earn up to $73 for study participation. Additionally, if all daily diary entries were completed, participants were entered into a raffle for a $50 Visa gift card drawn at the close of the study.

2.4 General Measures and Data Handling

Demographic measures collected include age, ethnicity, education, gender identity, biological sex and SES. Substance use patterns were assessed through a 60-day Timeline Follow-Back (TLFB)[145] assessment detailing daily substance use, as well as questionnaires targeted to participants' specified substance use group. The AO group completed the Alcohol Use Disorder Identification Test (AUDIT)[21] and the Alcohol Dependence Scale (ADS)[146,147], multi-item scales measuring alcohol use and the extent of symptoms and existing problems related to alcohol use and dependence. CO group members completed the Marijuana Dependence Scale (MDS)[148], a screenings used to measure frequency of use and symptoms of dependency. Participants were also asked to specify details about the strain and potency of the cannabis they typically use.

To account for participant variation in diet and eating habits, data from a food frequency questionnaire (FFQ)[149] (modified for American equivalents) was used to calculate a Recommended Food Score (RFS)[150–152] for each participant. Consumption frequencies for specific foods were taken from the FFQ and points were assigned to food categories based on how often individuals reported consuming the respective foods. Recommended food categories included whole grains (multigrain/brown rice, wholegrain bread), nuts and legumes (nuts, peanut butter, beans, lentils, tofu), whole fruits (apples, pears, oranges, grapefruit, bananas, grapes, melon, peaches, strawberries, canned fruit, dried fruit), vegetables (carrots, spinach, broccoli, sprouts, cabbage, peas, green beans, squash, cauliflower, parsnips, leeks, peppers, onions, bean sprouts, green salad, watercress, tomatoes, sweet corn, beetroot, coleslaw, avocado), low-fat milk, and fish. In total, there were 41 recommended items, totaling a possible 82 points: if an item was consumed less than once per week, it received 0 points; at least once per week received 1 point, and at least once per day received 2 points. Not recommended food categories included red/processed meat (beef, beef burgers, pork, lamb, bacon, ham, corned beef, sausage, meat soup), sugary beverages (cocoa, malted milk, soda and fizzy drinks), sodium-rich foods (fries, chips, pizza, popcorn, ramen, mac & cheese), and sugar/sweets (cookies, ice cream, chocolates, chocolate bars, sweets, sugar). There were 22 items in this list, totaling up to 22 points, 1 point awarded for each item consumed less than once per week. Altogether, possible RFS scores range from 0-104, with higher numbers indicating a higher quality diet.

For the quantification of biological measures, each participant consented to providing a single blood sample and a fecal swab sample. They were instructed to collect the fecal sample within one day of their scheduled lab session and to note the time and date of collection. Upon delivery, samples were stored in the laboratory freezer at -80°C. Trained researchers drew blood

intravenously at the beginning of the laboratory session and stored samples in a small refrigerator for the duration of the session (approximately 1.5 hours) until processing. Immediately after the session ended, blood samples were transported to the wet lab and vials were spun at 1000 RCF for 10 minutes to separate plasma, which was then stored at -80°C until analysis.

All electronic and biological data collected was de-identified and only accessed by trained and approved lab personnel. Electronic data was securely collected via REDCap and Qualtrics and stored on a password-protected university server. Participants chose whether or not to allow excess biological data to be used for exploratory analyses beyond the current study.

General analyses were conducted in RStudio[153]. Demographic variables were assessed for group differences prior to running extensive analyses. Full factorial models with pairwise comparisons between each group were used for all analyses; significant demographic variables were controlled for. Analyses of (co)variance [A(C)NOVA] were followed by LSD post-hoc analyses for pairwise comparisons. Because this project acts as a pilot study in which we focus on patterns and effect sizes for future guidance, we did not correct for multiple comparisons in effort to capture all relationships that warrant further investigation in larger confirmatory studies[154,155]. As multiple comparison corrections have the potential to overshadow real effects, we aimed to look at the data without overly stringent statistical corrections in this initial stage of analysis[154–156]. This was a deliberate choice in order to avoid interpretation errors with data derived from natural observation, where complex, interrelated variables might be at play[154]. For transparency, all effect sizes were measured and reported alongside confidence intervals. Age and sex were also assessed as moderators according to associated differences in the endocannabinoid system, immune system and gut microbiome, and used as covariates where significant[157,158].

Chapter 3. Personality and Mental Health Profiles

Aim 1. Explore trait depression and anxiety symptoms and diagnoses among the four groups with validated indices (e.g. Beck's depression and anxiety inventories)**.**

Hypothesis 1. The highest levels of depression and anxiety are predicted to be seen in the AO group compared to the other groups, while the CO group is expected to show the lowest levels overall.

3.1 Background & Rationale

National survey data indicate that approximately 62.5% of individuals over age 12 reported drinking alcohol in the past year, with 10.2% meeting criteria for an alcohol use disorder[1]. As legalization and access have expanded, daily cannabis use has become even more prevalent than daily or near-daily alcohol use[159]. Many adults report regular co-use of both substances either simultaneously so that intoxicating effects overlap, or concurrently in a way that the effects do not overlap[160]. While results are mixed, use of alcohol and cannabis on their own has been linked to higher rates of anxiety and mood disorders[161,162]. Emerging research suggests that co-use, especially simultaneous use, may increase these rates even further[163,164]. One longitudinal study in young adults links co-use with psychosis, externalizing, and conduct problems[163], and simultaneous use with more depressive symptoms and poorer general health relative to exclusive alcohol use[164].

Across research, patterns of substance use and mental health appear to influence one another. Many individuals report beginning regular cannabis use during periods of depression[60,165], and anxiety reduction is frequently cited as a motivator for continued use[122,166]. Depression is commonly comorbid with alcohol use and misuse[167], and social anxiety disorder co-occurs at high rates with alcohol use disorders[168]. Yet longitudinal analyses suggest that

adolescent cannabis use does not significantly increase adult risk for depression, anxiety disorders, or suicidal ideation[169]. Moreover, several studies indicate potential anxiolytic effects of cannabis compounds at specified doses[59,61,71,170]. These mixed findings underscore an unresolved question in the literature of what conditions might co-use be associated with additive harms versus neutral or even protective effects. Some evidence suggests that using both substances can be associated with lower consumption of alcohol and cannabis compared with single-substance use[171,172], although findings are limited and conflicting[173].

Mechanistically, the ECS provides a plausible framework for understanding these clinical and behavioral observations. Psychological and behavioral factors such as anxiety, depression, and substance use are influenced by endocannabinoid signaling[14,15,49,74,75]. Brain regions with higher CB1 receptor concentration, including structures critical for emotion processing such as the basal ganglia and hippocampus, map onto networks implicated in emotion regulation[14,107–109]. Individuals reporting depressive symptoms exhibit reduced circulating endocannabinoids in the brain and periphery[110]. CB1-mediated regulation of synaptic transmission shapes the release of mood-relevant neurotransmitters (e.g., serotonin and dopamine)[111], and global CB1 antagonists have produced serious adverse psychiatric effects in humans, including severe anxiety and depressive symptoms even in those without prior depression[75,110,113,114]. In contrast, THC administration in imaging studies is associated with decreased amygdala responses to negative emotional content, increased responses to positive content, and an overall shift toward positive affective bias[75,115–118]. Collectively, these data suggest a regulatory role for CB1 signaling in symptoms of anxiety and depression. Consistent with this, cannabinoids in the cannabis plant may work collectively to acutely ameliorate mood and anxiety symptoms[71], although effective clinical treatments for depression and anxiety that utilize the ECS have not yet emerged.

Conversely, the literature surrounding alcohol more consistently documents detrimental effects on mood and psychological functioning, including dysregulation of mood and sleep, increased aggression and impulsivity, cognitive impairments, and disrupted stress responses[174–180]. With these conclusions, it is an important unresolved question as to whether co-use presents additive risk compared to single-substance use, or whether cannabis may offer some protection against alcohol-related harms in specific scenarios. Several factors may contribute to the outcome of these questions, including individual differences.

One prominent individual-difference factor is personality. Traits such as impulsivity, sensation seeking, and emotional instability are linked with substance use and mental health diagnoses[181]. Alcohol use disorders are associated with high impulsivity and sensation seeking, along with lower conscientiousness and agreeableness[182,183]. Cannabis use on the other hand has been linked to higher neuroticism and openness, and lower conscientiousness and agreeableness[184]. Although fewer studies have examined personality in co-use, findings thus far indicate associations with impulsive personality traits[185]. Clarifying how personality profiles intersect with alcohol use, cannabis use, and co-use may help explain variability in mental health outcomes across users.

Most prior research has examined alcohol and cannabis use individually, however, given the prevalence of co-use and its potential to increase risk, direct comparisons across AO, CO, co-use and control groups are needed. Such comparisons can determine whether cannabis and alcohol have distinct effects on mental health and whether co-use differs in these outcomes when compared with single-substance use. Clarifying these differential risks will inform prevention, intervention, and harm-reduction strategies. The current aim addresses this question by directly

comparing individuals from the four substance-use groups across a battery of psychological questionnaires assessing depression, anxiety, stress, impulsiveness and personality variables.

3.2 Aim-specific Methods

Psychological variables were taken from a collection of self-report questionnaires that ask in detail about state and trait depression and anxiety, as well as trait impulsivity. In addition, specific questions regarding psychiatric diagnoses, personal health and prescribed medication were asked. The Ten Item Personality Inventory (TIPI)[186] measures the expression of validated traits including extraversion, agreeableness, openness, conscientiousness and emotional stability. To measure stress, anxiety and depression, several batteries were analyzed. The Depression Anxiety Stress Scale (DASS)[187] is a 21-item survey measuring depression, anxiety, and stress. The Beck Anxiety Inventory (BAI)[188] and Beck Depression Inventory (BDI)[189] each consist of 21 items describing common symptoms of anxiety and depression (respectively) over the week prior to testing. The Difficulties in Emotion Regulation Scale short form (DERS)[190] is an 18-item survey used to measure emotional regulation across 6 domains, including limited access to or knowledge of strategies to regulate emotions, non-acceptance of negative emotional response, difficulty with goal-directed behavior (i.e. accomplishing tasks) during distress, impulsiveness when experiencing negative emotions, lack of emotional awareness, and lack of clarity about emotions being experienced.

For impulsivity and sensation seeking, two additional measures were included. The Barratt Impulsiveness Scale (BIS)[191] measures trait impulsivity tendencies, which can be correlated with substance use patterns. The short Urgency-Premeditation-Perseverance-Sensation Seeking-Positive urgency survey is used to measure expression of the five dimensions of impulsivity listed in the name (UPPS-P)[192].

Each of these assessments are reliable and commonly used measures, the combination of which allow for a broad analysis of participants' psychological traits as they relate to substance use patterns and biological outcomes. Scores were calculated for each scale and subscale where appropriate and then compared by substance use group in analyses of variance (ANOVA) models. Sex and age were assessed for significant influence on each outcome and used as covariates where significant (ANCOVA). ANCOVA models were followed by LSD post-hoc analyses for pairwise comparisons. To further illustrate unadjusted group differences (i.e., without controlling for covariates or multiple comparisons), independent *t*-tests were also conducted as needed.

3.3 Results

A total of 170 participants were assigned to substance use groups based on the aforementioned criteria: AO (n = 41), CO (n = 29), co-use (n = 58), and control (n = 44). Participant demographic data for this outcome is summarized in Table 3.1. Sex had a fairly even split among groups, the co-use group being the exception with just 38% females though this difference was not statistically significant. The CO group was the least educated with 34.5% of its participants having earned a bachelor's degree or higher, contrasting with the AO group being the most educated with 83% of its participants earning at least a bachelor's degree. At least 50% of the members in each group reported annual earnings under $50,000, again with the exception of the AO group at just 24% of its participants earning less. Due to these demographic differences, income and education were covaried in all analyses.

Table 3.1. Participant demographics by substance use pattern. Significant group differences are indicated by subscript letters. Different letters indicate groups that are statistically distinct, whereas shared letters indicate no differences between groups.

	AO (n=41)	CO (n=30)	Co-use (n=77)	Control (n=45)	Total (n=193)
Demographics					
Age, mean years (SD)	32.5 (9.7)	32.6 (10.2)	31.1 (9.4)	33.9 (10.3)	32.3 (9.8)

Assigned female at birth, no. (%)	21 (51.2)	14 (46.7)	28 (36.4)	25 (55.6)	88 (45.6)
Bachelor's degree or higher, no. (%)	**34 (82.9)$_{a}$**	**10 (33.3)$_{b}$**	**35 (45.5)$_{b}$**	**34 (75.6)$_{a}$**	113 (58.5)
Income, <$50,000/yr, no. (%)	**13 (31.7)$_{a}$**	**18 (60)$_{b}$**	**52 (67.5)$_{b}$**	23 (51.1)$_{a,b}$	106 (54.9)
Race, white, no. (%)	**38 (92.7)$_{a}$**	26 (86.7)$_{a,b}$	67 (87.0)$_{a,b}$	**32 (71.1)$_{b}$**	163 (84.5)
Psychological Assessment Data					
AUDIT total, mean (SD)	**8.8 (3.6)$_{a}$**	-	**10.1 (5.0)$_{b}$**	-	-
MDS total, mean (SD)	-	2.9 (2.0)	2.8 (2.4)	-	-
BDI total, mean (SD)	7.4 (5.8)$_{a,b}$	**10.4 (8.5)$_{a}$**	7.9 (7.1)$_{a,b}$	**5.6 (6.0)$_{b}$**	7.7 (6.9)
BAI total, mean (SD)	5.4 (5.9)$_{a,b}$	**7.7 (6.7)$_{a}$**	**4.6 (5.3)$_{b}$**	**4.9 (5.8)$_{b}$**	5.3 (5.8)
14-day Daily Diary Reports					
% drinking days, mean (SD)	57.3 (27.9)	7.9 (11.2)	39.8 (30.6)	2.5 (9.9)	29.7 (32.0)
avg drinks/drinking day, mean (SD)	3.2 (1.8)	.97 (2.2)	3.1 (2.6)	.16 (.6)	2.1 (2.4)
% cannabis use days, mean (SD)	1.8 (5.1)	27.6 (30)	45.1 (32.9)	0 (0)	20.4 (30.4)
avg cannabis g/use day, mean (SD)	.01 (.03)	**1.42 (1.75)$_{a}$**	**.54 (.76)$_{b}$**	0 (0)	.43 (.96)
avg exercise minutes/day, mean (SD)	38 (25.9)	38.1 (37.7)	35.5 (35.1)	42.7 (39.6)	38.3 (34.8)

Group Differences in Depression, Anxiety and Stress

Group differences emerged in measures of depression across multiple scales (Figure 3.1). An ANCOVA with total DASS scores as the dependent variable showed significant group effects ($F_{(5,188)} = 3.42$, $p = 0.01$; $\eta^2 = .068$). While ANCOVA models did not identify significant differences between groups, independent t-tests revealed pairwise differences. The CO group scored significantly higher than the AO group in total DASS scores ($t_{(69)} = -1.72$, p=0.04, *Cohen's d* = .442). On the Depression subscale of the DASS, the ANCOVA revealed significant condition effects ($F_{(5,188)} = 4.02$, p=0.04; $\eta^2 = .047$). Pairwise comparisons showed the co-use group scoring higher than the AO group ($t_{(97)} = -2.03$, p=0.022, *Cohen's d* = .337) and the control group ($t_{(87)} = 1.81$, p<0.036, *Cohen's d* = .315).

An ANCOVA also revealed significant group differences in BDI scores ($F_{(5,164)} = 4.03$, p=0.002; $\eta^2 = .109$; Figure 3.1C), with the CO group scoring the highest and the control group scoring the lowest. Post hoc LSD pairwise comparisons revealed significant differences between the CO and control groups, with the CO group scoring significantly higher than the control group (p=0.003).

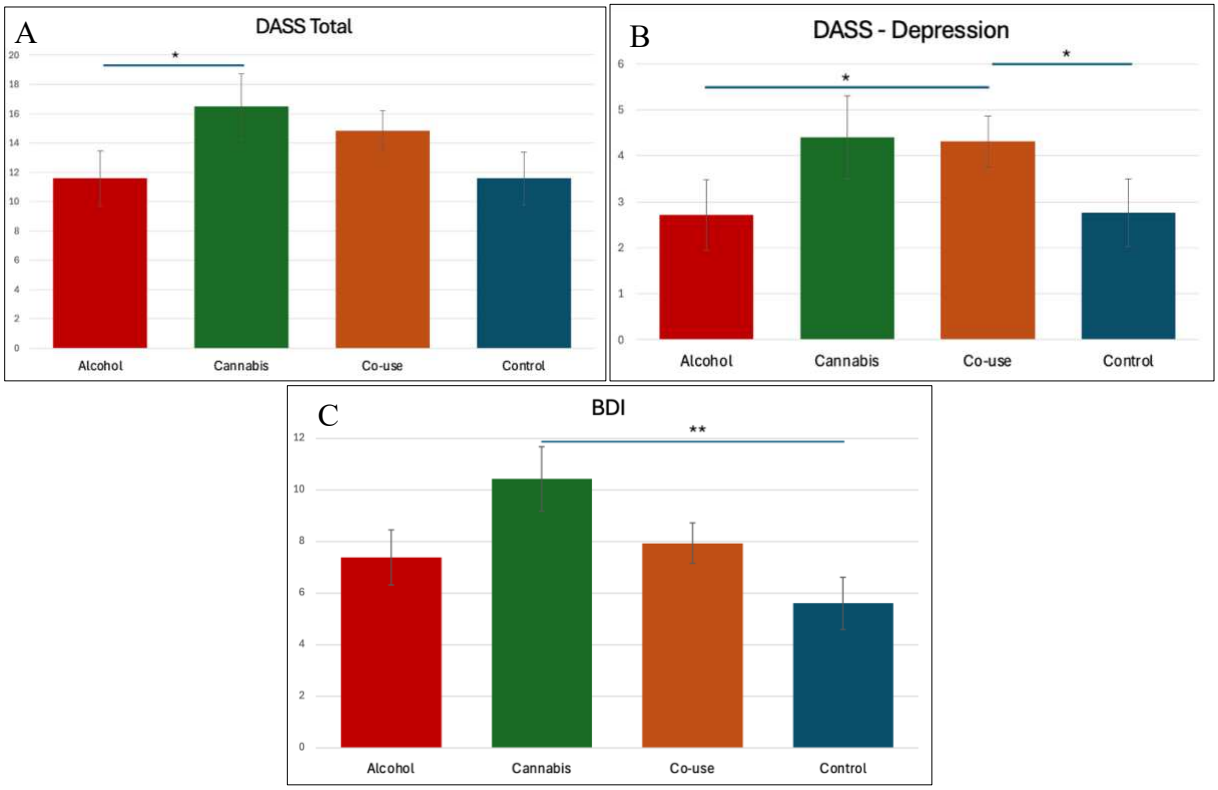

Figure 3.1. Group differences in rates of depression measures across scales. A) DASS total score; B) Depression subscale from the DASS; C) Beck's Depression Inventory total score.
Error bars = +/- 1 SE; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

Emotion regulation was quantified via the DERS and its subscales where higher scores indicate more difficulty with managing emotions. The subscales specify areas in which difficulties are seen. ANCOVA results for the DERS total score did not show significant group effects, however statistical differences did emerge in the subscale indicating lack of emotional clarity ($F_{(5,187)} = 2.94$, p=0.01; $\eta^2 = .073$), with independent t-tests revealing the AO group scoring lower than the CO ($t_{(69)} = -2.29$, p=0.01, *Cohen's d* = .546) and co-use ($t_{(97)} = -1.74$, p=0.04, *Cohen's d* = .292) groups (figure 3.2). No significant pairwise differences were seen in the other DERS subscales, or on the Stress subscale of the DASS.

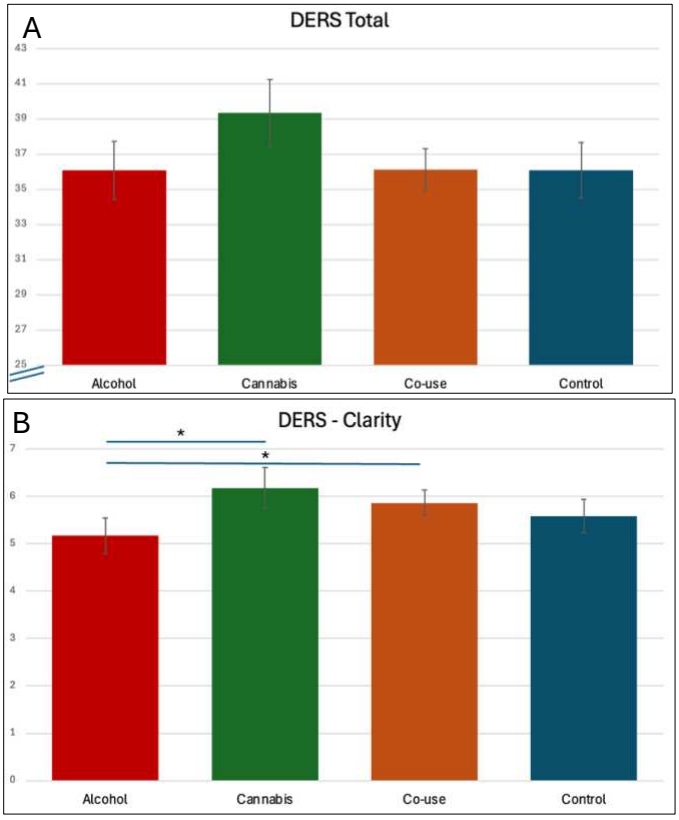

Figure 3.2. Emotion regulation outcomes from the DERS. A) DERS total score (no significant group differences); B) CO group scored highest in lack of emotional clarity DERS subscale.
Error bars = +/- 1 SE; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

Anxiety measures included the BAI and the Anxiety subscale of the DASS (Figure 3.3). Sex, age and income were covaried in these models. An ANCOVA revealed group differences in the DASS Anxiety subscale ($F_{(6,186)} = 5.33$, $p < 0.001$; $\eta^2 = 0.147$), where the CO group reported higher anxiety than the AO ($p = 0.03$) and control ($p = 0.04$) groups. ANCOVA results examining BAI total scores were significant ($F_{(6,186)} = 6.29$, $p < 0.001$; $\eta^2 = .169$) with post hoc LSD tests revealing the CO group to have scored higher than the control ($p = 0.02$) and co-use ($p = 0.01$) groups (figure 3.3B).

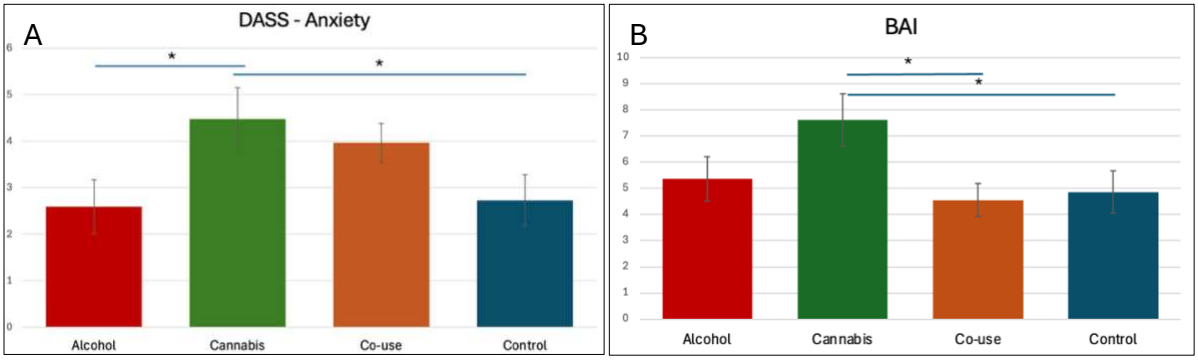

Figure 3.3. Group differences in rates of anxiety measures across scales. A) DASS anxiety subscale; B) BAI total score. Error bars = +/- 1 SE; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

Group Differences in Impulsivity

Total BIS scores differed by group, with the highest scores being endorsed by the co-use group and the lowest by the AO and control groups ($F_{(5,187)} = 5.27$, $p<0.001$; $\eta^2 = .123$). Subscales measure different facets of impulsiveness including non-planning, motor and attentional, as detailed in Figure 3.4. ANCOVA results for the Non-planning subscale covaried for sex, age, income and education and were significant ($F_{(7,185)} = 6.86$, $p<0.001$; $\eta^2 = .206$). This was the only subscale in which the CO group scored the highest. In Non-planning, the AO and control groups were not significantly different, and both scored significantly lower than the CO group as well as the co-use group. There were no differences between the co-use and CO groups. Motor subscale ANCOVA results also revealed group differences ($F_{(4,188)} = 2.79$, $p = 0.04$; $\eta^2 = .043$), with the co-use group scoring higher than the AO ($p = 0.03$) and CO ($p = 0.02$) groups. Finally, ANCOVA results revealed group differences in the Attentional subscale as well ($F_{(5,187)} = 2.53$, $p = 0.03$; $\eta^2 = .063$), with the control group exhibiting lower scores than the co-use group ($p = 0.007$).

Group differences were seen in subscales of the UPPS-P, with the co-use group consistently exhibiting the highest scores overall (Figure 3.5). Sex was also used as a covariate in a model for sensation seeking ($F_{(6,186)} = 11.27$, $p < 0.001$; $\eta^2 = .232$), where no differences were

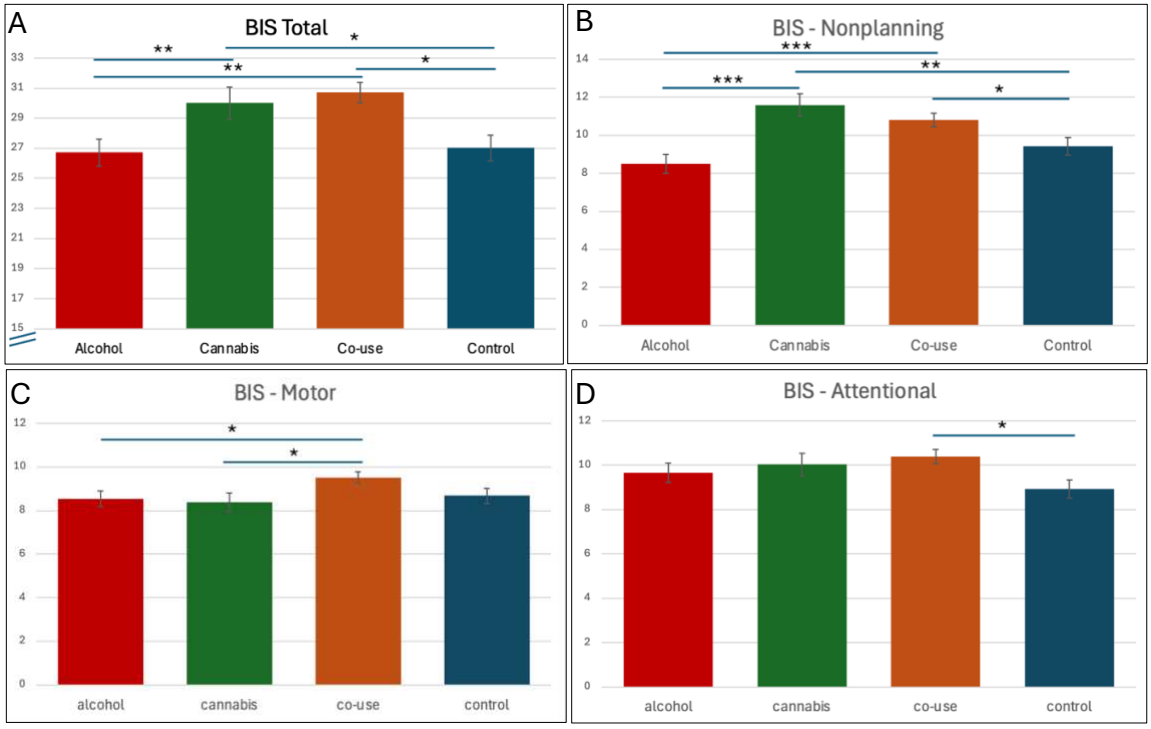

Figure 3.4. Group differences in the BIS measure of impulsiveness. A) BIS total; B) BIS Non-planning subscale results; C) BIS Motor subscale results; D) BIS Attentional subscale results. Error bars = +/- 1 SE; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

seen between the AO and co-use groups, but both scored significantly higher than the CO and control groups ($p < 0.01$; figure 3.5A). In the negative urgency subscale (measuring the tendency to act impulsively based on negative emotions), the co-use group scored the highest ($F_{(6,161)} = 2.84$, $p = 0.03$; $\eta^2 = .043$), endorsing higher scores than both the AO and control groups ($p = 0.02$; figure 3.5B). The positive urgency subscale (measuring the tendency to act impulsively based on positive emotions) revealed the same patterns ($F_{(5,187)} = 4.07$, $p = 0.003$; $\eta^2 = .080$), with the co-use group once again scoring higher than the AO ($p = 0.03$) and control ($p = 0.005$) groups (figure 3.5C). No differences were seen in the Perseverance or Premeditation subscales of the UPPS-P.

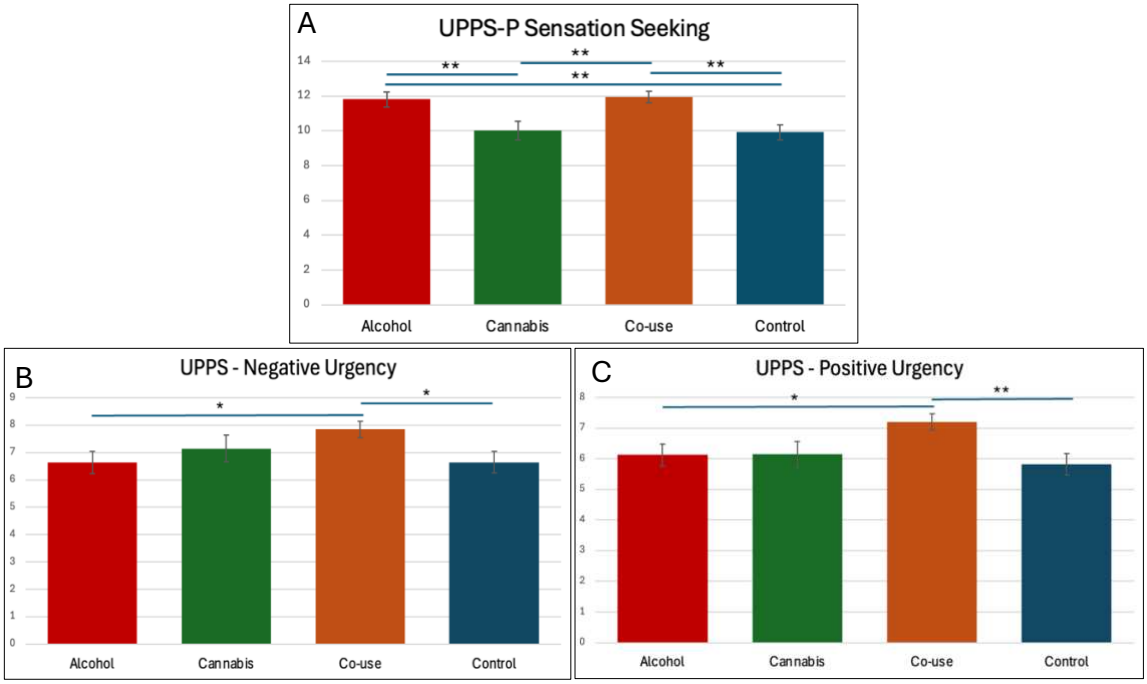

Figure 3.5. Group differences in measures of impulsiveness. A) UPPS-P Sensation Seeking subscale; B) UPPS-P negative emotional urgency subscale; C) UPPS-P positive emotional urgency subscale. Error bars = +/- 1 SE; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

Group Differences in Personality Traits

Significant group differences were seen in the TIPI (figure 3.6). An ANCOVA on the Conscientiousness subscale covaried for sex, age, income and education, and found the AO and control groups rated significantly higher than the co-use group ($F_{(7,185)} = 3.34$, $p = 0.002$; $\eta^2 = .112$). The Agreeableness subscale also demonstrated group differences, covarying for sex and race, with the control group also rating significantly higher than the CO and co-use groups ($F_{(5,187)} = 4.59$, $p < 0.001$; $\eta^2 = .109$). Further pairwise comparisons showed that the control group scored higher in Agreeableness than the CO group ($p = 0.008$) and the co-use group ($p = 0.03$). Significant group differences also emerged in Emotional Stability, with age, race and education covaried, where the CO group rated significantly lower than each of the other three groups ($F_{(6,186)} = 4.29$, $p < 0.001$; $\eta^2 = .122$). Pairwise comparisons revealed the CO group scored significantly lower than the AO ($p = 0.002$), control ($p < 0.001$), and co-use ($p = 0.004$) groups. Finally, group differences were seen in Openness where income and education were covaried

($F_{(5,187)} = 3.10$, $p = 0.01$; $\eta^2 = .077$), with the co-use group scoring significantly higher than the CO group ($p = 0.04$). Surprisingly, no condition effects were seen in extraversion.

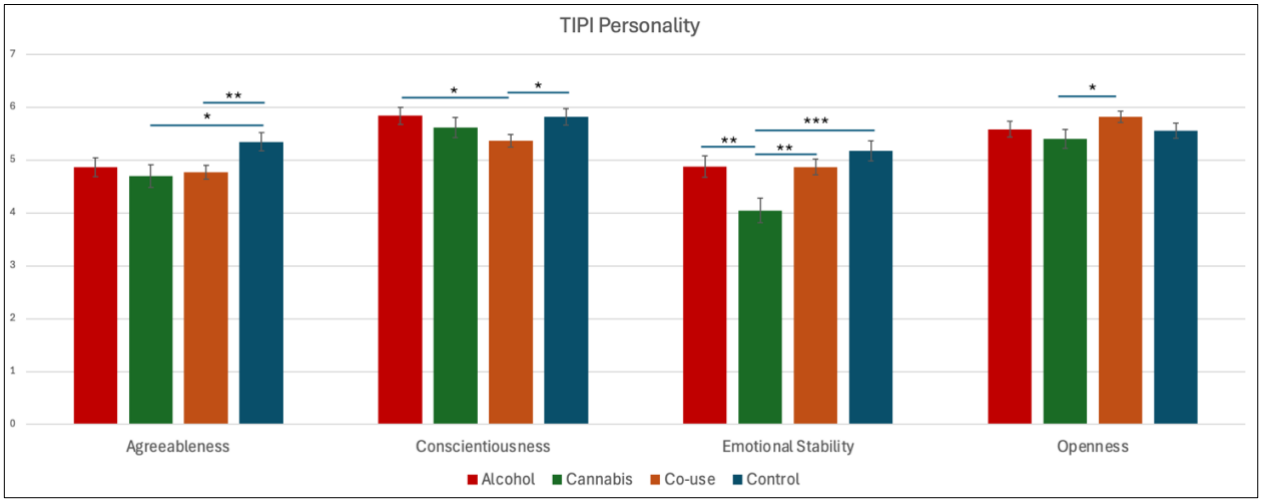

Figure 3.6. Significant group differences in TIPI personality subscales of conscientiousness, agreeableness, emotional stability and openness; Error bars = +/- 1 SE; * = p < 0.05, ** = p < 0.01

Group Differences in Subjective Health Ratings

Finally, participants were asked to rate their overall health on a 5-point Likert scale. The CO and co-use groups rated their health significantly lower than the AO and control groups ($F_{(3,189)} = 3.43$, $p = 0.01$; $\eta^2 = .052$). The CO group rated their health significantly poorer than the AO ($p = 0.02$) and control groups ($p = 0.004$) and the co-use group rated their health significantly poorer than the control group ($p = 0.04$).

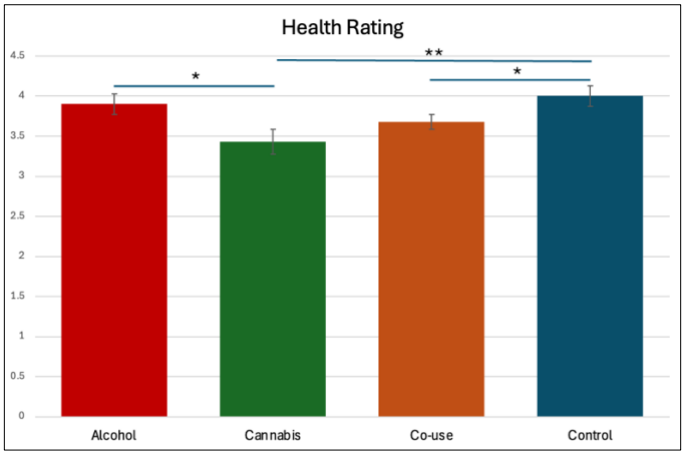

Figure 3.7. Group differences in responses to a general question rating their perception of their overall health. Error bars = +/- 1 SE; * = p < 0.05, ** = p < 0.01

Follow-up Cannabis Use Motives Questionnaire

Upon study completion and first look at these data, the trends were surprising, specifically in the CO and AO groups, in that they countered our hypotheses given the existing literature. In effort to widen our understanding and interpretation of the data, we obtained IRB approval to send a follow-up questionnaire to the CO group regarding motives for use and psychiatric diagnoses. We offered an additional $10 cash for completed responses. Out of the total 29 CO participants, 18 responded completing the motives questionnaire. Table 4.2 details the responses from this survey. Eleven (61%) of the 18 respondents said they had been diagnosed with one of the following psychiatric conditions: major depressive disorder, generalized anxiety disorder, attention deficit/hyperactivity disorder (ADHD), autism spectrum disorder, obsessive compulsive disorder or post-traumatic stress disorder. Of the 7 individuals that reported never receiving a psychiatric diagnosis, 5 individuals suspect they would or should be diagnosed with at least one of the conditions listed. Of the 18 CO respondents, 56% reported regularly using cannabis to help manage one or more of these conditions, whether suspected or diagnosed. Of the 58 co-use participants, 28 (48%) reported having been diagnosed with a psychiatric disorder including depression, anxiety or ADHD.

Table 3.2. Follow up frequency data for cannabis use motives for individuals who reported using cannabis for the given reason at least half of the time.

	MOTIVE FOR CANNABIS USE	**ENDORSED AT LEAST HALF OF THE TIME**
PSYCHOLOGICAL	to forget worries	7
	to relieve depression	8
	to relieve anxiety	10
	stress relief/escape	10
PHYSICAL	acute pain	11
	chronic pain	10
	to sleep	13
	appetite/nausea	8

MOOD	confidence	6
	motivation	6
	focus/creativity	10
	to feel happier	8
	improve overall mood	6
EUPHORIA	like the feeling	15
	to get high	12
	pleasant feeling	17

3.4 Discussion

Across 170 participants, group differences emerged in several key domains of mental health, impulsivity, personality, and perception of health. Demographic patterns showed the AO group to be the most educated with the highest annual earnings, while the CO group was the least educated with the lowest annual earnings. In measures of depression and anxiety, the CO group consistently scored higher than other groups, while the co-use group showed elevated depressive symptoms relative to AO and control participants. Not surprisingly, given their high scores on depression and anxiety measures, emotion regulation difficulties were most pronounced in the CO group, particularly concerning limited access to effective strategies, whereas the AO group reported fewer difficulties. Impulsivity measures revealed the highest scores in the co-use group in both the BIS and UPPS-P, with notable differences in sensation seeking and negative and positive emotional urgency. In contrast, the AO and control groups reported the lowest impulsivity scores, with the exception of sensation seeking in which the AO group scored among the highest. These results highlight important distinctions between people based on their alcohol and cannabis use. Most notably, mental health challenges were most pronounced in the CO group.

Personality trait differences between substance use groups are also evident and map on to group differences in mental health as described above. For example, the CO and co-use groups

scored lower in in conscientiousness and agreeableness relative to the AO and control groups, and the CO group also demonstrated the lowest emotional stability. Similarly, ratings of self-perception of health diverged significantly by substance use pattern, where the CO and co-use participants reported the poorest health while AO and control participants reported significantly better health. Across all participants, lower income was associated with higher symptoms of depression, anxiety, and impulsivity, which is consistent with existing data[193–195].

Cannabis Use Motives and the Self-Medication Hypothesis

Eighteen of the 29 CO group members responded to the follow-up Cannabis Use Motives questionnaire, providing some insight into potential reasons for differences in mental health outcomes. More than half of these respondents endorsed sleep and pain as motives for using cannabis at least half of the time[196]. Ten respondents reported using cannabis to relieve anxiety or stress at least half of the time. This form of self-medication is not surprising as studies demonstrate that consumers find cannabis or cannabis extracts (e.g. CBD) to be effective at achieving the desired result and is preferable to over-the-counter or prescription medications[196–199]. In fact, one study showed that market sleep aid sales decline with increased availability and access to legal-market cannabis[196]. Within our sample, data indicate that near-daily cannabis use in the absence of regular alcohol use may reflect a purposeful effort to obtain relief from specific physical or mental health issues. For example, many of the participants who reported using cannabis to aid with sleep only consumed an edible before they went to bed and did not report any other cannabis use. This substance use behavior differed in the co-use group, where timing and method of use was less predictable and consistent day to day.

With this additional information, the higher scores in measures of depression, anxiety, and stress symptoms in the CO group is understandable. Several participants reporting cannabis

use primarily as a tool to manage these symptoms is consistent with the self-medication hypothesis[200], which suggests that individuals turn to substances to alleviate undesirable negative affective states. Prior research has reported similar trends, with cannabis users frequently endorsing motives related to anxiety and depression relief[46,168,201]. However, research also suggests that despite these motives, long-term heavy cannabis use is often associated with higher risk of anxiety, depression and suicidal ideation[202,203], raising questions about the efficacy of cannabis as a coping mechanism and whether continued use may exacerbate the very symptoms individuals aim to reduce.

At the same time, the possibility of reverse causation cannot be excluded. It remains unclear whether cannabis contributes to negative mental health outcomes, as indicated by some research[202,203], or whether individuals with pre-existing psychological susceptibilities are more likely to use cannabis as a coping strategy[203]. This ambiguity highlights the importance of longitudinal and experimental designs to investigate competing causal directions.

Endocannabinoid and Gut-Brain Pathways

The findings in this chapter may also reflect underlying biological mechanisms. As previously discussed in the introduction of this work, dysregulation of the ECS has been implicated in mood and anxiety disorders, with evidence that CB1 receptor activity modulates neurotransmitters critical to emotional regulation, including serotonin and dopamine[204,205]. Chronic cannabis exposure may disrupt ECS signaling, producing long-term consequences for affective regulation, even while acute use may appear to be anxiolytic. Additionally, recent research highlights possible gut-brain pathways, whereby alcohol and cannabis use influence the gut microbiome, systemic inflammation, and neuroimmune signaling[35,206]. These pathways may

provide a mechanistic explanation for the link between substance use and altered mood states observed in the present study data.

Socioeconomic Disparities and Substance Use Patterns

It cannot be ignored that the AO group was notably more educated and less likely to fall into lower-income brackets. This demographic distinction is important, as socioeconomic status is strongly tied to access to healthy living, general health outcomes, stress exposure, and healthcare access[207]. Thus, the mental health profile of the AO group seen here may at least partially reflect socioeconomic advantages that perhaps outweigh the deleterious effects of regular alcohol use itself. It is also possible that because alcohol use is more socially normative and integrated into cultural contexts of socialization and leisure[208], there is reduced stigma and stress associated with its use compared to cannabis use. The drinking culture in certain regions such as Fort Collins, CO, (which is home to dozens of craft breweries), may further normalize and encourage alcohol consumption, whereas cannabis use, despite increasing legalization, still carries greater variability in social acceptability, legal risk and the option of public use.

Personality as a Common Factor

Across groups, certain personality traits shared trends correlating with both substance use and mental health. Regular cannabis use in the CO group was associated with lower agreeableness, conscientiousness, and emotional stability, whereas AO and control participants displayed higher levels of these traits. Participants in the co-use group displayed the lowest overall scores in conscientiousness and highest in openness while also endorsing the highest levels of impulsivity. These findings are consistent with prior literature linking impulsivity, neuroticism, and sensation seeking to substance use and substance use disorders[209–211]. From the clinical perspective of common factors, personality may represent a common vulnerability that

predisposes individuals both to substance use and mental health issues[210]. For example, low conscientiousness may increase the likelihood of risky or compulsive behaviors through sensation seeking, while high neuroticism may heighten susceptibility to distress, thus providing motivation to use substances as a coping strategy. Future research should investigate whether personality functions as a mediator or moderator of the link between substance use and mental health, potentially clarifying causal pathways.

Alignment of Results with Existing Theoretical Models

Several theoretical frameworks have been proposed to explain the complex relationship between substance use and mental health, each emphasizing different mechanisms of risk and maintenance. The present findings can be contextualized within these broader models. Biologically focused causal models propose that chronic exposure to substances such as alcohol disrupts neurobiological pathways that regulate mood, stress, reward and impulse control, including dopaminergic and endocannabinoid systems, ultimately increasing vulnerability to depression, anxiety, and impulsive behavior[205,212,213] . This model describes an allostatic shift as a result of repeated substance use in which there is a progressive dysregulation of stress and reward pathways that leads to both emotional instability and increased compulsive substance use[214]. In contrast, psychologically focused causal models pose that individuals with pre-existing tendencies toward traits such as impulsivity, neuroticism, or sensation-seeking may seek out substance use to alleviate distress or enhance stimulation. This model supports self-medication and reinforcement-based treatments for addiction[200,215].

Common factor models, as discussed previously, add to these frameworks by suggesting that certain common vulnerabilities such as genetic predisposition, early-life adversity and childhood trauma, or specific personality traits create an underlying risk for both substance use

and psychopathology[210]. Similarly, dual-process models of addiction highlight the tension between impulsive, reward-driven systems and deliberate, regulatory control systems[216,217]. Within this model, heightened impulsivity and decreased executive control (both observed in the current study's co-use group) may contribute to automatic tendencies toward substance use, and reinforcing cycles of substance use and emotion dysregulation.

Finally, bidirectional models integrate these biological and psychological frameworks, proposing that substance use and mental health symptoms influence each other over time. Substance use may temporarily reduce negative affect but exacerbate susceptibility to mental health conditions, creating a positive feedback loop of dysregulation[218]. The pattern of increased impulsivity, depressive symptoms, and substance use behavior (specifically co-use and exclusive cannabis use) in these results is consistent with an interaction between underlying vulnerabilities and substance-driven physiological and psychological stress, further influenced by disruptions in neural pathways related to stress response and reward seeking. Although we cannot determine causality in this observational design, these converging theoretical frameworks highlight the importance of longitudinal and mechanistic research to clarify how neurobiological, psychological, and behavioral systems all contribute to the development and maintenance of substance use and accompanying mental health challenges.

Implications

The findings within this chapter may have direct relevance in terms of development of interventions for mood and anxiety disorders. For example, treatment providers should carefully assess patterns of regular substance use, especially cannabis and alcohol co-use, given its association with poorer mental health outcomes. Screening for personality characteristics may also better target intervention to the individual and increase the potential for successful

treatment, as traits such as impulsivity or low conscientiousness could help develop individualized behavioral strategies. Further, given that many CO participants endorsed therapeutic motives for cannabis use, the role of individual motive and expectation should be considered. Education regarding the current state of research and the limited evidence for cannabis as an effective long-term treatment for mood or anxiety symptoms should be implemented in any intervention or treatment effort. Integrating education on ECS and gut-brain mechanisms may also increase understanding of why cannabis use may paradoxically worsen mood over time.

Future Directions

Future research should explicitly test these competing theoretical models in samples of people who use alcohol and cannabis. Incorporation of biological data would provide unique and novel insight. Specifically, large studies incorporating measures of personality, socioeconomic status, motives for use, and biological pathways (e.g., ECS signaling, gut microbiome markers) could help clarify the directionality of effects. This type of research would provide critical insight to intervention, prevention and harm reduction strategies, particularly given the increasing social acceptance of both alcohol and cannabis.

Limitations

Certain limitations should be considered when interpreting the findings of this study. First, the cross-sectional design prevents strong conclusions about causality or directionality. While regular cannabis use was associated with poorer mental health, it remains unclear whether cannabis use contributed to these outcomes or whether individuals with greater psychiatric burden were more likely to use cannabis as a coping mechanism, which seems likely considering

the follow-up motivational data collected from the CO group. Longitudinal and experimental studies are necessary to disentangle these dynamics.

This study relied on self-report measures of substance use, mental health, and motives. Although self-report is widely used in this field, it may be influenced by recall bias, social desirability, or differing perceptions of use and symptom severity. Incorporating objective biomarkers of use (e.g., THC metabolites, alcohol metabolites) and clinical interviews for confirmation would add to the validity of data gathered.

It cannot be ignored that the characteristics that emerged from this sample may limit generalizability. The groups differed in their socioeconomic status, with the AO group reporting higher income and education, which could independently influence physical and mental health outcomes and access to coping resources. Future research should account for these variables more systematically or recruit samples matched on socioeconomic indicators. Additionally, the sample size within some groups limited statistical power for detecting nuanced differences set apart by substance use.

Finally, the findings reflect data from a specific cultural and geographic context. Norms around drinking and cannabis use vary widely across regions, potentially shaping motives for use, social acceptability, and psychological outcomes. Cross-cultural or multi-site studies would greatly benefit the ability to determine whether these patterns are consistent beyond the present sample.

Despite these limitations, this chapter provides an important step in clarifying the distinct and combined effects of alcohol and cannabis use on psychological functioning and vice versa. Future research that incorporates longitudinal designs, biological markers, and diverse samples

will be essential for refining theoretical models, further understanding risk factors and informing clinical practice.

Chapter 4. Circulating Endocannabinoid Levels

Aim 2. Examine baseline circulating levels of endocannabinoids and differences in relation to substance use patterns.

Hypothesis 2. Endogenous cannabinoid levels should be elevated with higher substance use, likely with the highest circulating levels in the co-use group with the use of both alcohol and cannabis. The control group should reflect the lowest circulating endocannabinoid levels.

4.1 Background & Rationale

One research focus that has received a great deal of attention in the recent decades is how ligands that bind CB1 and CB2 receptors within the ECS can modulate various biological system interactions, and vice versa. In addition to exogenous cannabinoids, alcohol is known modulator of the ECS. In transgenic mouse models, CB1 knockout mice demonstrated a marked decrease in alcohol consumption compared to wildtype mice[219]. Animal studies also suggest that chronic alcohol exposure and withdrawal may alter levels of prominent endogenous cannabinoids, with a short-term decrease in receptor signaling followed by long-term up-regulation of receptor expression and endocannabinoid production[220,221]. Similarly, cannabis (specifically high doses of THC) alters endocannabinoid levels as well, but rather competes for receptor binding and leads to an acute increase in circulating endocannabinoids, followed by a sharp decline below baseline levels[222–224]. This change is observed to ultimately cause an increased level of circulating endocannabinoids[68,223,225].

Anandamide (AEA) and 2-arachidonoyl glycerol (2-AG) are two prominent endocannabinoids that are frequently cited in the literature. Both are lipid mediators that are highly involved in several inflammatory and metabolic diseases[226]. Specifically, both 2-AG and AEA are shown to play important roles in autism spectrum disorder, major depression and

inflammatory bowel disease[226–229]. With these insights, understanding how circulating endocannabinoid levels differ with substance use could create pathways for novel treatment and clinical intervention.

4.2 Aim-specific Methods

Plasma was separated and assayed in duplicate via enzyme-linked immunosorbent assay (ELISA) for quantification of circulating 2-AG and AEA. Data from just a small subgroup of samples ($N = 94$ for 2-AG and $N = 75$ for AEA) was analyzed in this chapter due to reasons discussed further in section 4.4. Given the skewness and heteroscedasticity of these data, we used non-parametric independent samples Kruskal-Wallis tests to analyze both measures.

4.3 Results

No differences were seen in circulating levels of 2-AG ($H_{(3,91)} = 5.91$, $p = 0.116$, $\varepsilon^2 = .064$) or AEA ($H_{(3,72)} = 1.27$, $p = 0.737$, $\varepsilon^2 = .017$).

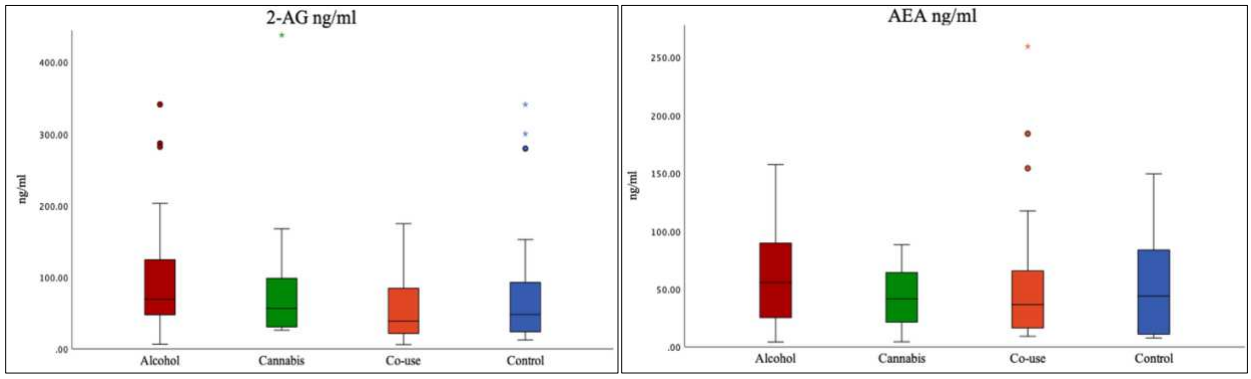

Figure 4.1 Circulating 2-AG and AEA concentrations in plasma

4.4 Discussion

Although group-level trends were observed, initial results were highly variable and lacked a clear, interpretable pattern once outlying values were considered. Importantly, at the time of these analyses we identified several critical pre-analytic and design limitations that substantially reduce confidence in the circulating endocannabinoid data seen here. These

limitations make accurate inference about group differences in this observational context unreliable. For these reasons, we suspended further pursuit of the endocannabinoid aim in the current project and instead focus on suggesting recommendations to improve endocannabinoid quantification methods for future work.

The primary concern with endocannabinoid quantification is pre-analytic variability. The literature highlights that lipid mediators like 2-AG and AEA are chemically volatile and can undergo enzymatic degradation after collection while remaining in whole blood samples[226,230]. 2-AG is also subject to rapid isomerization in whole blood, and the isomers would not bind in an assay targeting a distinct molecular arrangement. In other words, 2-AG quickly changes its shape in whole blood, and those altered forms (although still 2-AG) cannot be detected by assays designed to bind only its original structure. Consequently, timing and handling from blood draw to separation and storage of plasma are critical for reliable quantification[226,230]. In our protocol, the interval between sample collection and processing was not standardized across participants due to differences between study protocols for the co-use study sample and the other three groups. While we mitigated these risks as much as possible by refrigerating samples at 4°C and processing samples immediately after the session ended, timing was not formally standardized. Particularly for the co-use group, samples were often stored for several hours before processing due to the difference in study design. Given the known enzymatic activity in fresh blood and the rapid isomerization of 2-AG, it is possible and even likely that this timing difference would create artificial variability in the data[226].

A second substantive limitation was assay sensitivity and specificity. Although ELISAs have been used in several studies and provide an economical alternative to more sophisticated quantification methods, they are prone to cross-reactivity and can be less accurate, especially

when detecting low concentrations that are typically seen in circulating endocannabinoids. Liquid Chromatography with Mass Spectrometry (LC-MS) is considered the gold standard for quantifying these lipids due to its ability to provide higher specificity, better limits of detection, and use stable isotope internal standards[231,232]. Unfortunately, LC-MS was cost-prohibitive for the current project.

Finally, as a biological variable, baseline heterogeneity among participants varies greatly. Endocannabinoid signaling is influenced by a number of factors including sex, metabolic state, time since last meal, recent physical activity, medication use, and stress, among other variables[226]. Thus, single time-point, observational measures in a heterogeneous sample make it difficult to find meaningful differences between groups. Future studies should control for as many of these confounds as possible or look into within-subject changes in experimental or longitudinal designs.

Altogether, these limitations motivated the decision not to pursue a test of Aim 2 in the full study sample. In summary, circulating endocannabinoids are promising biomarkers for understanding substance-related neuroimmune interactions, but must be tightly controlled and precisely measured. Future studies addressing these limitations would increase interpretability and may help to reveal a clearer picture of how ECS signaling interacts with gut-mediated inflammation and mental health outcomes.

Chapter 5. Gut Microbiome & Intestinal Permeability

Aim 3. Investigate the effects of regular cannabis and alcohol consumption on the gut microbiome and intestinal permeability in the substance use groups compared to controls.

Hypothesis 3a. 16S gene sequencing is expected to reveal lowest gut microbial diversity and lowest abundance of commensal bacteria (e.g. Bifidobacteria[142]) and the highest levels of bacteria associated with inflammation and dysbiosis (e.g. Proteobacteria[35]) in the AO group, followed by the co-use group, while the CO and control groups will show the highest levels of diversity and commensal bacteria with diminished levels of opportunistic bacteria.

Hypothesis 3b. The AO group is predicted to show the highest levels of intestinal permeability (via plasma LBP and sCD-14 levels), followed by the co-use and CO groups, with the control group showing the lowest levels.

5.1 Background & Rationale

The gut microbiome plays a critical role in human health, contributing to digestion, immune signaling, and intestinal barrier function[233–235]. Disruption of healthy microbial balance (i.e. dysbiosis) can compromise gut barrier integrity, induce inflammation, and contribute to a variety of chronic diseases, from IBD to metabolic and neuropsychiatric conditions[37,38,96,236,237]. Substances that alter gut homeostasis may give rise to systemic pathophysiology through pathways mediated by the gut microbiome.

Chronic alcohol consumption is a deleterious disruptor of gut homeostasis. Alcohol damages intestinal epithelial tissue, reducing protein expression in tight junction and thereby increasing intestinal permeability and leading to what is commonly referred to as “leaky gut”[37,38,96]. Compromised tight junctions within the intestinal lining allows for microbial

translocation into blood circulation. This triggers widespread inflammation through activation of immune pathways and the release of pro-inflammatory cytokines such as IL-1β and IL-6[37–39,96]. Human studies have found that heavy alcohol use is associated with decreased alpha diversity, increased abundance of Proteobacteria, and relative loss of symbiotic taxa such as members of the families Lachnospiraceae and Ruminococcaceae[238].

In contrast, a steady accumulation of evidence suggests that cannabis and cannabinoid extracts such as cannabidiol (CBD) and Δ9-tetrahydrocannabinol (THC) may have protective or modulatory effects on intestinal barrier integrity and gut microbiota composition[86,239]. In vitro models show that CBD mitigates cytokine-induced permeability in intestinal epithelial cells and stabilizes tight junction proteins even under inflammatory conditions[86,239]. Additional studies report that cannabinoids can regulate gut motility, support barrier integrity, and have therapeutic potential in treating gastrointestinal diseases[96]. Novel data suggests that cannabis exposure can shift microbiome composition, although the effects are mixed depending on dose, mode of consumption, and health status[92].

Given the contrasting effects of alcohol and cannabis, this study aims to examine potential differences between substance use groups in gut microbiome diversity, evenness, and composition. Very little research has been published on the effects of cannabis and alcohol co-use on the health and function of the gut microbiome. By comparing taxon-level composition (i.e. differential abundance) as well as diversity and evenness metrics, I aim to discover whether regular cannabis use might offset negative effects of alcohol in the gut microbiome.

Diversity and Composition Metrics in Microbiome Analyses

Microbial diversity is typically described using two approaches – alpha diversity and beta diversity. Alpha diversity measures the diversity within a sample, capturing both the number of

distinct taxa (known as richness) and how evenly those taxa are distributed (evenness). Common richness metrics include Observed Features [Amplicon Sequence Variants (ASVs) or Operational Taxonomic Units (OTUs)] and Faith's Phylogenetic Diversity, which incorporates phylogenetic relationships among taxa[240]. Metrics such as Shannon's Index and Simpson's Dominance account for both richness and evenness, while Pielou's and Simpson's Evenness specifically quantify the equal distribution of taxon abundances.

In contrast, beta diversity describes variation between samples or groups of samples, essentially measuring how different microbial communities are from one another. Beta diversity is often assessed using distance or dissimilarity metrics such as Bray-Curtis, which incorporates information about presence/absence and abundance of identified taxa[241].

Before diversity analyses, sequencing data are typically rarefied, a process of subsampling without replacement to a read depth across all samples determined by minimum read count of samples. This normalization corrects for unequal sequencing depth, (since more deeply sequenced samples are likely to appear more diverse simply by chance), and ensures that diversity comparisons are not biased by read count differences[242]. While other normalization methods exist, rarefaction remains a standard and widely accepted approach in 16S rRNA analyses, allowing for further comparison of total abundance or read counts of individual taxa.

To visualize and interpret beta diversity, ordination techniques such as Principal Coordinates Analysis (PCoA) are used to reveal clustering patterns among samples. Statistical testing of microbiome differences can then be performed using methods such as Permutational Multivariate Analysis of Variance (PERMANOVA)[243] to assess differences in composition, and Permutational Analysis of Multivariate Dispersion (PERMDISP)[244] to test for differences in variability within groups.

Finally, Analysis of Composition of Microbiomes (ANCOM)[245] can be used to determine differential abundance between groups. This analytical approach accounts for the compositional nature of microbiome data, which is represented by taxa abundances of groups relative to a comparison group (such as a control) using an additive log-ratio (ALR) transformation. ANCOM is often preferred as it maintains high statistical power, reduces false discoveries, and is able to account for covariates[245].

Alongside these diversity and differential abundance metrics, investigation of abundance through total number of reads or rarefied counts of individual taxa between groups can be insightful as well. Relative abundance enrichment or depletion (as seen in ANCOM) cannot be relied upon to represent increases or decreases in overall taxa abundances within groups and thus, some researchers suggest that both relative and absolute abundance (or total number of reads from non-quantitative methods) should be analyzed for a fuller understanding and interpretation of microbiome comparisons[246,247]. Measurement and analysis of absolute taxa abundance can provide a more precise illustration of existing differences that isn't captured by traditional diversity metrics[247].

5.2 Aim-specific Methods

Microbiome Data Collection and Processing

Participants were given detailed instructions for collecting a fecal sample within 24 hours prior to their laboratory session. Researchers provided each participant with a collection kit which included two sterile, media-free swabs, a biohazard labeled zip-lock plastic bag and printed instructions with illustrations. Participants brought their samples to the lab and they were immediately stored at -80°C until sample processing for DNA extraction. At the study's conclusion, samples were thawed and processed to extract DNA using Qiagen QIAamp DNA

Microbiome kits (catalog no. 51704). DNA samples were then delivered to Colorado State University's Sequencing Core where they were they underwent amplification and high throughput sequencing of the 16S rRNA gene. Problematic sequences (e.g. short, chimeric, ambiguous, or low-quality) were removed and genes were classified according to at least 97% similarity into OTU (species) and ASV.

Microbiome Analysis

Sequenced data were processed, cleaned and analyzed in QIIME2[248] and RStudio[153]. Microbiome composition was determined using classification libraries, and data were assessed for microbial abundance and diversity by taxonomy across groups. Demultiplexed paired-end sequence files were trimmed to 244 base pairs (bp) on the forward reads and 223 bp on the reverse, and denoised using DADA2[249]. A feature table was then created and taxonomy was assigned to the ASVs using the QIIME2 feature classifier sklearn plugin[250] on the GreenGenes pre-trained classifier[251]. ASVs that corresponded to mitochondria or chloroplasts were filtered out and control samples were removed. Sequences were rarefied to 36,000 reads per sample for standardization. No outlying data were removed.

Alpha diversity was assessed through a number of validated measures to determine richness and evenness of ASVs within groups. Total number of observed features was first examined among samples within groups. The Shannon Index was used to compare richness and evenness. Faith's phylogenetic diversity was used to measure richness based on phylogenetic relationships. Simpson's Evenness provided values for how evenly species are distributed within samples, and Pielou's Evenness provided values for species distribution through diversity and evenness within samples. Simpson's Diversity measured taxa richness and evenness together, with higher scores indicating higher diversity, while Simpson's Dominance captured the

frequency of dominant species within samples. Finally, Fisher's Alpha calculated the number of species to their relative abundance within samples.

Beta diversity measured with PCoA based on Bray-Curtis dissimilarity metrics was used determine differences in microbial community composition between groups. PERMANOVA[243] with Kruskal-Wallis analysis was also used to compare taxa abundance across groups, while PERMDISP[244] was used to analyze the dispersion of taxa abundance within group clusters. Differential abundance of ASVs between groups was assessed using ANCOM[245] in QIIME2. Features with significant W-statistics were interpreted as differentially abundant.

In order to account for variability in substance use within group assignment (i.e., the fact that individuals assigned to the CO group did report some low levels of alcohol use, and those in the alcohol use did report some low level cannabis use, along with differing amounts of use within the co-use group), we investigated substance use amount as a continuous variable via linear regression analysis where data met assumptions of linearity. Data was taken from the 14-day daily diary record and number of standard alcoholic drinks per day and grams of cannabis consumed per day were used to predict total rarefied counts of specific taxa. The control group was removed from these analyses so as to avoid zero-inflation, given that they did not report alcohol or cannabis use. To address remaining zeros within the CO, AO and co-use groups, substance use data was transformed using the log(x+1) method. Data that did not meet assumptions of linearity are not presented in the present report.

Finally, we explored group differences in individual taxa abundance on rarefied sequences using ANCOVA with RFS as a covariate in every model. Data were either square root or log transformed and checked for assumption violations before analyses were run. As many bacterial species occur at relatively low levels in healthy individuals, two composite variables

were created consisting of several bacteria known to be either commensal or associated with inflammation, dysbiosis or pathogenesis, especially when overgrown in the gut. The variable capturing genera associated with disease and inflammation included total rarefied counts of Moraxella, Campylobacter, Mogibacterium, Megamonas, and Desulfovibrio, as well as Escherichia coli species[233,252–263]. The variable capturing commensal bacterial species included total rarefied counts of Clostridium butyricum, Bifidobacterium longum, Akkermansia mucinophila, Faecalibacterium prausnitzii, Bacteroides uniformis, and Prevotella copri[264–279].

Intestinal Permeability Analysis

Intestinal barrier integrity was assessed by protein quantification of soluble CD-14 and LBP in plasma via ELISA kits from Fisher Scientific. Optical density data output was processed and transformed into appropriate units of measurement for each protein and statistically analyzed using ANOVA. Due to expected biological differences among human subjects, no outlying data were removed. Assumption violations were corrected through square root transformation for sCD-14.

5.3 16S Microbiome Data Results

Samples from 116 participants were analyzed for microbiome composition. Group assignment was as follows: AO (n = 25), CO (n = 22), co-use (n = 32), and control (n = 37). Demographic data is summarized by group in Table 5.1. Note that sample sizes reported in table 5.1 differ from those in tables 3.1 and 6.1 due to unprovided biological samples, insufficient material for processing and attrition. Sex differed between the control group and the AO and co-use groups with more individuals assigned female at birth belonging to the control group. The AO and control groups housed more individuals who had earned at least a bachelor's degree than the CO and co-use groups. The AO group also reported higher annual income than the CO and

co-use groups. Finally, there were significantly more white individuals in the AO group than the control group. Given these differences, demographics were correlated with each outcome and were covaried where significant.

Table 5.1. Participant demographics by substance use pattern. Significant group differences are indicated by subscript letters. Different letters indicate groups that are statistically distinct, whereas shared letters indicate no differences between groups.

	AO (n=25)	**CO (n=22)**	**Co-use (n=32)**	**Control (n=37)**	**Total (n=116)**
Demographics					
Age, mean years (SD)	33.2 (10.2)	32.3 (8.9)	29.2 (8.8)	32.3 (10.0)	32.3 (9.6)
Assigned female at birth, no. (%)	**12 (48)$_{a}$**	12 (54.5)$_{a,b}$	15 (46.9)$_{a,b}$	**21 (56.8)$_{b}$**	61 (48.8)
Bachelor's degree or higher, no. (%)	**22 (88)$_{a}$**	**8 (36.4)$_{b}$**	**9 (28.1)$_{b}$**	**25 (67.6)$_{a}$**	64 (55.2)
Income, <$50,000/yr, no. (%)	**14 (63.6)$_{a}$**	**10 (45.5)$_{b}$**	**7 (21.9)$_{b}$**	17 (48.6)$_{a,b}$	48 (43.2)
Race, white, no. (%)	**23 (92)$_{a}$**	17 (77.3)$_{a,b}$	28 (87.5)$_{a,b}$	**27 (73)$_{b}$**	95 (81.9)
Ethnicity, Hispanic, no. (%)	5 (20)	6 (27.3)	5 (15.6)	4 (10.8)	20 (17.2)
14-day Daily Diary Data					
% drinking days, mean (SD)	60 (23.5)	8 (10.2)	51 (24.3)	3 (8.7)	28 (31.1)
avg drinks/day, mean (SD)	2.0 (1.2)	.09 (.14)	1.9 (1.5)	.07 (.22)	0.9 (1.3)
% cannabis use days, mean (SD)	4 (9.9)	91 (18.5)	69 (29.5)	3 (16.8)	35 (42.8)
avg cannabis g/day, mean (SD)	.001 (.004)	**1.35 (1.74)$_{a}$**	**.47 (.64)$_{b}$**	.05 (.34)	.38 (.97)
avg exercise minutes/day, mean (SD)	37.7 (29.1)	32.9 (39.7)	28.2 (27.7)	42.1 (31.3)	36.1 (31.8)
RFS score, mean (SD)	32.6 (7.2)	30.1 (6.5)	30.3 (8.3)	30.0 (7.3)	30.7 (7.4)

Alpha Diversity

No differences were seen in total number of observed features, Shannon's Entropy, Faith's Phylogenetic Diversity or Fisher's Alpha. However, Kruskal Wallis (KW) non-parametric tests revealed significant differences in number of dominant species within microbiomes via Simpson's Dominance ($H_{(3,112)} = 8.92$, $p = .03$, $\varepsilon^2 = .053$), indicating differences between the control and AO ($p = .01$) and control and co-use ($p = .02$) groups (see figure 5.1A). Differences in Simpson's Diversity were also detected ($H_{(3,112)} = 8.36$, $p = .03$, $\varepsilon^2 = .048$), revealing differences between AO and control ($p = .02$), co-use and control ($p = .03$) groups, and near-significant differences between the AO and CO groups ($p = .05$; see figure 5.1B).

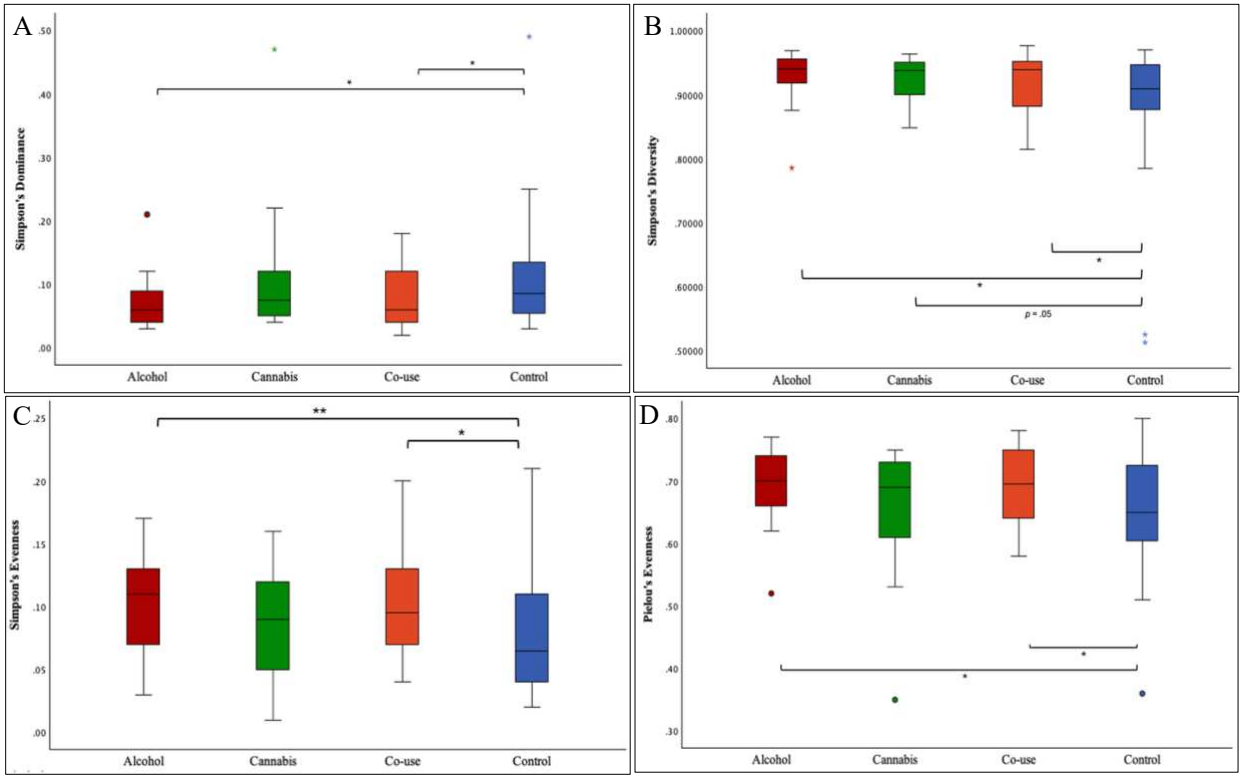

Figure 5.1. Alpha Diversity measures by group; colored stars and dots signify outlying data. Horizontal brackets with one asterisk (*) indicate $p < 0.05$, two asterisks (**) indicate $p < 0.01$. A.) Simpson's Dominance; B.) Simpson's Diversity (richness and evenness); C.) Simpson's Evenness; D.) Pielou's Evenness

Group differences in Simpson's Evenness ($H_{(3,112)} = 10.67$, $p = .01$, $\varepsilon^2 = .068$) with pairwise comparisons showing differences between the control and co-use ($p = .007$) and control and AO ($p = .01$) groups (see figure 5.1C). Pielou's Evenness was also analyzed as a secondary measure of evenness. KW results were significant ($H_{(3,112)} = 8.54$, $p = .03$, $\varepsilon^2 = .049$), showing differences between the control and AO ($p = .01$) and control and co-use ($p = .02$) groups (see figure 5.1D).

Beta Diversity

PERMANOVA results from Bray-Curtis distance data indicated significant differences between group clusters ($pseudoF_{(3,112)} = 1.41$, $p = 0.01$, 999 permutations) although the effect size was modest at $R^2 = 0.051$. Pairwise comparisons showed group differences between AO and co-use ($p = 0.03$), and CO and co-use groups ($p = 0.04$).

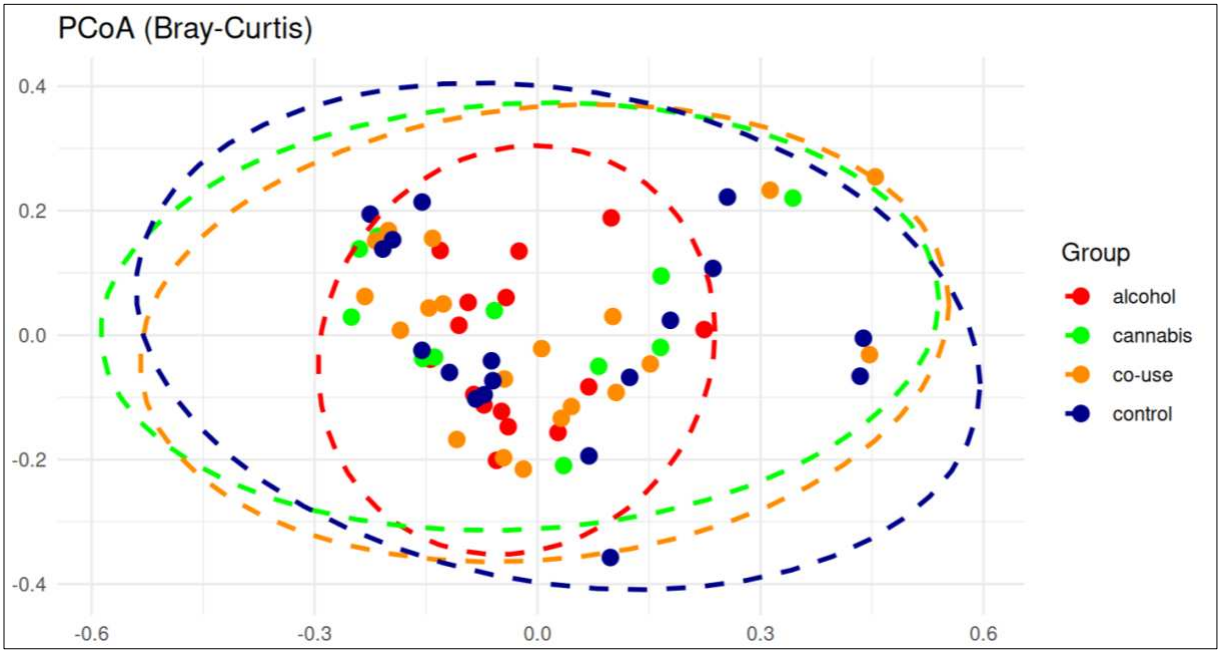

Figure 5.2. PCoA plot created from Bray-Curtis distance matrix illustrating dissimilarity between participants within groups. Each point represents an individual sample. Dashed lines represent average distance from the central point of group data.

Permutational multivariate analysis of dispersion (PERMDISP) revealed no differences in dispersion within group clusters, suggesting that differences are unlikely due to heterogeneity of dispersion, and PERMANOVA results likely reflect true condition effects ($F_{(3,112)} = .384$, $p = 0.764$, 999 permutations). A Principle Coordinates Analysis (PCoA) plot was generated to illustrate these results (see figure 5.2).

Table 5.2. ANCOM results of taxa either enriched or depleted in substance use groups compared to control.

W	clr	Family Taxon	Phylum	Differential Group
101	0.9854	Ruminococcaceae	Firmicutes	Depleted in Co-use
66	0.9421	Lachnospiraceae	Firmicutes	Depleted in AO and Co-use
19	0.9949	Bacteroidaceae	Bacteroidetes	Depleted in CO and Co-use
12	0.9652	Veillonellaceae	Firmicutes	Depleted in AO and Co-use
6	0.9760	Enterobacteriaceae	Proteobacteria	Enriched in AO, Depleted in CO and Co-use
5	0.9997	Streptococcaceae	Firmicutes	Depleted in AO

ANCOM analysis in Qiime2 found six taxa to be significantly differentially abundant at the family level with the control group set as the comparison (Table 5.2). Ruminococcaceae was

depleted in the co-use group while Streptococcaceae was depleted in the AO group. Lachnospiraceae was depleted in all three substance use groups compared to control, Bacteroidaceae, and Veillonellaceae was depleted the AO and co-use groups. Streptococcaceae was depleted in the AO group, while Enterobacteriaceae was enriched in the AO group and depleted in the CO and co-use groups.

Total Rarefied Counts: Group differences in number of reads in individual taxa

No differences were seen in total rarefied counts (TRC) between groups in the two most prominent phyla (Bacteroidetes and Firmicutes) in the gut microbiome[255], however the Actinobacteria phylum – typically considered commensal in the gut microbiome[280] – showed higher TRC in the CO group overall in an ANCOVA covarying for income ($F_{(4,106)} = 3.23$, $p = 0.01$, $\eta^2 = 0.109$; see figure 5.3). Upon further analysis at the genus level, Bifidobacterium – a commensal genus belonging to the Actinobacteria phylum often found in probiotic supplements[267] – appeared to be driving these group differences ($F_{(4,106)} = 2.67$, $p = 0.03$, $\eta^2 = 0.091$), with the highest TRC in the CO group and lowest in the AO group (see figure 5.3). Both RFS and income were covaried in these models as they were significantly correlated with the outcome.

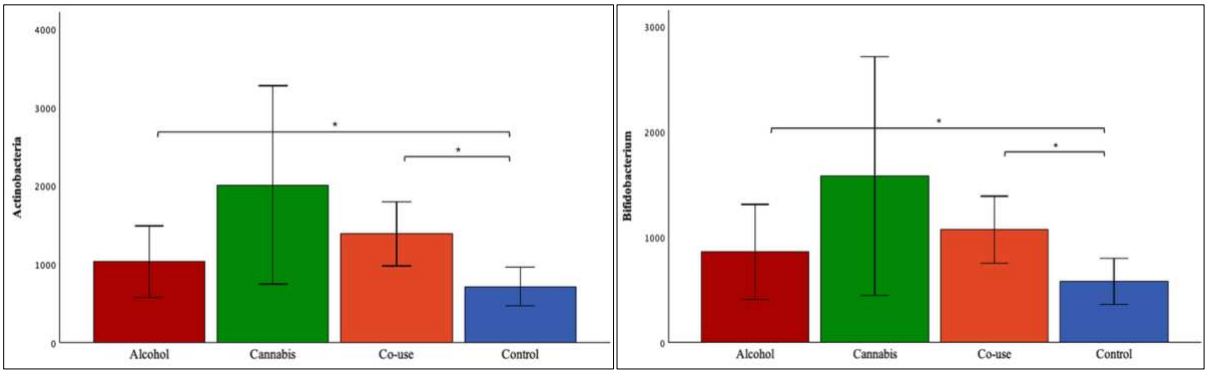

Figure 5.3. Group differences in total rarefied counts in Phylum Actinobacteria and Genus Bifidobacterium; Error bars = +/- 1 SE; * = p < 0.05, ** = p < 0.01

Bacteroidia is shown to be highly beneficial within the human gut. Significant group differences were seen in the TRC of this class ($F_{(4,103)} = 3.80$, $p = 0.007$, $\eta^2 = .141$), but this time with the CO group displaying the lowest TRC overall (see figure 5.4).

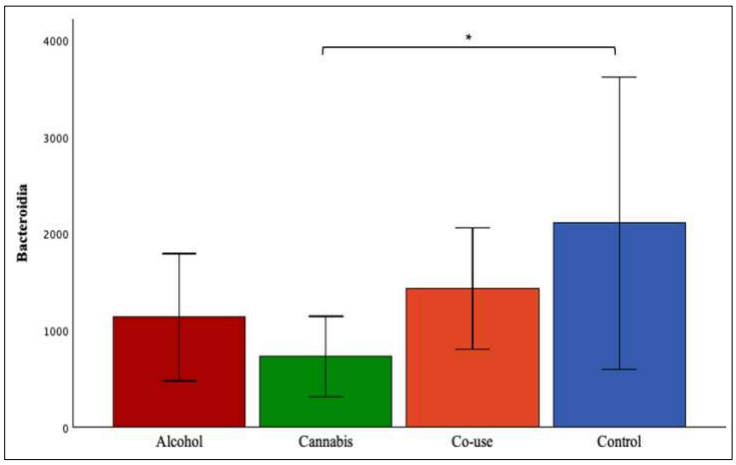

Figure 5.4. Group differences in total rarefied counts in Bacteroidia Class;
Error bars = +/- 1 SE; * = p < 0.05, ** = p < 0.01

An ANCOVA model of TRC of commensal bacterial species proved significant ($F_{(4,103)} = 2.93$, $p = 0.03$, $\eta^2 = .079$). Although no significant pairwise comparisons emerged, a trend can be seen in the AO group displaying lower TRC of these species than the co-use group (figure 5.5).

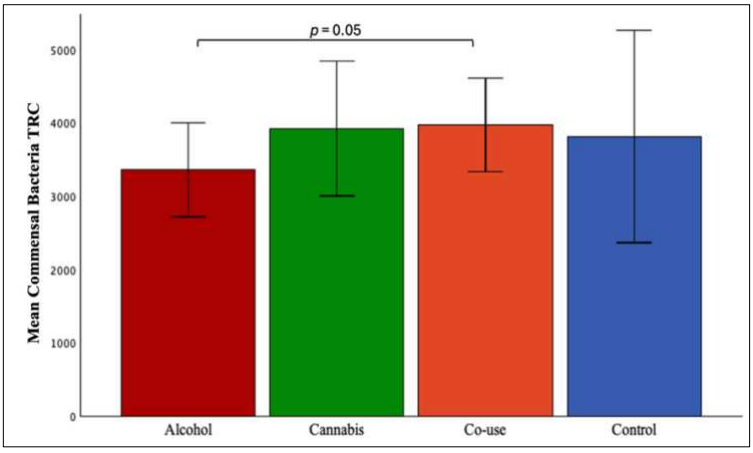

Figure 5.5. Differences in group means of total rarefied counts of commensal bacteria;
Error bars = +/- 1 SE; * = p < 0.05, ** = p < 0.01

Significant differences were seen between groups in TRC of inflammation and dysbiosis-associated (i.e. opportunistic) bacteria ($F_{(4,95)} = 3.08$, $p = 0.03$, $\eta^2 = 0.089$), revealing higher TRC in the AO group than all other groups (see figure 5.6).

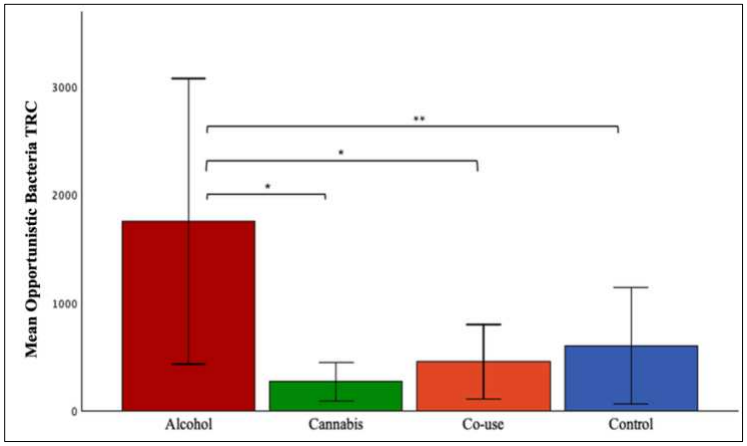

Figure 5.6. Differences in group means of total rarefied counts of opportunistic bacteria; Error bars = +/- 1 SE; * = p < 0.05, ** = p < 0.01

ANCOVA results showed significant group differences in TRC of Proteobacteria ($F_{(4,105)}$ = 4.29, p = 0.007, η^2 = .111), revealing the AO group to have significantly higher TRC than each other group. Further, linear regression analysis found that increased grams of cannabis use per day negatively predicted TRC of Proteobacteria in microbiomes ($F_{(2,69)}$ = 11.44, p = 0.001, R^2 = .168; see figure 5.7).

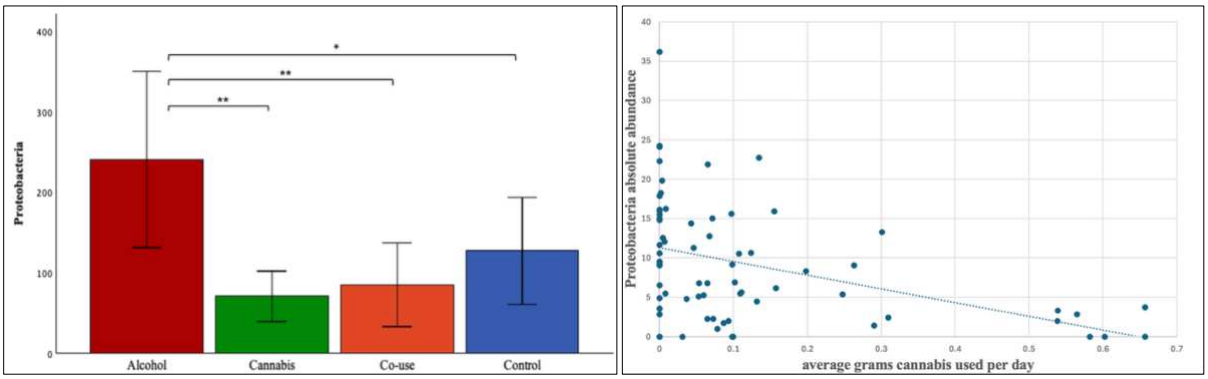

Figure 5.7. Bar graph displays group differences in TRC of Phylum Proteobacteria; Linear trendline visualizes the significant negative relationship between grams of cannabis consumed per day and TRC of Proteobacteria in individuals using cannabis with or without alcohol (control group excluded). Error bars = +/- 1 SE; * = p < 0.05, ** = p < 0.01

Another linear regression revealed that increased number of standard drinks per day positively predicted TRC of bacteria in the Erysipelotrichaceae family ($F_{(1,69)}$ = 5.08, p = 0.02, R^2 = .082; figure 5.8). Sex was significantly correlated with this outcome and was thus covaried

alongside RFS in an ANCOVA model for Erysipelotrichaceae, which was significant ($F_{(5,101)}$ = 4.13, p = 0.004, η^2 = .139; see figure 5.8).

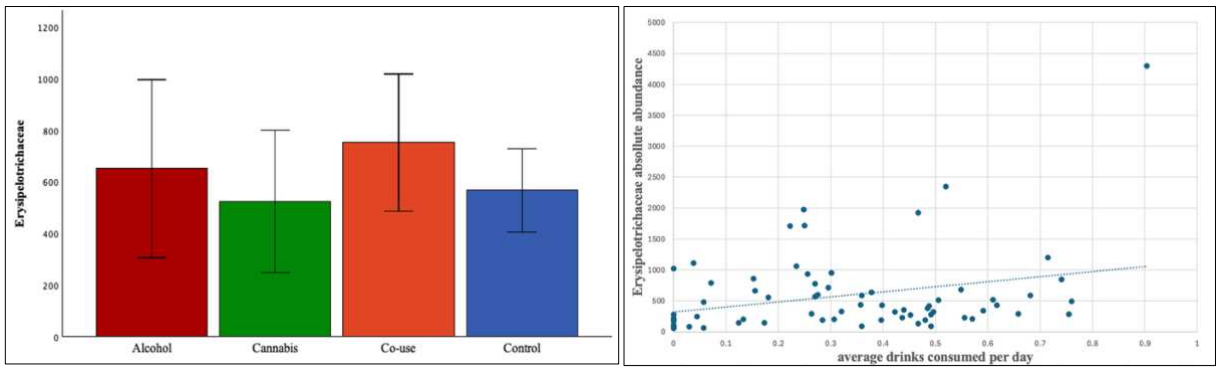

Figure 5.8. Bar graph shows TRC of family Erysipelotrichaceae by group. Linear trendline visualizes the significant negative relationship between standard drinks consumed per day and TRC of Erysipelotrichaceae in individuals using alcohol with or without cannabis (control group excluded).
Error bars = +/- 1 SE; * = p < 0.05, ** = p < 0.01

5.4 Intestinal Permeability Results

No significant differences were found in circulating LBP levels between groups. Differences were seen in soluble CD-14 levels in an ANCOVA covarying for sex ($F_{(4,152)}$ = 15.57, p < 0.001; η^2 = 0.291), with the AO and control groups expressing higher concentrations than the CO group. The co-use group had the lowest CD-14 concentration overall and was significantly lower than the AO group.

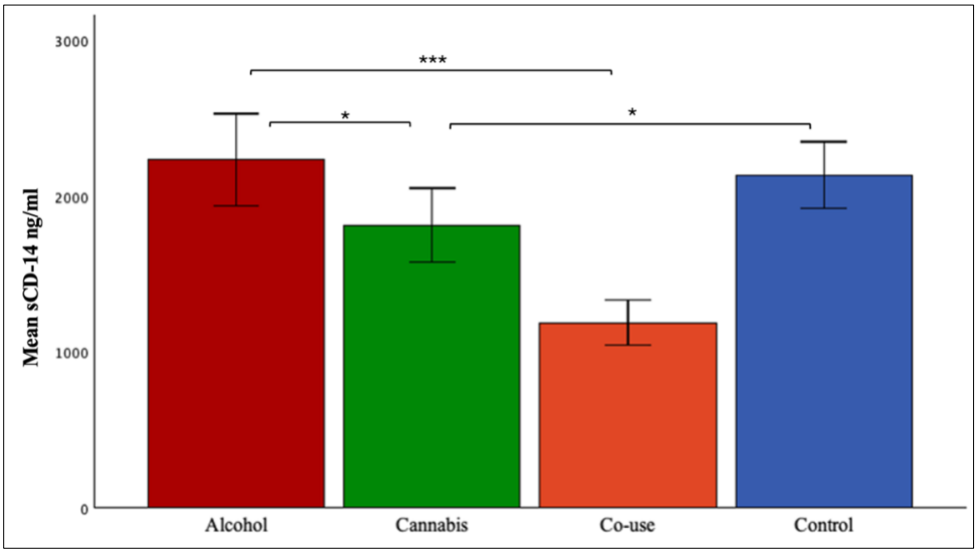

Figure 5.9. Group differences in plasma levels of soluble CD-14;
Error bars = +/- 1 SE; * = p < 0.05, ** = p < 0.01, *** = p < 0.001

5.5 Discussion

Alpha Diversity and Composition of Microbiomes

Although overall microbial richness and diversity (Observed Features, Shannon's Entropy, Faith's Phylogenetic Diversity, and Fisher's Alpha) did not differ between groups, indices measuring dominance and overall diversity (richness and evenness combined) revealed significant group differences in the gut microbiome. Simpson's Dominance and Simpson's Diversity both differed across groups, such that the lower values in these measures unexpectedly belonged to the control and CO groups, which we hypothesized would have greater diversity compared to the AO and co-use groups. Similarly, both Simpson's Evenness and Pielou's Evenness differed significantly between the control and AO and control and co-use groups, indicating that microbial communities were less evenly distributed in the control group, which again was unexpected.

Differential abundance testing with ANCOM supported these results, revealing significant contrasts among several dominant bacterial families. The most affected taxa included Ruminococcaceae, Lachnospiraceae, Bacteroidaceae, Veillonellaceae, Enterobacteriaceae, and Streptococcaceae, which collectively accounted for much of the observed community variation. Notably, Ruminococcaceae, Lachnospiraceae and Veillonellaceae are important short-chain fatty acid (SCFA)-producing families associated with gut health and implicated in cognitive performance[281–284], and showed diminished abundance in AO and co-use groups relative to the control group. This may suggest alcohol-induced disruption of balance in taxa involved in important SCFA metabolism, impacting metabolic and immune function[285,286]. The primarily commensal family Bacteroidaceae is known for aiding in digestion and its role in development

and maintenance of the immune system[287], and was depleted in the CO and co-use groups, calling into question the effect of cannabis use on this particular domain.

In contrast, the Enterobacteriaceae family is frequently linked to inflammation and alcohol-associated dysbiosis[288,289], and showed enrichment in the AO group compared to control appearing to reflect responses to alcohol exposure. The Streptococcaceae family is a more complex family as it is home to commensal species that contribute to immune homeostasis and epithelial and mucosal defense, but elevated levels have been linked to health issues such as IBD and pancreatic cancer[290,291]. Studies show that alcohol use disrupts mucin production and compromises tight junctions[292], finding reduced abundance of protective intestinal Streptococcus species with alcohol-related gut dysbiosis [35,293]. Given this research, depletion of Streptococcaceae in the AO group could indicate a loss of mucosal resilience and immune regulation through diminished levels of commensal species, creating an environment more supportive of opportunistic or inflammatory taxa.

It is important to note that relative abundance and TRC reflect two different measurements. The enrichment or depletion seen in substance use groups compared to the control group is calculated by the total read counts of specific taxa within those groups. However, when analyzing TRC of the same taxa, group differences may not statistically emerge due to mean values being more balanced among groups[246]. This indicates that differences in differential abundance analysis were driven by a smaller number of individuals within groups rather than the group as a whole, emphasizing the importance of considering diversity and compositional analyses wholistically and complimentary to one another.

Beta Diversity

While there may be biologically relevant group effects, these results leave room for interpretation. While group differences exist (likely driven by the clustering in the AO group), the overall variation explained by group membership ($R^2 = 0.051$) was modest in these data. Given the effect size and the significant overlap of group coordinates, it is important to consider heterogeneity within groups. The control and CO groups specifically displayed large within group variability, suggesting the likelihood that additional factors that were unaccounted for in the present study contributed to individual differences in microbiome composition aside from substance use alone. Environmental exposures (e.g. toxins or pollutants in the air), infrequent medication use, acute stress, lifestyle, genetics, medical history, unknown or undiagnosed conditions and sleep are just a few of the numerous factors that have the potential to account for variability in the composition of the gut microbiome. In order to capture differences caused by substance use, much tighter control in a clinical setting is needed. For example, participants could be studied for a period while living full-time in an inpatient unit where important factors such as diet, sleep, environmental exposures and medication use could be controlled and measured.

Ultimately, beta diversity analyses show the AO group to have a more homogenous profile, tightly clustered with little variance. While there is obvious overlap with the other groups, the AO group clearly stands out (see Fig 5.2). The CO group on the other hand is highly variable with a broad spread rather than distinct clustering, suggesting heterogeneity that makes group differentiation more difficult. The co-use group, while exhibiting interesting and unexpected patterns in several areas, overlaps both AO and CO groups which is consistent with the fact that the co-use group reflects both alcohol and cannabis use. Finally, (and somewhat

surprisingly), the control group is the most variable, capturing individuals with diverse profiles despite their lack of substance use and diminishing contrast from other groups. One possible explanation for this group variability is that these individuals may refrain from substance use for specific, health-related reasons (e.g. managing a chronic condition that could impact gut health). While we did screen for immune disorders and other medical conditions, we did not assess control individuals' reasons for abstinence. These data would be important to collect in future studies.

Differences in Total Rarefied Counts of Individual Taxa

While we can glean interesting and potentially useful information from these findings, further investigation is needed to identify reasons for observed microbiome differences. A closer look at composition through TRC of specific taxa across groups revealed intriguing distinctions in the microbiomes of different substance use groups.

The CO group displayed heightened TRC of Bifidobacterium (phylum Actinobacteria), well-known for its roles in maintaining gut homeostasis, supporting healthy digestion, and regulating immune responses[280]. This elevation in reads suggests a potential supportive or protective effect of cannabis, specifically in the absence of alcohol, on these commensal microbes. Similarly, increased daily cannabis use predicted decreased TRC of Proteobacteria, a phylum associated with dysbiosis, inflammation, and conditions such as IBD and metabolic syndrome[130,143]. Conversely, increased daily alcohol consumption positively predicted higher TRC of bacteria in the Erysipelotrichaceae family, a bacterial domain consistently linked to inflammation and metabolic disease[294,295]. This suggests cannabis may promote the growth of commensal bacteria while reducing opportunistic taxa, while alcohol may support growth of taxa associated with metabolic and inflammatory stress.

These results highlight that alcohol and cannabis differentially affect the gut microbiome. Alcohol use does show support for many commensal taxa but rather, it appears to foster environments favoring the growth of opportunistic taxa that may promote inflammation and pathogenesis. Cannabis on the other hand shows a more nuanced pattern, generally appearing to support mutualistic microbes (e.g. Bifidobacterium) while suppressing the enrichment of lineages that support inflammation and disease (e.g. Proteobacteria). However, the CO group also exhibits diminished TRC of Bacteroidia, a symbiotic class of bacteria aiding in metabolic health, energy production and epithelial maintenance[296,297], indicating that these effects are not necessarily universally positive and may depend on additional factors. One plausible explanation is varying nutrient availability in the gut according to differential substance use patterns. Alcohol and cannabis may alter the intestinal environment in a way that allows for certain taxa to flourish and others to decline, perhaps via changes in mucosal production or host metabolism for instance[92,298].

Given the existing literature, we cannot ignore the antimicrobial nature of cannabis itself. Some cannabinoids (e.g., cannabidiol) have well-documented properties which might selectively suppress certain bacterial taxa[299–302]. It's also worth investigating whether the mode of cannabis use (e.g. smoking vs. oral ingestion) leads to different effects on gut microbiota, given the general implication of smoking on inflammation. This could be relevant because inhalation of smoke is highly inflammatory[303–305] along with the fact that the gut is home to a wealth of endocannabinoid receptors[306,307]. The oral-intestinal route of cannabis consumption causes a lower rate of cannabinoid binding and absorption as it moves through the gastrointestinal tract and initiates ECS signaling[92]. Enzymes produced by gut microbiota also metabolize certain cannabinoids making them more potent[92,308], which altogether, could potentially have stronger

modulatory effects on the gut microbiome. This could imply that edible cannabis products have greater influence on the microbiome and provide more ample systemic anti-inflammatory benefits than the method of smoking. Research specific to the effects of cannabis smoke compared to edible ingestion on microbiome composition and inflammation is limited but should be explored to a larger degree.

Intestinal Permeability

In consideration of gut permeability biomarkers, no differences were seen in circulating levels of LBP. This is likely due to the fact that participants were relatively healthy and the amount of cannabis and alcohol used was variable within each substance use group. The concentration of LBP in plasma was within normal range for all groups. If we remove the co-use group from comparison, results for CD-14 were as expected, with elevated levels in the AO group and diminished levels in the CO group. However, we did not anticipate seeing the lowest levels in the co-use group. It is important to note that CD-14 is acutely sensitive to variables like time since last meal, diet composition, recent exposure to inflammatory stimuli, medication use, and variability in substance use. Because a 24-hour abstinence period prior to sample collection was imposed in the co-use group due to design constraints specific to that study, CD-14 levels may have been acutely modulated[36,309], potentially masking or diluting the longer-term effects of alcohol use. This period of abstinence may have also altered acute microbial dynamics, reducing the ability to detect the underlying effects of chronic substance use.

Limitations

Some important methodological caveats should be considered with these data. There was large variability in sample collection by participants and several fecal swab samples were unusable due to insufficient amount of sample material. Sparse sampling may fail to capture less

abundant taxa and alter community composition estimates. Moreover, the time from collection to freezer storage for co-use group samples was variable and not standardized to match storage procedures for the other groups. Differential handling can introduce bias or microbial drift and hinder accurate comparison of microbiomes with other groups.

Additionally, the demographic differences in sex, race, income, and education between groups are potentially confounding as these are all factors known to be associated with microbiome composition. Although we attempted to account for these differences statistically, residual effects may still confound results. Future designs should aim to mitigate these discrepancies through frequent checks on randomization across these covariates.

The study's smaller group sizes and heterogeneity among group participants inevitably introduces challenges, whereas a larger sample of participants would provide greater power to detect subtler microbiome effects. Further, cannabis dose (grams per day) and alcohol consumption varied widely within the co-use group, making interpretation of group differences more challenging.

Implications and future directions

Despite the noted challenges, the findings indicate that substance use exerts taxon-specific effects on the gut microbiome that may not be apparent in typical broad diversity metrics. The enrichment of commensal taxa with exclusive cannabis use juxtaposed against the selective growth of taxa associated with inflammation and dysbiosis with exclusive alcohol use suggests a potential influence of substance use on human health (whether metabolic, immune or neurologic) that is mediated by the microbiome.

Understanding these nuanced effects can be relevant for SUD treatment, especially given emerging investigation into MGBA interventions. For example, targeted microbiome therapies or

potent probiotics might help mitigate dysbiosis associated with alcohol craving and even relapse. Additionally, specifying mode of cannabis consumption, dose, and any duration of abstinence may help clarify when cannabis has protective vs. disruptive effects on the microbiome. The present chapter highlights the importance of moving beyond overall diversity and toward composition and taxon-level analysis when investigating how substances shape the overall gut microbiome. Future studies that are well-powered, tightly controlled, longitudinal, and mechanistic are needed to confirm causality, illuminate pathways, and identify therapeutic targets.

Chapter 6. Inflammatory Markers

Aim 4: Explore inflammatory markers (C-reactive protein and cytokines) in plasma for differences based on substance use patterns.

Hypothesis 4. The AO group was expected to show higher levels of pro-inflammatory cytokines and lower levels of anti-inflammatory cytokines overall. The control group was expected to display moderate levels of cytokines, lower pro-inflammatory and higher anti-inflammatory than the AO group, while the co-use group was anticipated to lie somewhere between the levels displayed by the AO and control groups. The CO group was expected to display low levels of pro-inflammatory cytokines compared to the AO and co-use groups, and possibly even lower than the control group, and higher levels of anti-inflammatory cytokines.

6.1 Background & Rationale

Inflammation is a primary component of the immune system's response to injury or infection, involving a complex network of signaling molecules. One category of such molecules is cytokines, a critical early messenger in the signaling cascade of an immune response[310]. Pro-inflammatory cytokines help initiate defenses against foreign bodies and pathogens. However, when cytokine levels remain chronically elevated due to disease states or other prolonged stressors, damage to tissue can occur and contribute to systemic disease[310]. Anti-inflammatory cytokines on the other hand act to dampen the immune response and restore homeostasis[311,312]. The balance between pro- and anti-inflammatory cytokines shapes how the body responds to injury, infection, and chronic stressors such as regular alcohol and cannabis use. Thus, both pro- and anti-inflammatory cytokines can indicate the status of the immune system, potentially pointing to issues from infection to autoimmune disease[313].

Disruptions in gut microbiome health are strongly implicated in systemic inflammation. Dysbiosis can compromise the intestinal barrier by breaking down tight junctions within the cell wall, creating a "leaky gut" in which endotoxins (such as lipopolysaccharide [LPS], a membrane component of gram-negative bacteria typically sequestered in the gut) enter the bloodstream[314]. This triggers an immune response and the release of inflammatory mediators, such as cytokines[310,314]. In response, the body produces lipopolysaccharide binding protein (LBP) to bind to LPS in effort to eliminate the threat of infection[315,316]. LBP then interacts with CD-14 (an innate immune system responder) which signals cytokine release, triggering the overall immune response. Given this sequence of events, each of these proteins serve as useful biomarkers of gut permeability[314–318]. Additionally, the liver responds to these disruptions by producing acute phase proteins, most notably C-reactive protein (CRP), which is widely used as an additional indicator of systemic inflammation[319]. Figure 6.1 illustrates the systemic multi-directional interactions discussed in this dissertation with these immune responses incorporated into the model.

The effects of inflammation also extend beyond physical health, with strong evidence of bidirectional interactions between systemic inflammation, gut health, and mental health. Dysbiosis can provoke inflammation, which in turn affects neuroinflammation and contributes to psychiatric symptoms[320,321]. These processes feed back into one another, creating a cycle that intertwines gastrointestinal, immune, and psychological outcomes.

Alcohol consumption is particularly relevant to these processes. Alcohol is inherently inflammatory, and disrupts microbial composition within the gut, contributing to compromised tight junctions. Thus, prolonged or chronic alcohol use heightens systemic immune activation and inflammation[37,316,322]. Cannabis, in contrast, has been associated with anti-inflammatory

properties, though effects vary depending on the cannabinoid profile and mode of administration[94,323]. Research suggests that whole-plant cannabis and concentrated extracts may differ in their physiological impact due to what is referred to as the "entourage effect". This idea centers around the well-established entourage effect, which refers to the interaction and synergy of the many cannabinoids that are naturally found in the cannabis plant, which produce differential effects compared to a single cannabinoid extract on its own[29,88,102]. Evidence suggests that cannabinoids can modulate cytokine activity. For example, activation of CB1 receptors by THC has shown to increase secretion of anti-inflammatory cytokine IL-10 and reduce pro-inflammatory cytokines[102]. Similarly, CBD-rich extracts have been shown to reduce levels of pro-inflammatory cytokines such as TNF-a and IL-1b while increasing IL-10, offering potential protective effects during dysregulated immune signaling[88].

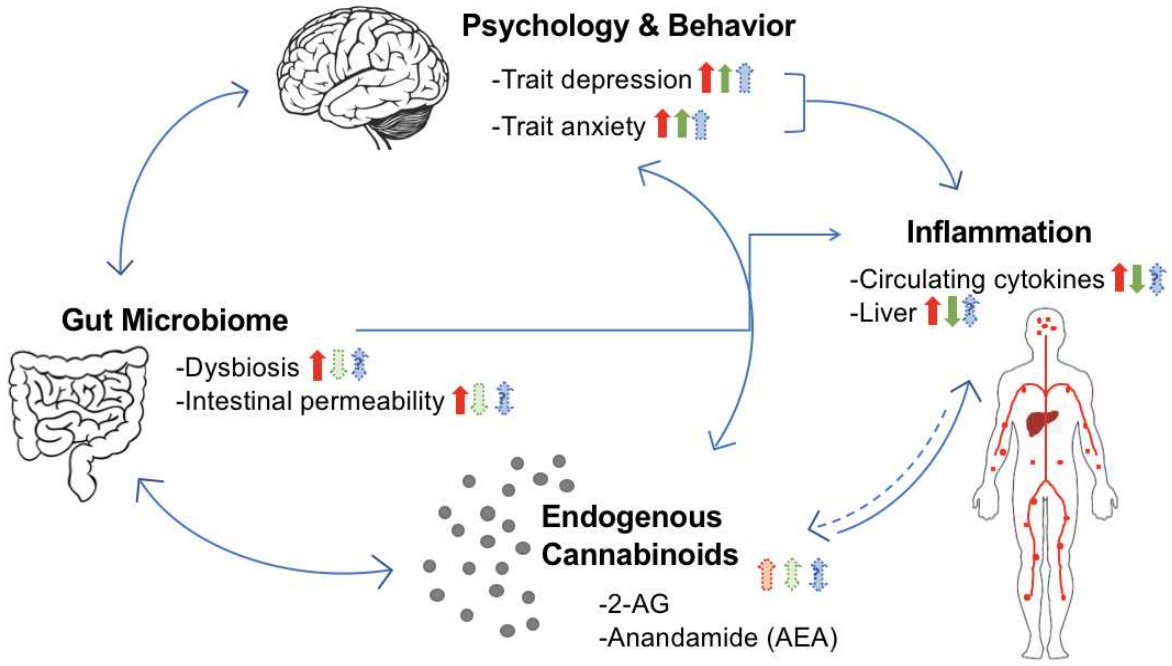

Figure 6.1. Interaction model of ECS-MGBA and immune system response based on current research

Red arrows represent alcohol, green arrows represent cannabis, and blue arrows represent alcohol & cannabis co-use.

Dark arrows indicate directional effects of respective substances on outcomes supported by existing literature. Light red, green and blue arrows indicate hypothesized effects that are not well-supported to date. Question marks in blue arrows indicate unexplored effects of co-use.

Solid blue lines indicate bidirectional interactions between systems supported by literature.

Together, these findings highlight the importance of exploring systemic inflammatory markers in relation to alcohol and cannabis use. Differences in cytokine and CRP levels across substance use patterns may clarify how these substances uniquely influence immune health and systemic inflammation. Considering the current data, we anticipate that individuals who consume alcohol only will show elevated levels of CRP and pro-inflammatory cytokines, reflecting immune system activation. By contrast, individuals who regularly use only cannabis are expected to display anti-inflammatory effects with lower levels of these circulating proteins. The combination of substance use in the co-use group is expected to cause levels of these biomarkers that lie somewhere between, higher levels of inflammatory biomarkers than the CO group but lower levels than the AO group.

6.2 Biomarkers of Interest

Table 6.1 summarizes the functions of four cytokines – interleukin-1 beta (IL-1β), interleukin-6 (IL-6), interleukin-10 (IL-10), and interleukin-22 (IL-22) – and CRP, highlighting their roles in systemic and neuroimmune signaling as well as the known effects of alcohol and cannabis use.

6.3 Aim-specific Methods

Researchers trained in phlebotomy collected a blood sample intravenously using glass EDTA tubes which were stored at 4°C until sample processing at the end of the study session. Samples were then spun at 1000 RCM for 10 minutes in order to separate plasma which was then stored at -80°C until assayed for desired protein concentration. Plasma concentration of circulating inflammatory markers was later assessed using ELISA kits.

Data analysis was conducted using ANCOVA and post-hoc pairwise comparison. Due to expected biological differences among human subjects, no outliers were removed.

Homoskedasticity and normality violations were corrected through square root data transformation for IL-10 and CRP.

Table 6.1 Inflammatory biomarkers of interest, their primary role in immune modulation, and effects of alcohol and cannabis use.

	Primary Role	Effect of Alcohol Use	Effect of Cannabis Use
IL-1β	Strong **pro-inflammatory** cytokine; initiates and sustains inflammation; influences neuro-inflammation and GABA signaling	Increases in the brain, especially amygdala; promotes binge-like drinking and alcohol dependence; linked to neuroinflammation and altered decision-making[324–327]	CBD suppresses IL-1β secretion in monocytes; THC modulates IL-1β bioactivity in macrophage-like cells[328]
IL-6	Generally **pro-inflammatory**, but context-dependent; central to acute-phase response (e.g. CRP)	Elevated in heavy and chronic drinkers; persists even after abstinence; contributes to systemic and liver inflammation[328–331]	CBD-rich extracts inhibit IL-6 expression; THC shows mixed effects (decreasing in some contexts, increasing in others)[332,333]
IL-10	Potent **anti-inflammatory** cytokine; inhibits IL-6 and TNF-α; maintains immune homeostasis, especially in the gut	Increased IL-10 production observed in alcohol dependence (possibly compensatory response to inflammation)[113,334]	Both THC and CBD enhance IL-10 release; THC upregulates IL-10 in endotoxemia, reducing systemic inflammation[88,94,102]
IL-22	Both **pro- and anti-inflammatory** properties; promotes epithelial repair, antimicrobial peptide production, and mucosal defense	Alcohol reduces IL-22 in gut and liver; supplementation restores barrier integrity, boosts beneficial bacteria (e.g. *Akkermansia*), and reduces endotoxemia[335,336]	THC may attenuate IL-22 production via CB2 signaling; possible implications for inflammatory bowel disease[337]
CRP	Acute-phase protein produced by liver in response to IL-6; general **marker of systemic inflammation**	Elevated in alcohol-related liver disease, including alcoholic steatohepatitis[338]	Limited direct evidence; cannabis may indirectly reduce CRP via IL-6 suppression[339,340]

6.4 Results

Plasma from a total of 161 participants was assayed for concentration of circulating inflammatory markers. Participants were sorted into substance use groups according to self-reported use patterns: AO (n = 40), CO (n = 29), co-use (n = 49), and control (n = 43). Summary demographic and substance use data for this outcome is summarized in Table 6.1 (sample size and data differs from previous chapters due to the number of processed plasma samples compared to number of processed fecal samples). The CO group was the least educated with 31% of its participants having earned a bachelor's degree or higher, while the AO group was the

most educated with 82.5% of members earning at least a bachelor's degree. Less than 50% of participants in the AO and control groups reported annual earnings under $50,000, while around 62% in the CO group and 59% in the co-use group reported an annual income of less than $50,000. The total sample was predominantly white and non-Hispanic.

Table 6.1. Participant demographics by substance use group. Significant group differences are indicated by subscript letters. Different letters indicate groups that are statistically distinct, whereas shared letters indicate no differences between groups.

	AO (n=40)	**CO (n=29)**	**Co-use (n=49)**	**Control (n=43)**	**Total (n=161)**
Demographics					
Age, mean years (SD)	32.0 (9.37)	31.9 (9.64)	30.9 (9.0)	34.2 (10.4)	32.3 (9.62)
Assigned female at birth, no. (%)	20 (50)	14 (48.3)	13 (26.5)	23 (53.5)	70 (43.5)
Bachelor's degree or higher, no. (%)	**33 (82.5)$_a$**	**9 (31)$_b$**	**24 (49)$_b$**	33 (76.7)$_{a,b}$	99 (61.5)
Income, <$50,000/yr, no. (%)	**18 (44)$_a$**	**18 (62.1)$_b$**	**29 (59.2)$_b$**	21 (48.8)$_{a,b}$	86 (53.4)
Race, white, no. (%)	**36 (90)$_a$**	25 (86)$_{a,b}$	43 (88)$_{a,b}$	**30 (70)$_b$**	134 (83.2)
14-day Daily Diary Reports					
% drinking days, mean (SD)	56.3 (27.4)	8.2 (11.3)	46.2 (29.7)	2.7 (10.1)	30.2 (32.2)
avg drinks/drinking day, mean (SD)	3.2 (1.8)	.10 (1.23)	3.5 (2.5)	.17 (.58)	2.1 (2.4)
% cannabis use days, mean (SD)	1.9 (5.13)	28.2 (30.3)	45.5 (33.8)	0 (0)	18.6 (29.7)
avg cannabis g/use day, mean (SD)	.01 (.03)	**1.46 (1.77)$_a$**	**.68 (.85)$_b$**	0 (0)	.47 (1.02)
avg exercise minutes/day, mean (SD)	36.3 (24.1)	36.8 (37.7)	35.7 (37.4)	41.9 (40.3)	37.8 (35.2)

Analysis of circulating inflammatory markers revealed significant group differences across cytokines (see figure 6.2).

There was a group effect in plasma concentrations of IL-1β ($F_{(3,150)} = 3.02$, $p = 0.03$, $\eta^2 = .057$). Pairwise comparisons showed the co-use group as having lower levels compared to both the AO ($t_{(82)} = 1.74$, $p = 0.04$, *Cohen's d* = .488) and CO ($t_{(69)} = 2.56$, $p = 0.007$, *Cohen's d* = .717) groups. A similar pattern was observed for IL-6, with a strong main effect for group ($F_{(3,154)} = 4.15$, $p = 0.007$, $\eta^2 = .075$). Post hoc tests revealed that the co-use group exhibited significantly lower IL-6 levels relative to the AO ($p = 0.005$), CO ($p = 0.005$), and control groups ($p = 0.01$).

By contrast, IL-10 demonstrated an opposite trend. A significant main effect was observed ($F_{(3,156)} = 3.49$, $p = 0.01$, $\eta^2 = .063$), and post hoc comparisons showed that the co-use

group had significantly higher IL-10 concentrations than the AO ($p < 0.001$), CO ($p = 0.001$), and control ($p = 0.01$) groups.

IL-22 also demonstrated a significant main group effect in an ANCOVA covarying for sex ($F_{(4,158)} = 2.92$, $p = 0.02$, $\eta^2 = .069$). Pairwise analyses indicated that the CO group exhibited significantly higher concentrations than the control group ($t_{(70)} = 2.35$, $p = 0.01$, *Cohen's d* = .659). The control group showed the lowest levels of IL-22 overall.

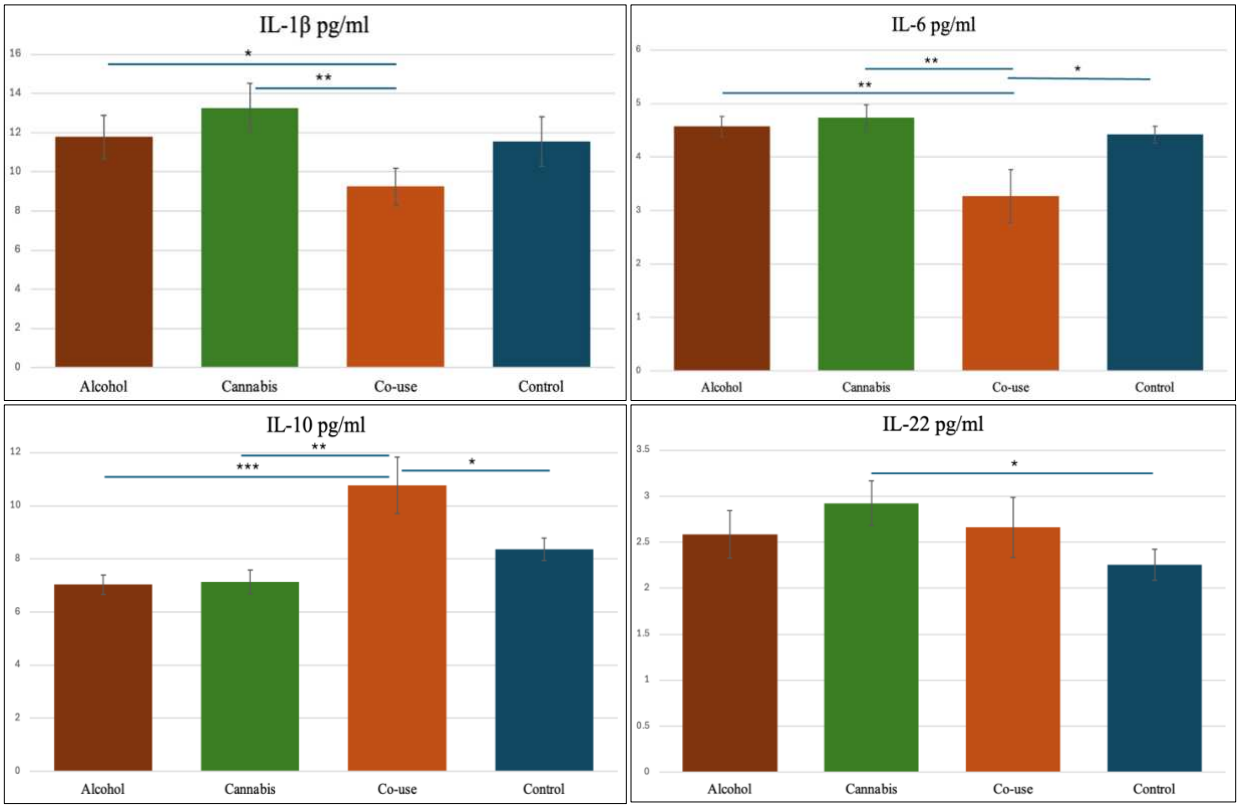

Figure 6.2 Plasma concentration of cytokines by substance use group;
Error bars = +/- 1 SE; * = p < 0.05, ** = p < 0.01

Finally, analyses of CRP revealed a group significant effect ($F_{(3,159)} = 3.77$, $p = 0.01$, $\eta^2 = .066$), with post hoc comparisons showing the co-use group to have significantly higher levels than the AO ($p = 0.036$), CO ($p = 0.017$), and control ($p = 0.02$) groups (see Figure 6.2).

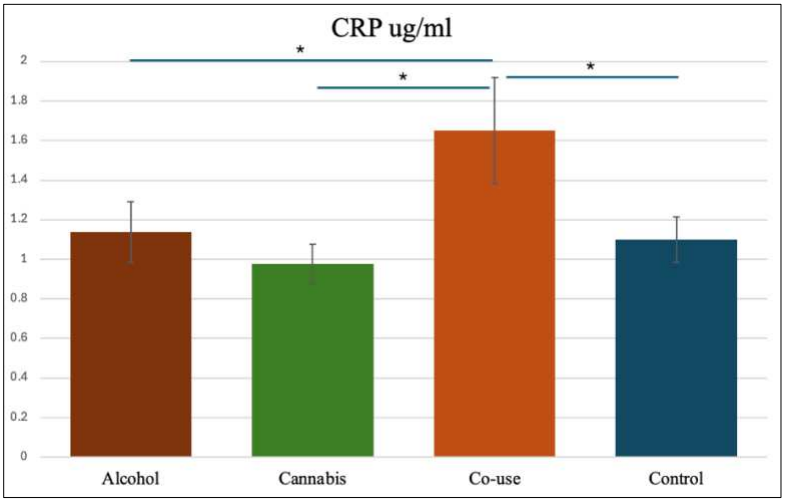

Figure 6.2 Plasma concentration of CRP by substance use group;
Error bars = +/- 1 SE; * = $p < 0.05$, ** = $p < 0.01$

6.5 Discussion

While patterns seen in the control group's levels of pro-inflammatory IL-1β and IL-6 were generally consistent with our expectations by trending lower than the AO and CO groups, the co-use group proved puzzling by displaying the lowest levels overall. Similarly, levels of IL-10, an anti-inflammatory cytokine, trended higher in the control group than the AO and CO groups while again, the co-use group displayed the highest levels overall. There were no significant differences between CO and AO groups in any of these measures. Differences in IL-22 however reveal the CO group to have significantly higher circulating levels than the control group. Finally, CRP levels were higher in the co-use group than each other group and lowest overall in the CO group (although the CO group was not significantly different from the AO and control groups).

Acute vs. Chronic Immune Activation

The co-use group exhibited lower circulating levels of IL-1β and IL-6, alongside higher IL-10 relative to other groups. While this pattern was unexpected in terms of alcohol use, the lower inflammatory cytokine levels are consistent with existing literature regarding cannabinoid-

mediated immunoregulation[99,341]. Research in preclinical models finds that THC and CBD can suppress pro-inflammatory cytokines including IL-1β and IL-6, while upregulating IL-10 via cannabinoid receptor signaling and downstream transcriptional control[88,323]. However, this does not account for the alcohol consumption in the co-use group or the disparity between the co-use and AO group levels. This also causes further confusion surrounding IL-6 and IL-1β specifically in the CO group, where concentrations were elevated compared to the other groups, opposite to what was expected.

One explanation for these unexpected findings may be that session procedures specific to the co-use group (but not the other 3 groups) required a 24-hour period of abstinence from alcohol. It is established that the effects of acute stressors such as alcohol on cytokine expression are dynamic, with notable fluctuations over a 6-24-hour window of time[342–344]. By implementing a 24-hour period of abstinence, it is possible that acute pro-inflammatory signals would be blunted at the time of sample collection even with regular or chronic consumption[337]. Acute alcohol use can also temporarily suppress immune responses in certain contexts, further complicating the interpretation of cytokine levels when the timing from each participant's last drink to sample to collection varies. It is possible that the period of abstinence within the co-use group may have diminished the expression of acute inflammatory markers we would expect to see from alcohol use at the time of sample collection, perhaps even allowing the effects of cannabis to dominate[88,94,343]. While co-use participants were abstinent from both alcohol and cannabis for 24 hours, research suggests that the anti-inflammatory effects of regular cannabis use can persist over the course of days or weeks[89,90].

The lack of differences observed between the AO, CO and control groups was also unexpected, although it may be explained in part by the non-clinical nature of the sample.

Individuals screened into this study could be considered fairly healthy given the eligibility criteria. Results may also be influenced by the modest group sizes in which there is not enough statistical power to detect subtle differences. Additionally, there are numerous factors that influence immune signaling which were not captured or accounted for in this observational design, including environmental exposures, sleep, chronic and acute stress, trauma history, among many others[345–348]. Inpatient studies are far more capable of tightly controlling for such variables that can influence inflammatory signaling.

The co-use group had the highest levels of CRP, a hepatic acute-phase protein that is notably driven largely by the presence of IL-6[349]. We hypothesize that the differing results for IL-6 and CRP may be due to the fact that the liver processes and integrates inflammatory activity over a longer period of time and thus, CRP can remain heightened as a result of inflammatory triggers (e.g. repeated alcohol exposure, microbial translocation from intestinal permeability) from days prior rather than just minutes or hours. Circulating IL-6 on the other hand can change on a much shorter timescale. This can be true even when IL-6 is low at the time of sampling[350,351]. Heavy alcohol use is commonly linked to higher CRP, consistent with the co-use group's high CRP levels. Observational data for cannabis alone are mixed, but the anti-inflammatory tendencies of cannabinoids that are supported by current research would not necessarily neutralize CRP levels elevated by chronic alcohol-related inflammation of the gut or liver[352,353].

Although pairwise comparisons for CRP weren't significant for AO vs. control, the overall pattern of elevated CRP in the co-use group aligns with extensive evidence that chronic alcohol use promotes gut permeability and endotoxemia, and downstream IL-6-driven acute-phase signaling, upholding the gut-liver axis that is strongly implicated in alcoholic liver injury

and systemic inflammation[351,354]. As AO cytokine levels were not consistently and distinctly elevated as expected, it is important to consider that timing effects (recent intake vs. abstinence) and daily variations in drinking patterns can conceal the detection of differences despite higher chronic inflammatory patterns that may be captured with CRP[355].

The CO group showed higher levels of IL-22 than controls, while not exhibiting the elevated CRP seen with alcohol use. IL-22 is a context-dependent cytokine that promotes epithelial repair, upregulates antimicrobial peptides, and strengthens barrier integrity within the gut[356–358] (see Table 6.1). When exogenous IL-22 is supplemented, it mitigates ethanol-induced gut and liver injury by re-establishing antimicrobial defenses and intestinal barrier function[359]. Conversely, lower endogenous IL-22 levels in the gut are linked to worse alcohol-related mucosal repair and defense[359].

Elevated IL-22 in CO participants could indicate adaptive mucosal repair and maintenance in response to a number of variables including lifestyle, diet, or microbiome traits displayed by this group. Cannabinoid signaling thus may modulate or mediate immune pathways that affect the expression of IL-22, particularly by epithelial cells. Considering the high concentration of cannabinoid receptors present in the gut, this cannabinoid-modulated epithelial mechanism is plausible, offering barrier support by way of IL-22. Comparatively, control participants displayed the lowest levels of IL-22, which is congruous with a profile of less epithelial stress and repair demand over time and overall lower systemic inflammation.

These prospective cannabinoid influences on epithelial repair may represent one pathway through which cannabis use contributes to broader anti-inflammatory outcomes. For instance, lower levels of systemic inflammation (i.e. CRP) in the CO group compared with individuals

who consume alcohol is consistent with cannabinoid anti-inflammatory effects reported in humans and animal models[88,94,102,337,351].

Altogether, the patterns of cytokine levels seen in the co-use group is compatible with the concept of ECS-linked immunoregulation, with CRP remaining elevated with alcohol exposure. In the CO group, higher IL-22 with relatively lower CRP may reflect barrier-supportive mechanisms rather than systemic pro-inflammatory response, though causal direction cannot be determined by the present data alone. These patterns suggest that cannabinoids may modulate acute inflammatory signaling without fully counteracting chronic alcohol-related inflammation, especially when it is mediated by the microbiome and liver.

Limitations and Other Considerations

Findings from the prior chapter's look into microbiome composition fit with the inflammatory patterns described here. Specifically, enrichment of Proteobacteria in alcohol-consuming groups and elevated CRP fit a framework that is detailed and consistent in the present literature concerning the deleterious effects of chronic alcohol consumption[35].

Once again, we must consider method of cannabis use, especially in the context of inflammation. Research shows that despite well-documented anti-inflammatory effects of cannabis, the smoke itself shares many compounds with that of cigarettes known for their adverse health effects from carcinogens, irritants and other toxins[304,305]. Smoking in general causes damage to the vascular system, triggering an immune response resulting in elevated levels of inflammatory markers, including CRP[360]. Research on oral administration of cannabis extracts, while not as prevalent, show equal if not greater protective effects against inflammation and permeability[7,87,91,92,361,362]. Considering that smoking is the most common method of cannabis use[304], it is possible that the inflammatory profile of the CO group is reflecting these

consequences to a larger degree than the protective anti-inflammatory effects typically observed with cannabis use. This would be consistent in the co-use group's results as well, as lower inflammatory markers might be illustrative of acute abstinence effects from smoking along with drinking.

Socioeconomic differences between groups could have a considerable impact on the inflammatory patterns seen in these data. The AO group had the highest education and income, whereas the CO and co-use groups had more participants earning less than $50,000 per year. Prior research demonstrates negative correlations between socioeconomic status and IL-6/CRP even after health behaviors are accounted for[363]. These differences have the potential to confound statistical analyses or mediate between-group differences and should be carefully considered when interpreting results[363]. Future investigations should seek to account for SES and education for more accurate group comparison.

There is concern within the field about the wide variability in findings regarding the effects of alcohol on peripheral cytokine levels and current researchers are pushing for standardization in order to mitigate these issues[341]. Some of the reasons for these inconsistencies include differences in sample collection, processing and storage protocols, bias in sample selection, as well as the selection of biomarkers and associated assay methods or quality assays currently available[341]. Of course, individual differences must also be considered, along with sensitivity to and metabolism of alcohol. As noted in a large-scale systematic review, there is abundant evidence supporting distinct cytokine profiles in AUD, and cytokine activity varies further within the different stages of AUD as well[364]. It is possible that among the relatively healthy AO and co-use groups, individual variability in cytokine expression obscures group-level differences that might otherwise emerge in larger or less healthy samples. Likewise, other health

behaviors such as sleep may significantly impact inflammatory profiles and should be measured to fully understand the effects of substance use.

Chapter 7. Exploratory Analyses, Comprehensive Discussion & Future Directions

7.1 Exploratory Analysis

Correlations across outcomes

We ran exploratory correlation analyses on several variables across psychological and biological measures to further investigate potentially important associations that may influence the results reported in the previous chapters. We used non-parametric Spearman's Rho correlations first with psychological outcomes and inflammatory markers, then with individual taxa abundance, along with daily cannabis and alcohol use. Figures 7.1 and 7.2 summarize these correlations. In the context of this project, while these correlations do not speak to causality and are not corrected for multiple testing due to the exploratory nature of this analysis, we can speculate on some of these significant associations and what further investigation of these variables might illuminate.

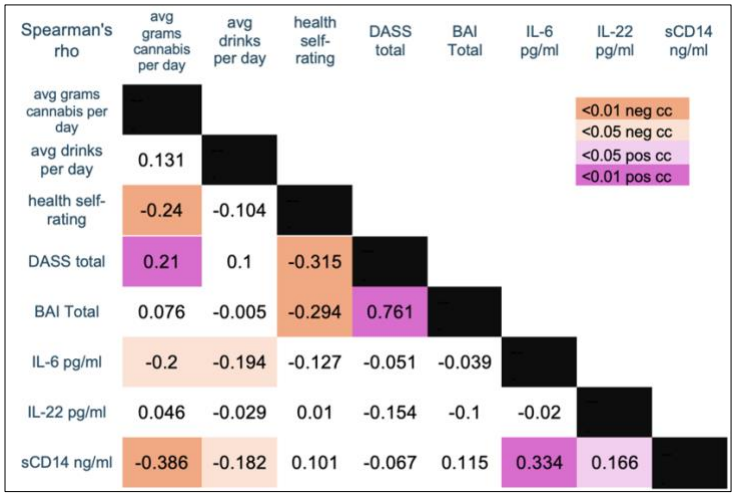

Figure 7.1 Spearman's Rho correlation coefficients for inflammation markers (see key for indication of significance)

Amount of daily cannabis use was positively associated with higher rates of depression and negatively associated with subjective health rating. Given the results from chapter 3, this is not surprising. However, increased daily cannabis use correlated with decreased levels of

circulating IL-6 and sCD-14, which is beneficial to physical health. Unexpectedly average number of standard drinks per day was also negatively correlated with IL-6 and sCD-14.

Figure 7.2 highlights additional correlations between personality or mental health and various taxa abundance that emerged. Agreeableness was found to be inversely correlated with the Clostridia class (belonging to the Firmicutes phylum) and subsequently, the Epulopiscium genus, which was also positively associated with sensation seeking. Openness was inversely correlated with the phylum Proteobacteria and positively correlated with the genus Streptococcus.

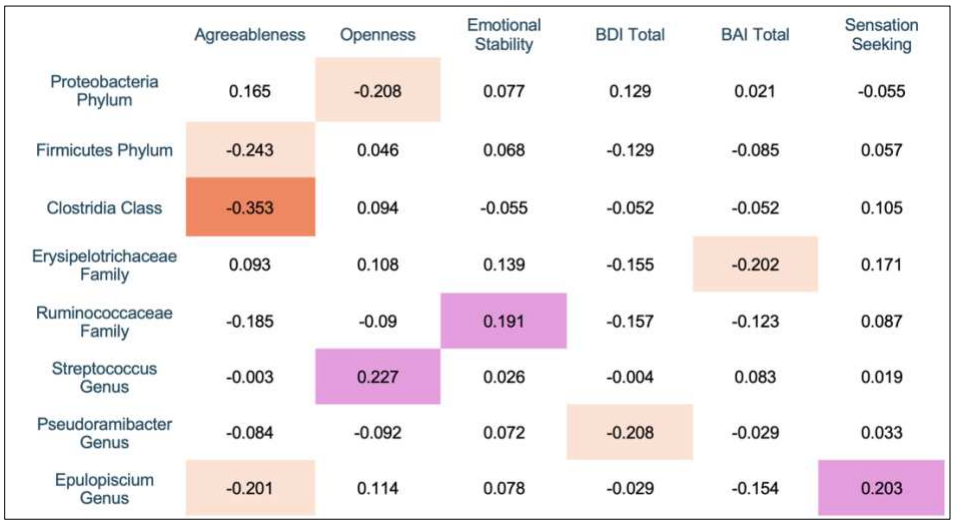

Figure 7.2 Spearman's Rho correlation coefficients (cc) for various taxa; light orange indicates significant negative correlations at the $p < 0.05$ level, dark orange indicates significant negative correlations at the $p < 0.001$ level, and purple indicates significant positive correlations at the $p < 0.05$ level.

Grams of cannabis consumed per day was also positively correlated with abundance of the genera Eubacterium ($\rho_{(rho)} = 0.202$, $p = 0.037$) and SMB53 ($\rho_{(rho)} = 0.222$, $p = 0.021$). Balanced abundance of Eubacterium has shown to bolster mucosal lining of the gut, reduce inflammation due to its production of butyrate, and aid in metabolic health[275,365]. Further research should explore whether cannabis or specific cannabinoids can help support healthy levels of Eubacterium in the gut. The SMB53 genus on the other hand is not well-researched but

has shown to be enriched with obesity and inflammation[252]. This association calls further into question the complex influence that cannabis may have on various gut microbiota.

Subjective heath rating was positively associated with abundance of Catenibacterium ($\rho_{(rho)} = 0.201$, $p = 0.038$) and negatively associated with Bacillus Cereus species abundance ($\rho_{(rho)} = -0.204$, $p = 0.035$). B. Cereus is an invasive common culprit of food poisoning[366], so it is not surprising that it correlates with poorer health ratings. Catenibacterium is a phylogenetically diverse genus and thus plays a variable role in the gut microbiome, with context-dependent factors as to whether its contribution is good or bad for gut health[367]. Imbalance and overgrowth of certain species and strains have been linked to disease, while other studies suggest healthy or even abundant levels can be protective against obesity and liver damage[367,368]. The correlation seen in these data may suggest a positive role for Catenibacterium in gut health.

Co-use Subgroups – Parsing Variability

Among all groups considered in the present work, outcomes from the co-use group were the most unexpected and highly variable. One notable observation is that substance use patterns varied widely within this group; although participants met heavy co-use criteria at screening, reported alcohol and/or cannabis use during the daily diary period was lower than initially reported for several individuals. To explore whether amount and ratio of alcohol and cannabis had any bearing on biological outcomes measured, we created 4 co-use subgroups based on substance use habits as reported in the 14-day Daily Diary (see figure 7.3). The subgroups included heavy co-use of cannabis and alcohol (individuals whose actual reported use during the daily diary period met the study's criteria of heavy and regular use, n=17), alcohol-heavy co-use (>2 drinks/day and <.2g cannabis/day, n=8), cannabis-heavy co-use (<1 drink/day and daily cannabis use of at least .2g/day, n=22), and light/moderate co-use (individuals who used a lesser

amount of each substance than noted above sporadically throughout the 14 days reported, *n*=12). We looked at various measures to see if these subgroups differed in their outcomes. We used ANOVA for some analyses, however subgroup sizes were unequal and variance between subgroups differed in some measures, in which case Kruskal-Wallis with pairwise analyses were used where appropriate. Interestingly, significant differences did emerge, though the focus of these exploratory analyses was to identify illuminating patterns within the co-use group rather than find statistical significance.

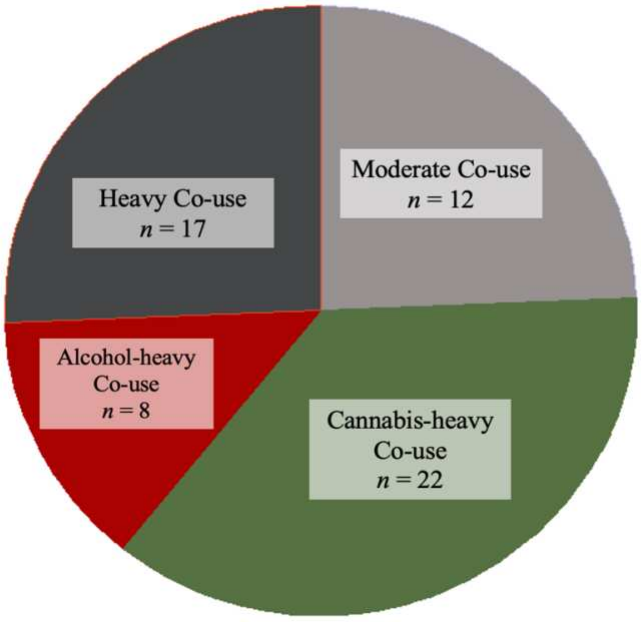

Figure 7.3 Co-use subgroup breakdown

AUDIT scores differed significantly by subgroup ($H_{(3,61)} = 14.28$, $p = 0.003$, $\varepsilon^2 = .188$), with the alcohol-heavy co-use group displaying higher scores than the cannabis-heavy ($p = 0.03$) and moderate co-use ($p < 0.001$) groups, and the heavy co-use group showing higher scores than the moderate co-use group ($p = 0.004$; figure 7.4). Although all participants in these subgroups regularly consume both cannabis and alcohol, no differences were seen in MDS scores.

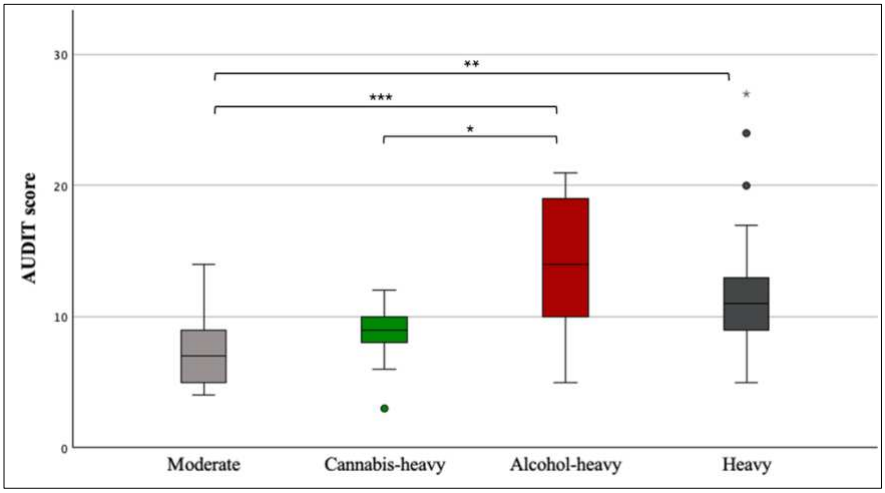

Figure 7.4 AUDIT scores by subgroup; colored dots and stars represent outlying data.
Horizontal brackets with * = $p < 0.05$, ** = $p < 0.01$, *** = $p < .001$

An ANOVA revealed differences in Openness ($F_{(3,69)} = 2.97$, p = 0.03, $\eta^2 = .114$) with the moderate co-use group scoring higher than the cannabis-heavy co-use group ($p = .02$), and the heavy co-use group scoring higher than the alcohol-heavy co-use group ($p = .01$). In the DASS Stress subscale, KW analyses were not significant, however, non-parametric pairwise comparisons showed the alcohol-heavy group as scoring higher than the heavy co-use ($p = .01$) and cannabis-heavy ($p = .03$) groups (see figure 7.5). This finding is interesting as no group differences were found between the original substance use groups.

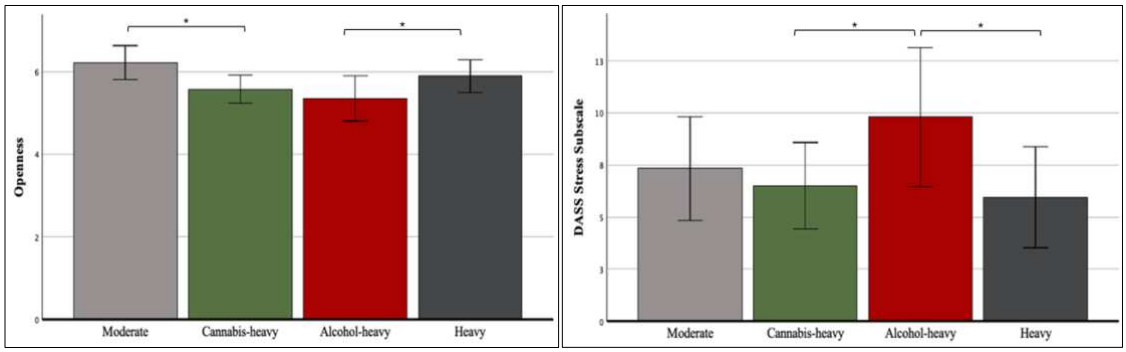

Figure 7.5 Openness and DASS Stress mean scores by subgroup;
Error bars = +/- 1 SE; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < .001$

IL-22 levels followed similar patterns seen in the original groups, although KW group analyses were not significant. Pairwise comparisons showed the cannabis-heavy co-use group to have higher plasma concentration of IL-22 than the alcohol-heavy co-use group ($p = .02$). The

KW test for IL-6 was significant ($H_{(3,58)} = 12.11$, p = 0.007, $\varepsilon^2 = .157$) with the moderate co-use group exhibiting lower levels than the cannabis-heavy ($p = .04$) group, and the heavy co-use group showing lower levels than the alcohol-heavy ($p = .01$) and cannabis-heavy ($p = .003$) co-use groups, although the heavy co-use group displays large within-group variance (see figure 7.6).

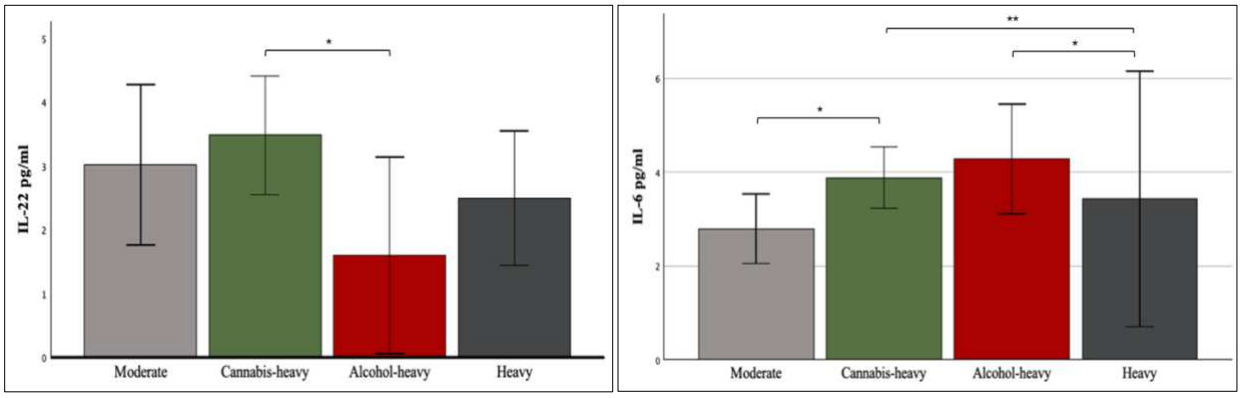

Figure 7.6 IL-22 and IL-6 concentration levels by subgroup;
Error bars = +/- 1 SE; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < .001$

A KW test for Fisher's Alpha (relative abundance) was significant ($H_{(3,37)} = 8.44$, p = 0.03 $\varepsilon^2 = .147$) revealing the highest values in the alcohol-heavy group compared to the heavy co-use ($p = 0.02$) and moderate co-use ($p = 0.006$) groups, and trending higher than the cannabis-heavy co-use group ($p = 0.06$; see figure 7.7).

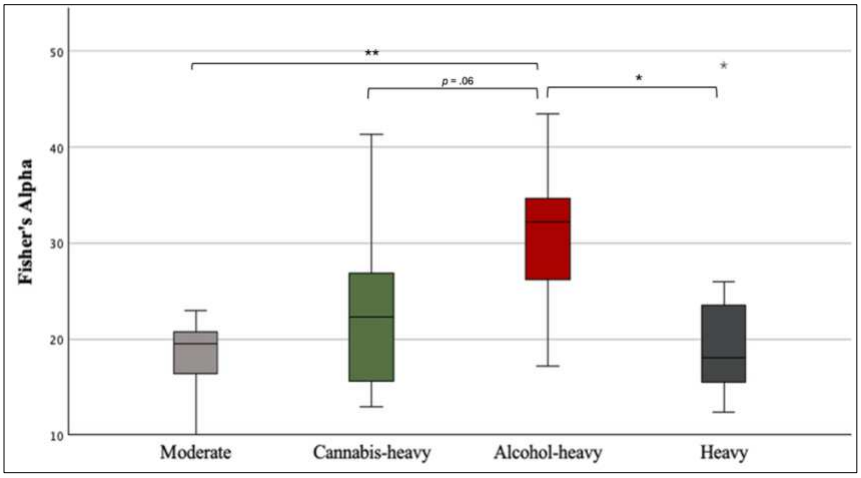

Figure 7.7 Differences in Fisher's Alpha value by co-use subgroup;
Horizontal brackets with * = $p < 0.05$, ** = $p < 0.01$, *** = $p < .001$

Finally, KW tests were performed on total rarefied counts of several taxa. While none were significant, pairwise differences emerged that followed the patterns of the original groups. First, TRC of Proteobacteria was higher in the alcohol-heavy group than both the heavy (p = 0.04) and moderate (p = 0.03) co-use groups, and trending toward significantly higher TRC than the cannabis-heavy group (p = 0.06). The alcohol-heavy group also showed higher TRC of opportunistic bacteria than the moderate co-use group (p = 0.03) and trended higher than the heavy (p = 0.05) and cannabis-heavy (p = 0.06) co-use groups. The higher Fisher's alpha values could be reflecting some of these differences, further supporting the analysis of both relative abundance and absolute abundance or TRC data in microbiome research.

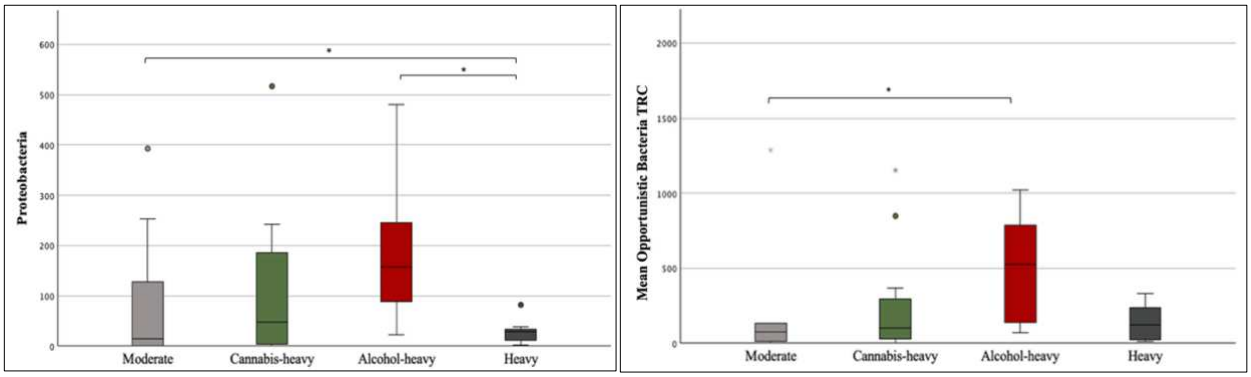

Figure 7.8 Differences in Proteobacteria and other harmful bacteria by co-use subgroup; colored dots and stars represent outlying data. Horizontal brackets with * = $p < 0.05$, ** = $p < 0.01$, *** = $p < .001$

These exploratory analyses follow similar patterns observed across the primary substance use groups in this study. Cannabis-heavy co-use demonstrated higher IL-22 levels and a lower abundance of opportunistic taxa associated with inflammation and dysbiosis, including Proteobacteria, supporting the theme of a more adaptive inflammatory and microbial profile. In contrast, alcohol-heavy co-use exhibited elevated IL-6 and greater TRC of bacteria associated with inflammation, consistent with alcohol's known disruptive effects on gut and immune homeostasis. Together, these trends provide a preliminary framework for understanding how

cannabis and alcohol may differentially influence inflammatory tone and microbiome health, establishing a foundation for more refined and targeted future research. Additionally, the exploratory correlations discussed highlight several possible associations that can help guide investigation in larger, hypothesis-driven studies.

7.2 Key Findings

Summary of Primary Outcomes

In the psychological domain (aim 1), we found notable group differences in mental health, personality, and subjective health rating. The co-use group exhibited greater impulsivity, openness, and depression than the AO and control groups, while the AO group scored the highest in sensation seeking. Overall, the CO group displayed the poorest mental health, with higher scores in depression and anxiety, and worse perceived health, consistent with self-medication patterns reported by several participants. Exploratory analyses revealed that the alcohol-heavy co-use group was less open and more stressed than heavy and cannabis-heavy co-use groups.

In microbiome analyses (aim 3), alpha diversity indices showed limited differences in richness, but measures of dominance and evenness signaled group-level divergence in microbial communities. Differential abundance analyses (via ANCOM) revealed significant family-level shifts in several key players inflammation regulation. Groups varied in absolute abundance of certain taxa, specifically the CO and control groups were more abundant in taxa known to be beneficial whereas the AO group tended to be more abundant in harmful and opportunistic taxa overall. The co-use group exhibited mixed results in individual taxa abundance.

Inflammatory biomarkers (aim 4) showed complex, and sometimes counterintuitive patterns. The CO group exhibited elevated IL-22, known to support gut health and repair. The co-use group exhibited higher levels of CRP but lower IL-1β and IL-6, possibly influenced by

acute abstinence effects. Exploratory correlations linked daily cannabis dose to lower levels of IL-6 and sCD14, along with higher abundance of beneficial taxa such as Eubacterium. Further associations with personality emerged including negative correlations between agreeableness and Clostridia, and openness and Proteobacteria.

Cannabis-mediated Gut Protection

A key mechanistic thread that has emerged in the prior chapters is the observation that the CO group had elevated IL-22 alongside enrichment of Bifidobacterium. IL-22 is known to promote epithelial regeneration, support mucosal defenses, and strengthen gut barrier integrity, allowing less translocation of bacteria into systemic circulation[356,357,369,370]. Elevated IL-22 in the CO group suggests that cannabis use may either stimulate mucosal repair mechanisms or mitigate low-level epithelial stress[337,369,371]. This, in turn, could sustain beneficial taxa like Bifidobacteria, and help to maintain a more resilient and balanced gut microbiome. The same may not be true for cannabis use when alcohol is introduced concurrently. This protective hypothesis is further supported by the observation that cannabis also appears to suppress harmful bacterial domains (e.g. Proteobacteria) and enhance other beneficial ones. At the same time, the reduction of abundance in certain beneficial classes (e.g. Bacteroidia) in the CO group advises that the effects are not uniformly beneficial and may vary by health status, dose or a number of other influences.

The Complexity of Co-use

The co-use group showed unexpected mixed patterns across outcomes. Briefly, overlap with the AO and CO groups was seen in microbial composition, and alpha diversity of the co-use group suggests microbial profiles that are higher in evenness and richness. This group also displayed elevated levels of CRP, contrasted by lower levels of pro-inflammatory cytokines. All

of this suggests that co-use may not simply be additive, but rather a unique state in which the potential protective influences of cannabis may be partially offset by the harmful effects of alcohol. It could also be the case that the interaction of the two substances influences which taxa or immune responses dominate. It is possible and even likely (given co-use subgroup differences) that timing, dosage, or sequence and frequency of cannabis versus alcohol exposure is more consequential, and that periods of abstinence (required of this group) can dampen acute cytokine expression[342–344].

Given the present data, definitive physiological effects of alcohol and cannabis co-use remain inconclusive. In the present sample, variability and inconsistency of substance use within this group (compared to exclusive consumers of either alcohol or cannabis) as well as methodological differences in data collection protocols between the co-use and other may further contribute to these mixed findings.

Common Themes

Across the aims of these chapters and exploratory analyses, a consistent theme emerges: alcohol and cannabis use differentially shape the gut microbiome and immune signaling in ways that may reasonably intersect with mental health and personality traits. While the results from this project cannot provide definitive causality, the converging evidence herein supports a nuanced model in which cannabis may exert partially protective or regulatory effects across the MGBA, perhaps by way of ECS activation, whereas alcohol tends toward dysbiosis and inflammation.

Altogether, these findings support an integrative model in which substance use, through its modulation of the ECS, gut microbiome, intestinal barrier integrity and immune signaling, may influence mental and physical health outcomes. Cannabis use may provide some protection

against microbiome disruption via ECS-mediated epithelial and immunomodulatory mechanisms, whereas alcohol appears to promote dysbiosis, microbial translocation, and systemic inflammation, contributing to the bidirectional gut-brain feedback system.

7.3 Overall Limitations

Several important limitations should be considered when interpreting these findings. First, the observational and cross-sectional design does not allow for causal inference. We cannot determine whether substance use directly drives the observed microbiome and immune differences, or whether preexisting microbiome or immune traits make individuals susceptible to certain patterns of substance use. Moreover, this study sample was non-clinical with participants who were relatively healthy. Effects of alcohol and cannabis use may be more pronounced in individuals with heavier use patterns or diagnosed substance use disorders (SUDs).

The 24-hour abstinence period required for the co-use group presents another limitation, as it may have altered acute expression of biomarkers of interest, particularly cytokines, thereby diminishing substance use-related increases. Similarly, methodological inconsistencies in blood and fecal sample collection and handling may have influenced results. Several samples were unable to be processed, and delays in freezing or uncertain handling conditions were more likely in the co-use group simply due to the nature of the study. These differences could potentially lead to compositional bias through microbial drift or differential survival of taxa[372].

Confounding demographic differences between groups also call for consideration. Variations in sex, race, income, and education, all known to influence both microbiome composition and immune function, introduce the potential for bias. We applied statistical adjustment to account for these differences, however, disparities in income and education across groups may have far-reaching consequences that we were unable to adequately measure or

account for in the present study. Equal variance between group demographics remains a critical consideration in studies addressing both overall health outcomes.

Additionally, there was substantial within-group heterogeneity in cannabis and alcohol co-use, including dose, frequency, and mode of administration (e.g., smoking vs. edibles), which is likely to impact outcomes and reduce the clarity of comparisons between groups. Power limitations present further constraints on interpretation. Larger sample sizes and longitudinal designs are needed to confirm these findings and better capture within-subject variability over time.

Design-related inconsistencies also represent a notable concern. Differences in timing of sample collection to processing and storage may have influenced protein concentrations detected in plasma. The co-use group, in particular, displayed unexpectedly lower biological measures compared to other groups. It is possible that this discrepancy reflects procedural variation between the F31 study and the sister K23 that supplied co-use data, as the latter required a more intensive, multi-week participation process involving mobile laboratory sessions lasting several hours. Although eligibility criteria were matched across studies, the fact that the K23 study involved a more in-depth protocol may mean that it attracted a different type of participant compared to the briefer observational F31 study. Future work should aim to collect samples from all groups within a single study protocol, using identical recruitment criteria, collection procedures, and processing protocols to ensure methodological consistency.

Finally, these data were derived from relatively healthy individuals, as participants were screened to exclude those with serious health complications, recent illnesses or medical procedures, immune disorders, and individuals seeking treatment for SUD. Given this, it is unsurprising that group differences in biological and psychological outcomes were generally

small. Collectively, these methodological and sample-related factors should guide the conceptual design of future studies to more accurately capture the biological effects of substance use on the microbiota-gut-immune axis.

7.4 Implications & Future Directions

Despite its limitations, this integrated analysis provides several important and clinically relevant insights into the relationship between substance use, the gut microbiome, and immune regulation. First, the findings point toward substance-specific microbiome signatures. Exclusive cannabis use may promote a microbial environment that is more resilient to dysbiosis, whereas exclusive alcohol use appears to shift the gut's ecosystem toward support of more harmful microbiota and greater inflammatory signaling. This group divergence emphasizes the differential physiological effects of alcohol and cannabis on gut health, with alcohol largely associated with microbial imbalance and inflammation, and cannabis exerting potential protective or modulatory effects on gut homeostasis.

Next, the concurrent elevation of IL-22 and enrichment of beneficial taxa in the CO group suggests a functional MGB-immune axis mechanism. IL-22's key role in epithelial barrier repair and mucosal defense, and its association with a more balanced microbiome[373] presents a clear cascade through which a healthy microbiome enhances epithelial resilience and further modulates systemic immune function. This relationship may present an efficacious intervention target for mitigating subsequent neuro-inflammation, which is particularly relevant for substance use and comorbid psychiatric disorders. Such insights may inform new therapeutic approaches involving microbiome-targeted interventions or treatments that incorporate cannabinoids aimed at restoring gut and immune balance.

Lastly, the co-use group represents a particularly interesting and clinically relevant population. The microbiome and immune profiles of this group did not align cleanly with either the AO or CO patterns. The potentially protective properties of cannabis may only partially counterbalance alcohol's deleterious effects, leaving co-users susceptible to ongoing subtle dysbiosis and low-grade inflammation. Clinically, this highlights the need for tailored interventions focused on enhancing microbiome resilience and barrier integrity, reducing inflammatory responses, and considering the timing and context of substance use behaviors.

Future research should build upon these findings through longitudinal and mechanistic approaches. Longitudinal studies following participants before, during, and after periods of substance use would allow for identification of causal direction and temporal sequencing of microbiome and immune changes. More rigorous methodological approaches are also needed to control for inevitable confounders such as diet, sleep, medication use, body composition, and socioeconomic status. Mode of cannabis use (e.g. vaping vs. smoking vs. oral administration) and precise quantification of doses and timing of use could help elucidate dose-response relationships as well.

Longitudinal human studies should be performed in which substance use patterns are tracked over a longer period of time, alongside the collection of multiple biological sampling points across varying contexts (e.g. significant life events, illness, travel, environmental allergies, variable physical activity, seasonal change, or stress) to assess how these factors influence the gut microbiome. Finally, extending this work to clinical populations, specifically individuals with active substance use disorders, will be critical to determine whether the observed microbial and immune alterations represent adaptive or maladaptive responses, and whether they can be targeted for therapeutic intervention.

These directions highlight the potential of integrating microbiome research into the study and treatment of substance use. Understanding the nuanced, bidirectional effects of cannabis and alcohol on gut and immune function could pave the way for personalized, microbiome-informed strategies to support both physical and mental health in individuals with varying substance use patterns.

7.5 Conclusion

In conclusion, the findings from this project suggest that alcohol and cannabis use have distinct and perhaps interactive influences across psychological, microbial, and immunologic domains, converging in a model in which these substances modulate an MGB-immune axis in ways that oppose each other. Exclusive cannabis use was associated with biological patterns indicative of greater microbial resilience and mucosal support, including elevated IL-22 and enrichment of commensal taxa such as Bifidobacterium. In contrast, exclusive alcohol use corresponded with dysbiotic shifts in the microbiome and inflammatory signatures consistent with barrier disruption and immune activation.

Meanwhile, the co-use group demonstrated a unique and complex profile rather than an additive blend of exclusive cannabis and alcohol use. Elevated CRP accompanied by reduced IL-6 and IL-1β may reflect a compensatory or blunted inflammatory state, potentially formed by the competing physiological effects of both substances. Variability within this group – driven by timing, dose, and frequency of alcohol and cannabis use – likely obscures and complicates clear group-level effects, emphasizing the consideration of alcohol and cannabis co-use as a distinct pattern of substance involvement that warrants further focused investigation.

Across aims, a consistent mechanistic theme emerges: the ECS likely mediates many of these bidirectional effects. Through its regulation of gut barrier integrity, microbial balance, and

immunologic tone, ECS activation by cannabinoids may partially counteract alcohol-induced inflammation and dysbiosis. Conversely, chronic alcohol exposure appears to disrupt these same regulatory pathways, promoting permeability and systemic inflammatory signaling that can feed back into psychological health. These converging biological and behavioral findings support a model in which substance use behavior, gut microbial composition, and immune signaling are dynamically interwoven, with mental health both shaping and being shaped by this biological network.

In this context, the results have broader implications for prevention and intervention for substance use disorders and comorbid mental health conditions by way of personalized medical treatment. Individuals may benefit from a microbiome-informed treatment plan that recognizes the gut and immune system as active mediators of substance effects on mood, emotion regulation, cognition, and motivation. Future ECS-based or microbiome-targeted interventions could hold potential for mitigating alcohol-related gut and immune dysfunction, restoring microbial balance, and ultimately improving psychological outcomes.

Altogether, this work contributes to a growing body of evidence that the MGB-immune axis (as seen in figure 6.1) is a critical mediator of substance-related health outcomes. Alcohol and cannabis engage overlapping yet divergent biological pathways whose interactions are not simply additive but dynamically intertwined. Recognizing these distinctions, particularly the role of cannabis within this system, offers a more nuanced framework for understanding the physiological and psychological consequences of substance use, pointing toward new and integrative targets for research and intervention.

References

1. SAMHSA. *National Survey on Drug Use and Health.* https://www.samhsa.gov/data/data-we-collect/nsduh-national-survey-drug-use-and-health (2024).
2. Subbaraman, M. S. Substitution and Complementarity of Alcohol and Cannabis: A Review of the Literature. *Subst Use Misuse* **51**, 1399–1414 (2016).
3. Bishehsari, F. *et al.* Alcohol and Gut-Derived Inflammation. *Alcohol Res* **38**, 163–171 (2017).
4. Patel, S. *et al.* Alcohol and the Intestine. *Biomolecules* **5**, 2573–2588 (2015).
5. Graczyk, M., Lewandowska, A. A. & Dzierżanowski, T. The Therapeutic Potential of Cannabis in Counteracting Oxidative Stress and Inflammation. *Molecules* **26**, 4551 (2021).
6. Pellati, F. *et al.* Cannabis sativa L. and Nonpsychoactive Cannabinoids: Their Chemistry and Role against Oxidative Stress, Inflammation, and Cancer. *Biomed Res Int* **2018**, 1691428 (2018).
7. Maayah, Z. H., Takahara, S., Ferdaoussi, M. & Dyck, J. R. B. The molecular mechanisms that underpin the biological benefits of full-spectrum cannabis extract in the treatment of neuropathic pain and inflammation. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* **1866**, 165771 (2020).
8. Picardo, S., Kaplan, G. G., Sharkey, K. A. & Seow, C. H. Insights into the role of cannabis in the management of inflammatory bowel disease. *Therap Adv Gastroenterol* **12**, 1756284819870977 (2019).
9. Lisano, J. K., Kisiolek, J. N., Smoak, P., Phillips, K. T. & Stewart, L. K. Chronic cannabis use and circulating biomarkers of neural health, stress, and inflammation in physically active individuals. *Appl Physiol Nutr Metab* **45**, 258–263 (2020).

10. Wang, Y. *et al.* Cannabidiol attenuates alcohol-induced liver steatosis, metabolic dysregulation, inflammation and neutrophil-mediated injury. *Sci Rep* **7**, 12064 (2017).
11. Zou, S. & Kumar, U. Cannabinoid Receptors and the Endocannabinoid System: Signaling and Function in the Central Nervous System. *Int J Mol Sci* **19**, 833 (2018).
12. Lu, H.-C. & Mackie, K. Review of the Endocannabinoid System. *Biol Psychiatry Cogn Neurosci Neuroimaging* **6**, 607–615 (2021).
13. Mackie, K. Distribution of cannabinoid receptors in the central and peripheral nervous system. *Handb Exp Pharmacol* 299–325 (2005) doi:10.1007/3-540-26573-2_10.
14. Ibarra-Lecue, I. *et al.* The endocannabinoid system in mental disorders: Evidence from human brain studies. *Biochem Pharmacol* **157**, 97–107 (2018).
15. Navarrete, F. *et al.* Endocannabinoid System Components as Potential Biomarkers in Psychiatry. *Front Psychiatry* **11**, 315 (2020).
16. Marco, E. M. & Laviola, G. The endocannabinoid system in the regulation of emotions throughout lifespan: a discussion on therapeutic perspectives. *J Psychopharmacol* **26**, 150–163 (2012).
17. Zhou, D. *et al.* Role of the endocannabinoid system in the formation and development of depression. *Pharmazie* **72**, 435–439 (2017).
18. Storr, M. A. & Sharkey, K. A. The endocannabinoid system and gut-brain signalling. *Curr Opin Pharmacol* **7**, 575–582 (2007).
19. Hungund, B. L., Szakall, I., Adam, A., Basavarajappa, B. S. & Vadasz, C. Cannabinoid CB1 receptor knockout mice exhibit markedly reduced voluntary alcohol consumption and lack alcohol-induced dopamine release in the nucleus accumbens. *J Neurochem* **84**, 698–704 (2003).

20. Mitrirattanakul, S. *et al.* Bidirectional alterations of hippocampal cannabinoid 1 receptors and their endogenous ligands in a rat model of alcohol withdrawal and dependence. *Alcohol Clin Exp Res* **31**, 855–867 (2007).

21. Serrano, A. *et al.* Deficient endocannabinoid signaling in the central amygdala contributes to alcohol dependence-related anxiety-like behavior and excessive alcohol intake. *Neuropsychopharmacology* **43**, 1840–1850 (2018).

22. Ellgren, M. *et al.* Dynamic changes of the endogenous cannabinoid and opioid mesocorticolimbic systems during adolescence: THC effects. *Eur Neuropsychopharmacol* **18**, 826–834 (2008).

23. Walter, C. *et al.* Exogenous Delta9-Tetrahydrocannabinol Influences Circulating Endogenous Cannabinoids in Humans. *Journal of Clinical Psychopharmacology* **33**, 699 (2013).

24. Thieme, U. *et al.* Quantification of anandamide and 2-arachidonoylglycerol plasma levels to examine potential influences of tetrahydrocannabinol application on the endocannabinoid system in humans. *Drug Test Anal* **6**, 17–23 (2014).

25. Trautmann, S. M. & Sharkey, K. A. The Endocannabinoid System and Its Role in Regulating the Intrinsic Neural Circuitry of the Gastrointestinal Tract. *Int Rev Neurobiol* **125**, 85–126 (2015).

26. Srivastava, R. K., Lutz, B. & Ruiz de Azua, I. The Microbiome and Gut Endocannabinoid System in the Regulation of Stress Responses and Metabolism. *Front Cell Neurosci* **16**, 867267 (2022).

27. Cuddihey, H., MacNaughton, W. K. & Sharkey, K. A. Role of the Endocannabinoid System in the Regulation of Intestinal Homeostasis. *Cellular and Molecular Gastroenterology and Hepatology* **14**, 947–963 (2022).

28. García-Bueno, B. & Caso, J. R. Chapter 68 - Cannabis, Cannabinoid Receptors, and Stress-Induced Excitotoxicity. in *Neuropathology of Drug Addictions and Substance Misuse* (ed. Preedy, V. R.) 731–737 (Academic Press, San Diego, 2016). doi:10.1016/B978-0-12-800213-1.00068-7.

29. Pandey, R., Mousawy, K., Nagarkatti, M. & Nagarkatti, P. Endocannabinoids and immune regulation. *Pharmacol Res* **60**, 85–92 (2009).

30. Pandey, S. *et al.* Endocannabinoid system in irritable bowel syndrome and cannabis as a therapy. *Complement Ther Med* **48**, 102242 (2020).

31. Zhu, X. *et al.* Microbiota-gut-brain axis and the central nervous system. *Oncotarget* **8**, 53829–53838 (2017).

32. Amato, K. R. An introduction to microbiome analysis for human biology applications. *Am J Hum Biol* **29**, (2017).

33. Mayer, E. A., Tillisch, K. & Gupta, A. Gut/brain axis and the microbiota. *J Clin Invest* **125**, 926–938 (2015).

34. Cani, P. D. *et al.* Endocannabinoids — at the crossroads between the gut microbiota and host metabolism. *Nat Rev Endocrinol* **12**, 133–143 (2016).

35. Leclercq, S. *et al.* Intestinal permeability, gut-bacterial dysbiosis, and behavioral markers of alcohol-dependence severity. *Proc Natl Acad Sci U S A* **111**, E4485–E4493 (2014).

36. Liangpunsakul, S. *et al.* Quantity of alcohol drinking positively correlates with serum levels of endotoxin and markers of monocyte activation. *Sci Rep* **7**, 4462 (2017).

37. Chen, G. *et al.* Gut microbiota dysbiosis: The potential mechanisms by which alcohol disrupts gut and brain functions. *Front Microbiol* **13**, 916765 (2022).

38. Kreimeyer, H. Influence of Alcohol on the Intestinal Immune System. *ARCR* **45**, 03 (2025).

39. Koponen, K. *et al.* Associations of alcohol with the human gut microbiome and prospective health outcomes in the FINRISK 2002 cohort. *Eur J Nutr* **64**, 153 (2025).

40. Cocetta, V. *et al.* Cannabidiol Isolated From Cannabis sativa L. Protects Intestinal Barrier From In Vitro Inflammation and Oxidative Stress. *Front Pharmacol* **12**, 641210 (2021).

41. Gigli, S. *et al.* Cannabidiol restores intestinal barrier dysfunction and inhibits the apoptotic process induced by Clostridium difficile toxin A in Caco-2 cells. *United European Gastroenterol J* **5**, 1108–1115 (2017).

42. Gorfinkel, L. R., Stohl, M. & Hasin, D. Association of Depression With Past-Month Cannabis Use Among US Adults Aged 20 to 59 Years, 2005 to 2016. *JAMA Netw Open* **3**, e2013802 (2020).

43. Dierker, L., Selya, A., Lanza, S., Li, R. & Rose, J. Depression and Marijuana Use Disorder Symptoms among Current Marijuana Users. *Addict Behav* **76**, 161–168 (2018).

44. Kuhns, L., Kroon, E., Colyer-Patel, K. & Cousijn, J. Associations between Cannabis Use, Cannabis Use Disorder and Mood Disorders: Longitudinal, Genetic, and Neurocognitive Evidence. *Psychopharmacology (Berl)* **239**, 1231–1249 (2022).

45. Ouellette, M. J. *et al.* Why Use Cannabis? Examining Motives for Cannabis Use in Individuals with Anxiety Disorders. *Behaviour Change* **40**, 223–239 (2023).

46. Glodosky, N. C. & Cuttler, C. Motives Matter: Cannabis use motives moderate the associations between stress and negative affect. *Addictive Behaviors* **102**, 106188 (2020).

47. Schuckit, M. A. Comorbidity between substance use disorders and psychiatric conditions. *Addiction* **101 Suppl 1**, 76–88 (2006).

48. Hasin, D. & Kilcoyne, B. Comorbidity of psychiatric and substance use disorders in the United States: current issues and findings from the NESARC. *Curr Opin Psychiatry* **25**, 165–171 (2012).

49. Foster, J. A. & McVey Neufeld, K.-A. Gut-brain axis: how the microbiome influences anxiety and depression. *Trends Neurosci* **36**, 305–312 (2013).

50. Rathore, K. *et al.* The Bidirectional Relationship Between the Gut Microbiome and Mental Health: A Comprehensive Review. *Cureus* **17**, e80810.

51. Mehta, I. *et al.* Gut Microbiota and Mental Health: A Comprehensive Review of Gut-Brain Interactions in Mood Disorders. *Cureus* **17**, e81447.

52. An, L., Wang, X. & Cederbaum, A. I. Cytokines in alcoholic liver disease. *Arch Toxicol* **86**, 1337–1348 (2012).

53. Crews, F. T. *et al.* Cytokines and alcohol. *Alcohol Clin Exp Res* **30**, 720–730 (2006).

54. Moura, H. F. *et al.* Inflammatory cytokines and alcohol use disorder: systematic review and meta-analysis. *Braz J Psychiatry* **44**, 548–556 (2022).

55. Stahre, M., Roeber, J., Kanny, D., Brewer, R. D. & Zhang, X. Contribution of excessive alcohol consumption to deaths and years of potential life lost in the United States. *Prev Chronic Dis* **11**, E109 (2014).

56. Sacks, J. J., Gonzales, K. R., Bouchery, E. E., Tomedi, L. E. & Brewer, R. D. 2010 National and State Costs of Excessive Alcohol Consumption. *Am J Prev Med* **49**, e73–e79 (2015).

57. Perou, R. *et al.* Mental health surveillance among children--United States, 2005-2011. *MMWR Suppl* **62**, 1–35 (2013).

58. Rodríguez-Cintas, L. *et al.* Factors associated with lifetime suicidal ideation and suicide attempts in outpatients with substance use disorders. *Psychiatry Res* **262**, 440–445 (2018).

59. Skelley, J. W., Deas, C. M., Curren, Z. & Ennis, J. Use of cannabidiol in anxiety and anxiety-related disorders. *J Am Pharm Assoc (2003)* **60**, 253–261 (2020).

60. Turna, J., Patterson, B. & Van Ameringen, M. Is cannabis treatment for anxiety, mood, and related disorders ready for prime time? *Depress Anxiety* **34**, 1006–1017 (2017).

61. Blessing, E. M., Steenkamp, M. M., Manzanares, J. & Marmar, C. R. Cannabidiol as a Potential Treatment for Anxiety Disorders. *Neurotherapeutics* **12**, 825–836 (2015).

62. Jonas, D. E. *et al.* Pharmacotherapy for adults with alcohol use disorders in outpatient settings: a systematic review and meta-analysis. *JAMA* **311**, 1889–1900 (2014).

63. Dutra, L. *et al.* A meta-analytic review of psychosocial interventions for substance use disorders. *Am J Psychiatry* **165**, 179–187 (2008).

64. Yang, W., Singla, R., Maheshwari, O., Fontaine, C. J. & Gil-Mohapel, J. Alcohol Use Disorder: Neurobiology and Therapeutics. *Biomedicines* **10**, 1192 (2022).

65. Priya, M. The Biological and Psychological Factors of Alcoholism Disease. *Journal of Alcoholism & Drug Dependence* **12**, 1–2 (2024).

66. NIAAA. *National Institute on Alcohol Abuse and Alcoholism Strategic Plan 2017-2021*.

67. Nelson, N. G. *et al.* Combined Δ9-tetrahydrocannabinol and moderate alcohol administration: effects on ingestive behaviors in adolescent male rats. *Psychopharmacology (Berl)* **236**, 671–684 (2019).

68. Izzo, A. A. & Sharkey, K. A. Cannabinoids and the gut: new developments and emerging concepts. *Pharmacol Ther* **126**, 21–38 (2010).

69. Purohit, V. *et al.* Alcohol, intestinal bacterial growth, intestinal permeability to endotoxin, and medical consequences: summary of a symposium. *Alcohol* **42**, 349–361 (2008).

70. Crews, F. T. *et al.* Neuroimmune Function and the Consequences of Alcohol Exposure. *Alcohol Res* **37**, 331–351 (2015).

71. Ferber, S. G. *et al.* The "Entourage Effect": Terpenes Coupled with Cannabinoids for the Treatment of Mood Disorders and Anxiety Disorders. *Curr Neuropharmacol* **18**, 87–96 (2020).

72. Reiman, A. Cannabis as a substitute for alcohol and other drugs. *Harm Reduction Journal* **6**, 35 (2009).

73. Foster, J. A. & McVey Neufeld, K.-A. Gut-brain axis: how the microbiome influences anxiety and depression. *Trends Neurosci* **36**, 305–312 (2013).

74. Franklin, J. M. & Carrasco, G. A. Cannabinoid receptor agonists upregulate and enhance serotonin 2A (5-HT(2A)) receptor activity via ERK1/2 signaling. *Synapse* **67**, 145–159 (2013).

75. Horder, J., Browning, M., Di Simplicio, M., Cowen, P. J. & Harmer, C. J. Effects of 7 days of treatment with the cannabinoid type 1 receptor antagonist, rimonabant, on emotional processing. *J Psychopharmacol* **26**, 125–132 (2012).

76. Ibarra-Lecue, I. *et al.* The endocannabinoid system in mental disorders: Evidence from human brain studies. *Biochem Pharmacol* **157**, 97–107 (2018).

77. Navarrete, F. *et al.* Endocannabinoid System Components as Potential Biomarkers in Psychiatry. *Front Psychiatry* **11**, 315 (2020).

78. Petra, A. I. *et al.* Gut-Microbiota-Brain Axis and Its Effect on Neuropsychiatric Disorders With Suspected Immune Dysregulation. *Clin Ther* **37**, 984–995 (2015).

79. Cryan, J. F. & Dinan, T. G. Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. *Nat Rev Neurosci* **13**, 701–712 (2012).

80. Ohlsson, L. *et al.* Leaky gut biomarkers in depression and suicidal behavior. *Acta Psychiatr Scand* **139**, 185–193 (2019).

81. Moura, H. F. *et al.* Inflammatory cytokines and alcohol use disorder: systematic review and meta-analysis. *Braz J Psychiatry* **44**, 548–556 (2022).

82. Gorky, J. & Schwaber, J. The Role of the Gut-Brain Axis in Alcohol Use Disorders. *Prog Neuropsychopharmacol Biol Psychiatry* **65**, 234–241 (2016).

83. Li, X. *et al.* Therapeutic Interventions of Gut-Brain Axis as Novel Strategies for Treatment of Alcohol Use Disorder Associated Cognitive and Mood Dysfunction. *Front Neurosci* **16**, 820106 (2022).

84. Reed, S. F., Porges, S. W. & Newlin, D. B. Effect of alcohol on vagal regulation of cardiovascular function: contributions of the polyvagal theory to the psychophysiology of alcohol. *Exp Clin Psychopharmacol* **7**, 484–492 (1999).

85. Wang, H. J., Zakhari, S. & Jung, M. K. Alcohol, inflammation, and gut-liver-brain interactions in tissue damage and disease development. *World J Gastroenterol* **16**, 1304–1313 (2010).

86. Alhamoruni, A., Wright, K., Larvin, M. & O'Sullivan, S. Cannabinoids mediate opposing effects on inflammation-induced intestinal permeability. *British Journal of Pharmacology* **165**, 2598–2610 (2012).

87. Mishra, V., Puri, A. & Sharma, K. The effect of full-spectrum cannabis leaf extract on pain, inflammation, and quality of sleep: Case series. *Journal of Integrative Medicine and Research* **2**, 91 (2024).

88. Aswad, M. *et al.* High-CBD Extract (CBD-X) Downregulates Cytokine Storm Systemically and Locally in Inflamed Lungs. *Front Immunol* **13**, 875546 (2022).

89. McDougall, J. J. & McKenna, M. K. Anti-Inflammatory and Analgesic Properties of the Cannabis Terpene Myrcene in Rat Adjuvant Monoarthritis. *Int J Mol Sci* **23**, 7891 (2022).

90. Moshfeghinia, R., Najibi, A., Moradi, M., Assadian, K. & Ahmadi, J. The association between hematological markers of inflammation and chronic cannabis use: a systematic review and meta-analysis of observational studies. *Front Psychiatry* **15**, 1438002 (2024).

91. Couch, D. G. *et al.* Palmitoylethanolamide and Cannabidiol Prevent Inflammation-induced Hyperpermeability of the Human Gut In Vitro and In Vivo-A Randomized, Placebo-controlled, Double-blind Controlled Trial. *Inflamm Bowel Dis* **25**, 1006–1018 (2019).

92. Vitetta, L., Nation, T., Oldfield, D. & Thomsen, M. Medicinal Cannabis and the Intestinal Microbiome. *Pharmaceuticals* **17**, 1702 (2024).

93. Burstein, S. Cannabidiol (CBD) and its analogs: a review of their effects on inflammation. *Bioorg Med Chem* **23**, 1377–1385 (2015).

94. Zaiachuk, M., Suryavanshi, S. V., Pryimak, N., Kovalchuk, I. & Kovalchuk, O. The Anti-Inflammatory Effects of Cannabis sativa Extracts on LPS-Induced Cytokines Release in Human Macrophages. *Molecules* **28**, 4991 (2023).

95. Kovalchuk, A. *et al.* Fighting the storm: could novel anti-TNFα and anti-IL-6 C. sativa cultivars tame cytokine storm in COVID-19? *Aging (Albany NY)* **13**, 1571–1590 (2021).

96. Crowley, K. *et al.* Effects of Cannabinoids on Intestinal Motility, Barrier Permeability, and Therapeutic Potential in Gastrointestinal Diseases. *International Journal of Molecular Sciences* **25**, 6682 (2024).

97. Adejumo, A. C. *et al.* Cannabis use is associated with reduced prevalence of progressive stages of alcoholic liver disease. *Liver Int* **38**, 1475–1486 (2018).

98. Adejumo, A. C., Akanbi, O., Adejumo, K. L. & Bukong, T. N. Reduced Risk of Alcohol-Induced Pancreatitis With Cannabis Use. *Alcohol Clin Exp Res* **43**, 277–286 (2019).

99. Karoly, H. C., Bidwell, L. C., Mueller, R. & Hutchison, K. Investigating the relationships between alcohol consumption, cannabis use and circulating cytokines: a preliminary analysis. *Alcohol Clin Exp Res* **42**, 531–539 (2018).

100. Sharkey, K. A. & Wiley, J. W. The role of the endocannabinoid system in the brain-gut axis. *Gastroenterology* **151**, 252–266 (2016).

101. Cani, P. D. *et al.* Endocannabinoids--at the crossroads between the gut microbiota and host metabolism. *Nat Rev Endocrinol* **12**, 133–143 (2016).

102. Joffre, J. *et al.* Activation of CB1R Promotes Lipopolysaccharide-Induced IL-10 Secretion by Monocytic Myeloid-Derived Suppressive Cells and Reduces Acute Inflammation and Organ Injury. *J Immunol* **204**, 3339–3350 (2020).

103. Mustafa, W., Elgendy, N., Salama, S., Jawad, M. & Eltoukhy, K. The Effect of Cannabis on the Clinical and Cytokine Profiles in Patients with Multiple Sclerosis. *Multiple Sclerosis International* **2021**, 6611897 (2021).

104. Wang, Y., Guo, J., Mao, Z. & Chen, Y. Symphony of the gut microbiota and endocannabinoidome: a molecular and functional perspective. *Front. Cell. Infect. Microbiol.* **15**, (2025).

105. Schiano Moriello, A., Di Marzo, V. & Petrosino, S. Mutual Links between the Endocannabinoidome and the Gut Microbiome, with Special Reference to Companion Animals: A Nutritional Viewpoint. *Animals* **12**, 348 (2022).

106. Hungund, B. L. & Basavarajappa, B. S. Role of endocannabinoids and cannabinoid CB1 receptors in alcohol-related behaviors. *Ann N Y Acad Sci* **1025**, 515–527 (2004).

107. Mato, S. *et al.* Selective up-regulation of cannabinoid CB1 receptor coupling to Go-proteins in suicide victims with mood disorders. *Biochem Pharmacol* **157**, 258–265 (2018).

108. Rubino, T. *et al.* CB1 receptor stimulation in specific brain areas differently modulate anxiety-related behaviour. *Neuropharmacology* **54**, 151–160 (2008).

109. Zimmermann, K. *et al.* Emotion regulation deficits in regular marijuana users. *Hum Brain Mapp* **38**, 4270–4279 (2017).

110. Hill, M. N., Hillard, C. J. & McEwen, B. S. Alterations in corticolimbic dendritic morphology and emotional behavior in cannabinoid CB1 receptor-deficient mice parallel the effects of chronic stress. *Cereb Cortex* **21**, 2056–2064 (2011).

111. Häring, M., Grieb, M., Monory, K., Lutz, B. & Moreira, F. A. Cannabinoid CB_1 receptor in the modulation of stress coping behavior in mice: the role of serotonin and different forebrain neuronal subpopulations. *Neuropharmacology* **65**, 83–89 (2013).

112. Vinod, K. Y. & Hungund, B. L. Role of the endocannabinoid system in depression and suicide. *Trends Pharmacol Sci* **27**, 539–545 (2006).

113. Patel, R. R. *et al.* IL-10 normalizes aberrant amygdala GABA transmission and reverses anxiety-like behavior and dependence-induced escalation of alcohol intake. *Progress in Neurobiology* **199**, 101952 (2021).

114. Van Gaal, L., Pi-Sunyer, X., Després, J.-P., McCarthy, C. & Scheen, A. Efficacy and safety of rimonabant for improvement of multiple cardiometabolic risk factors in overweight/obese patients: pooled 1-year data from the Rimonabant in Obesity (RIO) program. *Diabetes Care* **31 Suppl 2**, S229-240 (2008).

115. Ballard, M. E., Bedi, G. & de Wit, H. Effects of delta-9-tetrahydrocannabinol on evaluation of emotional images. *J Psychopharmacol* **26**, 1289–1298 (2012).

116. Bossong, M. G. *et al.* The endocannabinoid system and emotional processing: a pharmacological fMRI study with Δ9-tetrahydrocannabinol. *Eur Neuropsychopharmacol* **23**, 1687–1697 (2013).

117. Segev, A., Rubin, A. S., Abush, H., Richter-Levin, G. & Akirav, I. Cannabinoid receptor activation prevents the effects of chronic mild stress on emotional learning and LTP in a rat model of depression. *Neuropsychopharmacology* **39**, 919–933 (2014).

118. Phan, K. L. *et al.* Cannabinoid modulation of amygdala reactivity to social signals of threat in humans. *J Neurosci* **28**, 2313–2319 (2008).

119. Beurel, E., Toups, M. & Nemeroff, C. B. The Bidirectional Relationship of Depression and Inflammation: Double Trouble. *Neuron* **107**, 234–256 (2020).

120. Felger, J. C. Role of Inflammation in Depression and Treatment Implications. *Handb Exp Pharmacol* **250**, 255–286 (2019).

121. Felger, J. C. Imaging the Role of Inflammation in Mood and Anxiety-related Disorders. *Curr Neuropharmacol* **16**, 533–558 (2018).

122. Buckner, J. D. & Zvolensky, M. J. Cannabis and related impairment: The unique roles of cannabis use to cope with social anxiety and social avoidance. *The American Journal on Addictions* **23**, 598–603 (2014).

123. Martin, A. M., Sun, E. W. & Keating, D. J. Mechanisms controlling hormone secretion in human gut and its relevance to metabolism. *J Endocrinol* **244**, R1–R15 (2019).

124. Mudyanadzo, T. A., Hauzaree, C., Yerokhina, O., Architha, N. N. & Ashqar, H. M. Irritable Bowel Syndrome and Depression: A Shared Pathogenesis. *Cureus* **10**, e3178 (2018).

125. Malinova, T. S., Dijkstra, C. D. & de Vries, H. E. Serotonin: A mediator of the gut-brain axis in multiple sclerosis. *Mult Scler* **24**, 1144–1150 (2018).

126. Chevalier, G. *et al.* Effect of gut microbiota on depressive-like behaviors in mice is mediated by the endocannabinoid system. *Nat Commun* **11**, 6363 (2020).

127. Miller, A. H. & Raison, C. L. The role of inflammation in depression: from evolutionary imperative to modern treatment target. *Nat Rev Immunol* **16**, 22–34 (2016).

128. Guan, Q. & Zhang, J. Recent Advances: The Imbalance of Cytokines in the Pathogenesis of Inflammatory Bowel Disease. *Mediators Inflamm* **2017**, 4810258 (2017).

129. Maydych, V. The Interplay Between Stress, Inflammation, and Emotional Attention: Relevance for Depression. *Front Neurosci* **13**, 384 (2019).

130. Shin, N.-R., Whon, T. W. & Bae, J.-W. *Proteobacteria*: microbial signature of dysbiosis in gut microbiota. *Trends in Biotechnology* **33**, 496–503 (2015).

131. Litvak, Y., Byndloss, M. X., Tsolis, R. M. & Bäumler, A. J. Dysbiotic *Proteobacteria* expansion: a microbial signature of epithelial dysfunction. *Current Opinion in Microbiology* **39**, 1–6 (2017).

132. Osimo, E. F. *et al.* Inflammatory markers in depression: A meta-analysis of mean differences and variability in 5,166 patients and 5,083 controls. *Brain Behav Immun* **87**, 901–909 (2020).

133. Kim, I.-B., Lee, J.-H. & Park, S.-C. The Relationship between Stress, Inflammation, and Depression. *Biomedicines* **10**, 1929 (2022).

134. Allen, J., Balfour, R., Bell, R. & Marmot, M. Social determinants of mental health. *Int Rev Psychiatry* **26**, 392–407 (2014).

135. Branchi, I. *et al.* Breaking free from the inflammatory trap of depression: Regulating the interplay between immune activation and plasticity to foster mental health. *Neuroscience Applied* **3**, 103923 (2024).

136. Slavich, G. M. & Irwin, M. R. From stress to inflammation and major depressive disorder: a social signal transduction theory of depression. *Psychol Bull* **140**, 774–815 (2014).

137. Cui, L. *et al.* Major depressive disorder: hypothesis, mechanism, prevention and treatment. *Sig Transduct Target Ther* **9**, 30 (2024).

138. Berk, M. *et al.* So depression is an inflammatory disease, but where does the inflammation come from? *BMC Med* **11**, 200 (2013).

139. Han, K.-M. & Ham, B.-J. How Inflammation Affects the Brain in Depression: A Review of Functional and Structural MRI Studies. *Journal of Clinical Neurology* **17**, 503–515 (2021).

140. Moriarity, D. P. *et al.* A systematic review of associations between emotion regulation characteristics and inflammation. *Neuroscience & Biobehavioral Reviews* **150**, 105162 (2023).

141. Han, K.-M. & Ham, B.-J. How Inflammation Affects the Brain in Depression: A Review of Functional and Structural MRI Studies. *J Clin Neurol* **17**, 503–515 (2021).

142. O'Callaghan, A. & van Sinderen, D. Bifidobacteria and Their Role as Members of the Human Gut Microbiota. *Front Microbiol* **7**, 925 (2016).

143. Rizzatti, G., Lopetuso, L. R., Gibiino, G., Binda, C. & Gasbarrini, A. Proteobacteria: A Common Factor in Human Diseases. *Biomed Res Int* **2017**, 9351507 (2017).

144. Machado, L., Thompson, L. M. & Brett, C. H. R. Visual analogue mood scale scores in healthy young versus older adults. *Int Psychogeriatr* **31**, 417–424 (2019).

145. Sobell, L. C. & Sobell, M. B. Timeline follow-back: A technique for assessing self-reported alcohol consumption. in *Measuring alcohol consumption: Psychosocial and biochemical methods* 41–72 (Humana Press/Springer Nature, Totowa, NJ, US, 1992). doi:10.1007/978-1-4612-0357-5_3.

146. Skinner, H. A. & Allen, B. A. Alcohol dependence syndrome: measurement and validation. *J Abnorm Psychol* **91**, 199–209 (1982).

147. Doyle, S. R. & Donovan, D. M. A Validation Study of the Alcohol Dependence Scale. *J Stud Alcohol Drugs* **70**, 689–699 (2009).

148. Stephens, R. S., Roffman, R. A. & Curtin, L. Marijuana Dependence Scale. https://doi.org/10.1037/t03579-000 (2000) doi:10.1037/t03579-000.

149. FFQ. https://www.epic-norfolk.org.uk/about-epic-norfolk/nutritional-methods/ffq/.

150. Coelho, L. G., Cândido, A. P. C., Machado-Coelho, G. L. L. & Freitas, S. N. de. Association between nutritional status, food habits and physical activity level in schoolchildren. *J Pediatr (Rio J)* **88**, 406–412 (2012).

151. Coelho, L. G., Cândido, A. P. C., Machado-Coelho, G. L. L. & Freitas, S. N. de. Food habits and risk of cardiovascular disease in schoolchildren from Ouro Preto, Minas Gerais. *Rev. Nutr.* **28**, 133–142 (2015).

152. Hajjar, M. & Rezazadeh, A. The Recommended Food Score and Healthy Nordic Food Index in cardiovascular disease and stroke: A systematic review. *ARYA Atheroscler* **16**, 248–257 (2020).

153. Team, R. RStudio: Integrated Development Environment for R.

154. Rothman, K. J. No Adjustments Are Needed for Multiple Comparisons. *Epidemiology* **1**, 43–46 (1990).

155. Teresi, J. A., Yu, X., Stewart, A. L. & Hays, R. D. Guidelines for Designing and Evaluating Feasibility Pilot Studies. *Med Care* **60**, 95–103 (2022).

156. Althouse, A. D. Adjust for Multiple Comparisons? It's Not That Simple. *Ann Thorac Surg* **101**, 1644–1645 (2016).

157. Craft, R. M., Marusich, J. A. & Wiley, J. L. Sex differences in cannabinoid pharmacology: A reflection of differences in the endocannabinoid system? *Life Sci* **92**, 476–481 (2013).

158. Viudez-Martínez, A., García-Gutiérrez, M. S. & Manzanares, J. Gender differences in the effects of cannabidiol on ethanol binge drinking in mice. *Addict Biol* **25**, e12765 (2020).

159. Caulkins, J. P. Changes in self-reported cannabis use in the United States from 1979 to 2022. *Addiction* **119**, 1648–1652 (2024).

160. Subbaraman, M. S. & Kerr, W. C. Simultaneous versus concurrent use of alcohol and cannabis in the National Alcohol Survey. *Alcohol Clin Exp Res* **39**, 872–879 (2015).

161. Puddephatt, J.-A., Irizar, P., Jones, A., Gage, S. H. & Goodwin, L. Associations of common mental disorder with alcohol use in the adult general population: a systematic review and meta-analysis. *Addiction* **117**, 1543–1572 (2022).

162. Rup, J., Freeman, T. P., Perlman, C. & Hammond, D. Cannabis and Mental Health: Adverse Outcomes and Self-Reported Impact of Cannabis Use by Mental Health Status. *Subst Use Misuse* **57**, 719–729 (2022).

163. Thompson, K., Holley, M., Sturgess, C. & Leadbeater, B. Co-Use of Alcohol and Cannabis: Longitudinal Associations with Mental Health Outcomes in Young Adulthood. *Int J Environ Res Public Health* **18**, 3652 (2021).

164. Fleming, C. B. *et al.* Young adult simultaneous alcohol and marijuana use: Between- and within-person associations with negative alcohol-related consequences, mental health, and general health across two-years. *Addict Behav* **123**, 107079 (2021).

165. Feingold, D., Weiser, M., Rehm, J. & Lev-Ran, S. The association between cannabis use and mood disorders: A longitudinal study. *Journal of Affective Disorders* **172**, 211–218 (2015).

166. Guillot, C. R., Blumenthal, H., Zvolensky, M. J. & Schmidt, N. B. Anxiety sensitivity components in relation to alcohol and cannabis use, motives, and problems in treatment-seeking cigarette smokers. *Addict Behav* **82**, 166–173 (2018).

167. Cornelius, J. R. *et al.* Double-Blind Placebo-Controlled Trial of Fluoxetine in Adolescents with Comorbid Major Depression and an Alcohol Use Disorder. *Addict Behav* **34**, 905–909 (2009).

168. Buckner, J. D., Ledley, D. R., Heimberg, R. G. & Schmidt, N. B. Treating Comorbid Social Anxiety and Alcohol Use Disorders. *Clin Case Stud* **7**, 208–223 (2008).

169. Gobbi, G. *et al.* Association of Cannabis Use in Adolescence and Risk of Depression, Anxiety, and Suicidality in Young Adulthood: A Systematic Review and Meta-analysis. *JAMA Psychiatry* **76**, 426–434 (2019).

170. Lee, J. L. C., Bertoglio, L. J., Guimarães, F. S. & Stevenson, C. W. Cannabidiol regulation of emotion and emotional memory processing: relevance for treating anxiety-related and substance abuse disorders. *Br J Pharmacol* **174**, 3242–3256 (2017).

171. Karoly, H. C., Conner, B. T. & Prince, M. A. Associations between alcohol and cannabis use order, frequency, quantity, and consequences in a college sample of individuals who co-use alcohol and cannabis. *Am J Addict* **32**, 283–290 (2023).

172. Coelho, S. G., Hendershot, C. S., Rueda, S. & Wardell, J. D. Daily associations between cannabis use and alcohol use among people who use cannabis for both medicinal and nonmedicinal reasons: Substitution or complementarity? *Psychol Addict Behav* **37**, 1006–1018 (2023).

173. Gunn, R. L., Aston, E. R. & Metrik, J. Patterns of Cannabis and Alcohol Co-Use: Substitution Versus Complementary Effects. *Alcohol Res* **42**, 04 (2022).

174. Koob, G. F. & Colrain, I. M. Alcohol use disorder and sleep disturbances: a feed-forward allostatic framework. *Neuropsychopharmacol.* **45**, 141–165 (2020).

175. Li, J. *et al.* Effect of alcohol use disorders and alcohol intake on the risk of subsequent depressive symptoms: a systematic review and meta-analysis of cohort studies. *Addiction* **115**, 1224–1243 (2020).

176. Sontate, K. V. *et al.* Alcohol, Aggression, and Violence: From Public Health to Neuroscience. *Front Psychol* **12**, 699726 (2021).

177. Coskunpinar, A., Dir, A. L. & Cyders, M. A. Multidimensionality in Impulsivity and Alcohol Use: A Meta-Analysis using the UPPS Model of Impulsivity. *Alcohol Clin Exp Res* **37**, 1441–1450 (2013).

178. MacKillop, J. *et al.* Hazardous drinking and alcohol use disorders. *Nat Rev Dis Primers* **8**, 80 (2022).

179. Sinha, R. Alcohol's Negative Emotional Side: The Role of Stress Neurobiology in Alcohol Use Disorder. *Alcohol Res* **42**, 12 (2022).

180. Yen, F.-S., Wang, S.-I., Lin, S.-Y., Chao, Y.-H. & Wei, J. C.-C. The impact of heavy alcohol consumption on cognitive impairment in young old and middle old persons. *Journal of Translational Medicine* **20**, 155 (2022).

181. Juchem, C. M. *et al.* Personality changes related to presence and treatment of substance use (disorders): a systematic review. *Psychol Med* **54**, 1905–1929 (2024).

182. Koudys, J. W., Cane, C., Nikolova, Y. S. & Ruocco, A. C. Transdiagnostic Impulsivity-Relevant Phenotypes and the Comorbidity of Personality Disorders with Substance and Alcohol Use Disorders. *Curr Addict Rep* **10**, 122–130 (2023).

183. Lui, P. P., Chmielewski, M., Trujillo, M., Morris, J. & Pigott, T. D. Linking Big Five Personality Domains and Facets to Alcohol (Mis)Use: A Systematic Review and Meta-Analysis. *Alcohol Alcohol* **57**, 58–73 (2022).

184. Winters, A. M., Malouff, J. M. & Schutte, N. S. The association between the five-factor model of personality and problem cannabis use: A meta-analysis. *Personality and Individual Differences* **193**, 111635 (2022).

185. Waddell, J. T., Blake, A. J. & Chassin, L. Relations between impulsive personality traits, alcohol and cannabis co-use, and negative alcohol consequences: A test of cognitive and behavioral mediators. *Drug and Alcohol Dependence* **225**, 108780 (2021).

186. Thørrisen, M. M. & Sadeghi, T. The Ten-Item Personality Inventory (TIPI): a scoping review of versions, translations and psychometric properties. *Front Psychol* **14**, 1202953 (2023).

187. Henry, J. D. & Crawford, J. R. The short-form version of the Depression Anxiety Stress Scales (DASS-21): construct validity and normative data in a large non-clinical sample. *Br J Clin Psychol* **44**, 227–239 (2005).

188. Beck, A. T., Epstein, N., Brown, G. & Steer, R. A. An inventory for measuring clinical anxiety: psychometric properties. *J Consult Clin Psychol* **56**, 893–897 (1988).

189. Beck, A. T., Steer, R. A. & Brown, G. Beck Depression Inventory–II. https://doi.org/10.1037/t00742-000 (1996) doi:10.1037/t00742-000.

190. Kaufman, E. A. *et al.* The Difficulties in Emotion Regulation Scale Short Form (DERS-SF): Validation and Replication in Adolescent and Adult Samples. *J Psychopathol Behav Assess* **38**, 443–455 (2016).

191. Patton, J. H., Stanford, M. S. & Barratt, E. S. Factor structure of the Barratt impulsiveness scale. *J Clin Psychol* **51**, 768–774 (1995).

192. Cyders, M. A., Littlefield, A. K., Coffey, S. & Karyadi, K. A. Examination of a Short Version of the UPPS-P Impulsive Behavior Scale. *Addict Behav* **39**, 1372–1376 (2014).

193. Azizabadi, Z., Aminisani, N. & Emamian, M. H. Socioeconomic inequality in depression and anxiety and its determinants in Iranian older adults. *BMC Psychiatry* **22**, 761 (2022).

194. Qian, Z. *et al.* Changes in anxiety and depression in patients with different income levels through the COVID-19 pandemic. *J Affect Disord* **338**, 17–20 (2023).

195. Madigan, A. & Daly, M. Socioeconomic status and depressive symptoms and suicidality: The role of subjective social status. *Journal of Affective Disorders* **326**, 36–43 (2023).

196. Doremus, J. M., Stith, S. S. & Vigil, J. M. Using recreational cannabis to treat insomnia: Evidence from over-the-counter sleep aid sales in Colorado. *Complementary Therapies in Medicine* **47**, 102207 (2019).

197. Berger, M., Amminger, G. P. & McGregor, I. S. Medicinal cannabis for the treatment of anxiety disorders. *Aust J Gen Pract* **51**, 586–592 (2022).

198. Stack, S. K., Wheate, N. J. & Schubert, E. A. Medicinal Cannabis for the Treatment of Anxiety Disorders: a Narrative Review. *Curr Treat Options Psych* **9**, 163–173 (2022).

199. Sharpe, L., Sinclair, J., Kramer, A., de Manincor, M. & Sarris, J. Cannabis, a cause for anxiety? A critical appraisal of the anxiogenic and anxiolytic properties. *J Transl Med* **18**, 374 (2020).

200. Khantzian, E. J. The self-medication hypothesis of substance use disorders: a reconsideration and recent applications. *Harv Rev Psychiatry* **4**, 231–244 (1997).

201. Thayer, R. E., Coromac-Medrano, J. & Neiderman, A. C. Cannabis Use Motives Associated with Mental Health Screening Among Older Adults. *Psychoactives* **4**, 36 (2025).

202. Meier, M. H. *et al.* Persistent cannabis users show neuropsychological decline from childhood to midlife. *Proc Natl Acad Sci U S A* **109**, E2657-2664 (2012).

203. Volkow, N. D. *et al.* Effects of Cannabis Use on Human Behavior, Including Cognition, Motivation, and Psychosis: A Review. *JAMA Psychiatry* **73**, 292–297 (2016).

204. Ruehle, S. *et al.* Cannabinoid CB1 Receptor in Dorsal Telencephalic Glutamatergic Neurons: Distinctive Sufficiency for Hippocampus-Dependent and Amygdala-Dependent Synaptic and Behavioral Functions. *J. Neurosci.* **33**, 10264–10277 (2013).

205. Koob, G. F. & Volkow, N. D. Neurobiology of addiction: a neurocircuitry analysis. *Lancet Psychiatry* **3**, 760–773 (2016).

206. Bostick, J. W., Schonhoff, A. M. & Mazmanian, S. K. Gut Microbiome-Mediated Regulation of Neuroinflammation. *Curr Opin Immunol* **76**, 102177 (2022).

207. Adler, N. E. & Rehkopf, D. H. U.S. disparities in health: descriptions, causes, and mechanisms. *Annu Rev Public Health* **29**, 235–252 (2008).

208. Room, R., Babor, T. & Rehm, J. Alcohol and public health. *Lancet* **365**, 519–530 (2005).

209. Waddell, J. T., Fairlie, A. M., Calhoun, B. H., Patrick, M. E. & Lee, C. M. Planned vs. Unplanned Drinking and Cannabis Use: Do Facets of Trait Impulsivity Influence Daily Risk? *Psychol Addict Behav* **37**, 341–352 (2023).

210. Kotov, R., Gamez, W., Schmidt, F. & Watson, D. Linking 'big' personality traits to anxiety, depressive, and substance use disorders: a meta-analysis. *Psychol Bull* **136**, 768–821 (2010).

211. Terracciano, A., Löckenhoff, C. E., Crum, R. M., Bienvenu, O. J. & Costa, P. T. Five-Factor Model personality profiles of drug users. *BMC Psychiatry* **8**, 22 (2008).

212. Semaan, A. & Khan, M. K. Neurobiology of Addiction. in *StatPearls* (StatPearls Publishing, Treasure Island (FL), 2025).

213. Volkow, N. D. & Blanco, C. Substance use disorders: a comprehensive update of classification, epidemiology, neurobiology, clinical aspects, treatment and prevention. *World Psychiatry* **22**, 203–229 (2023).

214. Koob, G. F. & Le Moal, M. Addiction and the brain antireward system. *Annu Rev Psychol* **59**, 29–53 (2008).

215. Fazzino, T. L., Bjorlie, K. & Lejuez, C. W. A systematic review of reinforcement-based interventions for substance use: Efficacy, mechanisms of action, and moderators of treatment effects. *Journal of Substance Abuse Treatment* **104**, 83–96 (2019).

216. Stacy, A. W. & Wiers, R. W. Implicit Cognition and Addiction: A Tool for Explaining Paradoxical Behavior. *Annu Rev Clin Psychol* **6**, 551–575 (2010).

217. Wiers, R. W., Havermans, R., Deutsch, R. & Stacy, A. W. A mismatch with dual process models of addiction rooted in psychology. *Behavioral and Brain Sciences* **31**, 460–460 (2008).

218. Moggi, F. Etiological Theories on the Relationship of Mental Disorders and Substance Use Disorders. https://karger.com/books/book/2907/chapter/5818227/Etiological-Theories-on-the-Relationship-of-Mental.

219. Hungund, B. L., Szakall, I., Adam, A., Basavarajappa, B. S. & Vadasz, C. Cannabinoid CB1 receptor knockout mice exhibit markedly reduced voluntary alcohol consumption and lack alcohol-induced dopamine release in the nucleus accumbens. *J Neurochem* **84**, 698–704 (2003).

220. Mitrirattanakul, S. *et al.* Bidirectional alterations of hippocampal cannabinoid 1 receptors and their endogenous ligands in a rat model of alcohol withdrawal and dependence. *Alcohol Clin Exp Res* **31**, 855–867 (2007).

221. Serrano, A. *et al.* Deficient endocannabinoid signaling in the central amygdala contributes to alcohol dependence-related anxiety-like behavior and excessive alcohol intake. *Neuropsychopharmacology* **43**, 1840–1850 (2018).

222. Walter, C. *et al.* Exogenous delta^{9}-tetrahydrocannabinol influences circulating endogenous cannabinoids in humans. *J Clin Psychopharmacol* **33**, 699–705 (2013).

223. Ellgren, M. *et al.* Dynamic changes of the endogenous cannabinoid and opioid mesocorticolimbic systems during adolescence: THC effects. *Eur Neuropsychopharmacol* **18**, 826–834 (2008).

224. Thieme, U. *et al.* Quantification of anandamide and 2-arachidonoylglycerol plasma levels to examine potential influences of tetrahydrocannabinol application on the endocannabinoid system in humans. *Drug Test Anal* **6**, 17–23 (2014).

225. Cluny, N. L., Keenan, C. M., Reimer, R. A., Le Foll, B. & Sharkey, K. A. Prevention of Diet-Induced Obesity Effects on Body Weight and Gut Microbiota in Mice Treated Chronically with Δ9-Tetrahydrocannabinol. *PLoS One* **10**, e0144270 (2015).

226. Kratz, D., Thomas, D. & Gurke, R. Endocannabinoids as potential biomarkers: It‘s all about pre-analytics. *Journal of Mass Spectrometry and Advances in the Clinical Lab* **22**, 56–63 (2021).

227. Aran, A. *et al.* Lower circulating endocannabinoid levels in children with autism spectrum disorder. *Molecular Autism* **10**, 2 (2019).

228. Hill, M. N., Miller, G. E., Carrier, E. J., Gorzalka, B. B. & Hillard, C. J. Circulating endocannabinoids and *N*-acyl ethanolamines are differentially regulated in major depression and following exposure to social stress. *Psychoneuroendocrinology* **34**, 1257–1262 (2009).

229. Grill, M. *et al.* Members of the endocannabinoid system are distinctly regulated in inflammatory bowel disease and colorectal cancer. *Sci Rep* **9**, 2358 (2019).

230. Hillard, C. J. Circulating Endocannabinoids: From Whence Do They Come and Where are They Going? *Neuropsychopharmacol.* **43**, 155–172 (2018).

231. Aydin, E. *et al.* UHPLC-ESI-MS/MS assay for quantification of endocannabinoids in cerebrospinal fluid using surrogate calibrant and surrogate matrix approaches. *Journal of Pharmaceutical and Biomedical Analysis* **222**, 115090 (2023).

232. Amir Hamzah, K., Turner, N., Nichols, D. & Ney, L. J. Advances in targeted liquid chromatography-tandem mass spectrometry methods for endocannabinoid and N-acylethanolamine quantification in biological matrices: A systematic review. *Mass Spectrom Rev* **44**, 513–538 (2025).

233. Thursby, E. & Juge, N. Introduction to the human gut microbiota. *Biochem J* **474**, 1823–1836 (2017).

234. Wiertsema, S. P., van Bergenhenegouwen, J., Garssen, J. & Knippels, L. M. J. The Interplay between the Gut Microbiome and the Immune System in the Context of Infectious Diseases throughout Life and the Role of Nutrition in Optimizing Treatment Strategies. *Nutrients* **13**, 886 (2021).

235. Colella, M. *et al.* Microbiota revolution: How gut microbes regulate our lives. *World J Gastroenterol* **29**, 4368–4383 (2023).

236. Dicks, L. M. T., Hurn, D. & Hermanus, D. Gut Bacteria and Neuropsychiatric Disorders. *Microorganisms* **9**, 2583 (2021).

237. Mallick, K., Khodve, G., Ruwatia, R. & Banerjee, S. Gut microbes: Therapeutic Target for neuropsychiatric disorders. *Journal of Psychiatric Research* **184**, 27–38 (2025).

238. Gurwara, S. *et al.* Alcohol use alters the colonic mucosa-associated gut microbiota in humans. *Nutr Res* **83**, 119–128 (2020).

239. Alhamoruni, A., Lee, A. C., Wright, K. L., Larvin, M. & O'Sullivan, S. E. Pharmacological effects of cannabinoids on the Caco-2 cell culture model of intestinal permeability. *J Pharmacol Exp Ther* **335**, 92–102 (2010).

240. Cassol, I., Ibañez, M. & Bustamante, J. P. Key features and guidelines for the application of microbial alpha diversity metrics. *Sci Rep* **15**, 622 (2025).

241. Walters, K. E. & Martiny, J. B. H. Alpha-, beta-, and gamma-diversity of bacteria varies across habitats. *PLoS One* **15**, e0233872 (2020).

242. Schloss, P. D. Rarefaction is currently the best approach to control for uneven sequencing effort in amplicon sequence analyses. *mSphere* **9**, e00354-23 (2024).

243. Tang, Z.-Z., Chen, G. & Alekseyenko, A. V. PERMANOVA-S: association test for microbial community composition that accommodates confounders and multiple distances. *Bioinformatics* **32**, 2618–2625 (2016).

244. Ty, M. *et al.* Performance of distinct microbial based solutions in a Campylobacter infection challenge model in poultry. *Anim Microbiome* **4**, 2 (2022).

245. Mandal, S. *et al.* Analysis of composition of microbiomes: a novel method for studying microbial composition. *Microb Ecol Health Dis* **26**, 27663 (2015).

246. Props, R. *et al.* Absolute quantification of microbial taxon abundances. *ISME J* **11**, 584–587 (2017).

247. Barlow, J. T., Bogatyrev, S. R. & Ismagilov, R. F. A quantitative sequencing framework for absolute abundance measurements of mucosal and lumenal microbial communities. *Nat Commun* **11**, 2590 (2020).

248. Caporaso, J. G. *et al.* QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* **7**, 335–336 (2010).

249. Callahan, B. J. *et al.* DADA2: High-resolution sample inference from Illumina amplicon data. *Nat Methods* **13**, 581–583 (2016).

250. Bokulich, N. A. *et al.* Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. *Microbiome* **6**, 90 (2018).

251. McDonald, D. *et al.* An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *ISME J* **6**, 610–618 (2012).

252. Zhang, H. *et al.* Distinct Gut Microbiota and Arachidonic Acid Metabolism in Obesity-Prone and Obesity-Resistant Mice with a High-Fat Diet. *Nutrients* **16**, 1579 (2024).

253. Zhang, Y.-J. *et al.* Impacts of Gut Bacteria on Human Health and Diseases. *Int J Mol Sci* **16**, 7493–7519 (2015).

254. Singh, S. B., Carroll-Portillo, A. & Lin, H. C. Desulfovibrio in the Gut: The Enemy within? *Microorganisms* **11**, 1772 (2023).

255. Karakasidis, E., Kotsiou, O. S. & Gourgoulianis, K. I. Lung and Gut Microbiome in COPD. *Journal of Personalized Medicine* **13**, 804 (2023).

256. Facciolà, A. *et al.* Campylobacter: from microbiology to prevention. *J Prev Med Hyg* **58**, E79–E92 (2017).

257. Costa, D. & Iraola, G. Pathogenomics of Emerging Campylobacter Species. *Clinical Microbiology Reviews* **32**, 10.1128/cmr.00072-18 (2019).

258. Jordan, V. & Naqvi, S. *Mogibacterium* bacteraemia in an elderly woman with severe periodontal disease. *New Microbes and New Infections* **65**, 101585 (2025).

259. Luo, J. *et al.* Relationship of Mogibacterium in the gut microbiota with early-stage lung cancer. *J. Appl. Microbiol.* **136**, lxaf130 (2025).

260. Harris, R., Drummond, S. P. A., Sletten, T. L. & Wolkow, A. P. 0351 Protective Factors Buffer the Impact of High Shift Work Disorder Risk on Health Outcomes. *Sleep* **47**, A150–A151 (2024).

261. Zhou, J. *et al.* The relationship of Megamonas species with nonalcoholic fatty liver disease in children and adolescents revealed by metagenomics of gut microbiota. *Sci Rep* **12**, 22001 (2022).

262. Wu, C. *et al.* Obesity-enriched gut microbe degrades *myo*-inositol and promotes lipid absorption. *Cell Host & Microbe* **32**, 1301-1314.e9 (2024).

263. Khorsand, B. *et al.* Overrepresentation of Enterobacteriaceae and Escherichia coli is the major gut microbiome signature in Crohn's disease and ulcerative colitis; a comprehensive metagenomic analysis of IBDMDB datasets. *Front. Cell. Infect. Microbiol.* **12**, (2022).

264. Ariyoshi, T., Hagihara, M., Takahashi, M. & Mikamo, H. Effect of Clostridium butyricum on Gastrointestinal Infections. *Biomedicines* **10**, 483 (2022).

265. Gomaa, E. Z. Human gut microbiota/microbiome in health and diseases: a review. *Antonie van Leeuwenhoek* **113**, 2019–2040 (2020).

266. Bäckhed, F. *et al.* Defining a Healthy Human Gut Microbiome: Current Concepts, Future Directions, and Clinical Applications. *Cell Host & Microbe* **12**, 611–622 (2012).

267. Wong, C. B., Odamaki, T. & Xiao, J. Beneficial effects of *Bifidobacterium longum* subsp. *longum* BB536 on human health: Modulation of gut microbiome as the principal action. *Journal of Functional Foods* **54**, 506–519 (2019).

268. Antony, M. A. *et al.* Gut microbiome supplementation as therapy for metabolic syndrome. *World J Diabetes* **14**, 1502–1513 (2023).

269. Stoeva, M. K. *et al.* Butyrate-producing human gut symbiont, Clostridium butyricum, and its role in health and disease. *Gut Microbes* **13**, 1907272 (2021).

270. Parsaei, M., Sarafraz, N., Moaddab, S. Y. & Ebrahimzadeh Leylabadlo, H. The importance of Faecalibacterium prausnitzii in human health and diseases. *New Microbes New Infect* **43**, 100928 (2021).

271. Dai, W. *et al.* Discovery of Bacteroides uniformis F18-22 as a Safe and Novel Probiotic Bacterium for the Treatment of Ulcerative Colitis from the Healthy Human Colon. *International Journal of Molecular Sciences* **24**, 14669 (2023).

272. Wang, C. *et al.* Roles of intestinal bacteroides in human health and diseases. *Critical Reviews in Food Science and Nutrition* **61**, 3518–3536 (2021).

273. Yang, C. *et al.* Prevotella copri alleviates hyperglycemia and regulates gut microbiota and metabolic profiles in mice. *mSystems* **9**, e00532-24 (2024).

274. Heinken, A. *et al.* Functional Metabolic Map of Faecalibacterium prausnitzii, a Beneficial Human Gut Microbe. *Journal of Bacteriology* **196**, 3289–3302 (2014).

275. Mukherjee, A., Lordan, C., Ross, R. P. & Cotter, P. D. Gut microbes from the phylogenetically diverse genus Eubacterium and their various contributions to gut health. *Gut Microbes* **12**, 1802866 (2020).

276. Ryu, S. W. *et al.* Gut Microbiota Eubacterium callanderi Exerts Anti-Colorectal Cancer Activity. *Microbiology Spectrum* **10**, e02531-22 (2022).

277. Rastogi, S. & Singh, A. Gut microbiome and human health: Exploring how the probiotic genus Lactobacillus modulate immune responses. *Front. Pharmacol.* **13**, (2022).

278. Costea, P. I. *et al.* Subspecies in the global human gut microbiome. *Molecular Systems Biology* **13**, 960 (2017).

279. Kho, Z. Y. & Lal, S. K. The Human Gut Microbiome – A Potential Controller of Wellness and Disease. *Front. Microbiol.* **9**, (2018).

280. Binda, C. *et al.* Actinobacteria: A relevant minority for the maintenance of gut homeostasis. *Digestive and Liver Disease* **50**, 421–428 (2018).

281. Portincasa, P. *et al.* Gut Microbiota and Short Chain Fatty Acids: Implications in Glucose Homeostasis. *Int J Mol Sci* **23**, 1105 (2022).

282. He, J. *et al.* Short-Chain Fatty Acids and Their Association with Signalling Pathways in Inflammation, Glucose and Lipid Metabolism. *Int J Mol Sci* **21**, 6356 (2020).

283. Nogal, A., Valdes, A. M. & Menni, C. The role of short-chain fatty acids in the interplay between gut microbiota and diet in cardio-metabolic health. *Gut Microbes* **13**, 1897212.

284. Dwojaczny, B., Złomańczuk, P., Bejtka, M. & Loska, D. The fecal content of Veillonellaceae family bacteria correlates with cognitive parameters in young healthy human subjects. *Medical Research Journal* **9**, 282–289 (2024).

285. Kuo, C., El-Omar, E., Kao, C., Lin, J. & Wu, C. Compositional and Metabolomic Shifts of the Gut Microbiome in Alcohol-Related Liver Disease. *J Gastroenterol Hepatol* **40**, 2176–2189 (2025).

286. Pohl, K., Moodley, P. & Dhanda, A. D. Alcohol's Impact on the Gut and Liver. *Nutrients* **13**, 3170 (2021).

287. Amini Khiabani, S., Haghighat, S., Tayebi Khosroshahi, H., Asgharzadeh, M. & Samadi Kafil, H. Diversity of Bacteroidaceae family in gut microbiota of patients with chronic kidney disease and end stage renal disease. *Health Promot Perspect* **13**, 237–242 (2023).

288. Moreira de Gouveia, M. I., Bernalier-Donadille, A. & Jubelin, G. Enterobacteriaceae in the Human Gut: Dynamics and Ecological Roles in Health and Disease. *Biology* **13**, 142 (2024).

289. Fairfield, B. & Schnabl, B. Gut dysbiosis as a driver in alcohol-induced liver injury. *JHEP Reports* **3**, 100220 (2021).

290. Sayols-Baixeras, S. *et al.* Streptococcus Species Abundance in the Gut Is Linked to Subclinical Coronary Atherosclerosis in 8973 Participants From the SCAPIS Cohort. *Circulation* **148**, 459–472 (2023).

291. Kaci, G. *et al.* Anti-Inflammatory Properties of Streptococcus salivarius, a Commensal Bacterium of the Oral Cavity and Digestive Tract. *Appl Environ Microbiol* **80**, 928–934 (2014).

292. Mutlu, E. A. *et al.* Colonic microbiome is altered in alcoholism. *Am J Physiol Gastrointest Liver Physiol* **302**, G966-978 (2012).

293. Dubinkina, V. B. *et al.* Links of gut microbiota composition with alcohol dependence syndrome and alcoholic liver disease. *Microbiome* **5**, 141 (2017).

294. Wu, J. *et al.* Isolation and genomic characterization of five novel strains of Erysipelotrichaceae from commercial pigs. *BMC Microbiology* **21**, 125 (2021).

295. Chen, L. *et al.* NLRP12 attenuates colon inflammation by maintaining colonic microbial diversity and promoting protective commensal bacterial growth. *Nat Immunol* **18**, 541–551 (2017).

296. Zafar, H. & Saier, M. H. Gut Bacteroides species in health and disease. *Gut Microbes* **13**, 1848158.

297. Cheng, J., Hu, J., Geng, F. & Nie, S. *Bacteroides* utilization for dietary polysaccharides and their beneficial effects on gut health. *Food Science and Human Wellness* **11**, 1101–1110 (2022).

298. Calleja-Conde, J. *et al.* The Immune System through the Lens of Alcohol Intake and Gut Microbiota. *Int J Mol Sci* **22**, 7485 (2021).

299. Karas, J. A. *et al.* The Antimicrobial Activity of Cannabinoids. *Antibiotics* **9**, 406 (2020).

300. Mahmud, M. S. *et al.* Antimicrobial and Antiviral (SARS-CoV-2) Potential of Cannabinoids and Cannabis sativa: A Comprehensive Review. *Molecules* **26**, 7216 (2021).

301. Sionov, R. V. & Steinberg, D. Anti-Microbial Activity of Phytocannabinoids and Endocannabinoids in the Light of Their Physiological and Pathophysiological Roles. *Biomedicines* **10**, 631 (2022).

302. Klahn, P. Cannabinoids-Promising Antimicrobial Drugs or Intoxicants with Benefits? *Antibiotics* **9**, 297 (2020).

303. Smoking and Inflammation. *PLoS Med* **2**, e198 (2005).

304. Aloufi, N. *et al.* Standardized Cannabis Smoke Extract Induces Inflammation in Human Lung Fibroblasts. *Front Pharmacol* **13**, 852029 (2022).

305. Graves, B. M. *et al.* Comprehensive characterization of mainstream marijuana and tobacco smoke. *Sci Rep* **10**, 7160 (2020).

306. DiPatrizio, N. V. Endocannabinoids in the Gut. *Cannabis Cannabinoid Res* **1**, 67–77 (2016).

307. Srivastava, R. K., Lutz, B. & Ruiz de Azua, I. The Microbiome and Gut Endocannabinoid System in the Regulation of Stress Responses and Metabolism. *Front. Cell. Neurosci.* **16**, (2022).

308. Watanabe, K. *et al.* Conversion of cannabidiol to Δ9-tetrahydrocannabinol and related cannabinoids in artificial gastric juice, and their pharmacological effects in mice. *Forensic Toxicol* **25**, 16–21 (2007).

309. Janicova, A. *et al.* Acute Alcohol Intoxication Modulates Monocyte Subsets and Their Functions in a Time-Dependent Manner in Healthy Volunteers. *Front Immunol* **12**, 652488 (2021).

310. Commins, S. P., Borish, L. & Steinke, J. W. Immunologic messenger molecules: Cytokines, interferons, and chemokines. *Journal of Allergy and Clinical Immunology* **125**, S53–S72 (2010).

311. Bhol, N. K. *et al.* The interplay between cytokines, inflammation, and antioxidants: mechanistic insights and therapeutic potentials of various antioxidants and anti-cytokine compounds. *Biomedicine & Pharmacotherapy* **178**, 117177 (2024).

312. D'Elia, R. V., Harrison, K., Oyston, P. C., Lukaszewski, R. A. & Clark, G. C. Targeting the "Cytokine Storm" for Therapeutic Benefit. *Clin Vaccine Immunol* **20**, 319–327 (2013).

313. Al-Qahtani, A. A., Alhamlan, F. S. & Al-Qahtani, A. A. Pro-Inflammatory and Anti-Inflammatory Interleukins in Infectious Diseases: A Comprehensive Review. *Tropical Medicine and Infectious Disease* **9**, 13 (2024).

314. Mohammad, S. & Thiemermann, C. Role of Metabolic Endotoxemia in Systemic Inflammation and Potential Interventions. *Front Immunol* **11**, 594150 (2021).

315. Schumann, R. R. *et al.* Structure and function of lipopolysaccharide binding protein. *Science* **249**, 1429–1431 (1990).

316. Wang, H. J., Zakhari, S. & Jung, M. K. Alcohol, inflammation, and gut-liver-brain interactions in tissue damage and disease development. *World J Gastroenterol* **16**, 1304–1313 (2010).

317. Bishehsari, F. *et al.* Alcohol and Gut-Derived Inflammation. *Alcohol Res* **38**, 163–171 (2017).

318. Engen, P. A., Green, S. J., Voigt, R. M., Forsyth, C. B. & Keshavarzian, A. The Gastrointestinal Microbiome. *Alcohol Res* **37**, 223–236 (2015).

319. Singh, B., Goyal, A. & Patel, B. C. C-Reactive Protein: Clinical Relevance and Interpretation. in *StatPearls* (StatPearls Publishing, Treasure Island (FL), 2025).

320. Rogers, G. B. *et al.* From gut dysbiosis to altered brain function and mental illness: mechanisms and pathways. *Mol Psychiatry* **21**, 738–748 (2016).

321. Ahmed, G. K., Ramadan, H. K.-A., Elbeh, K. & Haridy, N. A. Bridging the gap: associations between gut microbiota and psychiatric disorders. *Middle East Current Psychiatry* **31**, 2 (2024).

322. Nagy, L. E. The Role of Innate Immunity in Alcoholic Liver Disease. *Alcohol Res* **37**, 237–250 (2015).

323. Henshaw, F. R., Dewsbury, L. S., Lim, C. K. & Steiner, G. Z. The Effects of Cannabinoids on Pro- and Anti-Inflammatory Cytokines: A Systematic Review of In Vivo Studies. *Cannabis Cannabinoid Res* **6**, 177–195 (2021).

324. Varodayan, F. P. *et al.* Chronic ethanol induces a pro-inflammatory switch in interleukin-1β regulation of GABAergic signaling in the medial prefrontal cortex of male mice. *Brain, Behavior, and Immunity* **110**, 125–139 (2023).

325. Marshall, S. A. *et al.* IL-1 receptor signaling in the basolateral amygdala modulates binge-like ethanol consumption in male C57BL/6J mice. *Brain Behav Immun* **51**, 258–267 (2016).

326. Patel, R. R. *et al.* Synaptic effects of IL-1β and CRF in the central amygdala after protracted alcohol abstinence in male rhesus macaques. *Neuropsychopharmacology* **47**, 847–856 (2022).

327. Muzamil, A. *et al.* Inflammatory Process and Role of Cytokines in Inflammation: An Overview. *Punjab Univ. J. Zool.* **36**, 235–250 (2022).

328. Sermet, S., LI, J., BACH, A., CRAWFORD, R. B. & KAMINKSI, N. E. Cannabidiol selectively modulates interleukin (IL)-1β and IL-6 production in toll-like receptor activated human peripheral blood monocytes. *Toxicology* **464**, 153016 (2021).

329. Xing, Z. *et al.* IL-6 is an antiinflammatory cytokine required for controlling local or systemic acute inflammatory responses. *J Clin Invest* **101**, 311–320 (1998).

330. Scheller, J., Chalaris, A., Schmidt-Arras, D. & Rose-John, S. The pro- and anti-inflammatory properties of the cytokine interleukin-6. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* **1813**, 878–888 (2011).

331. Blaine, S. K. *et al.* IL-6, but not TNF-α, response to alcohol cues and acute consumption associated with neural cue reactivity, craving, and future drinking in binge drinkers. *Brain Behav Immun Health* **31**, 100645 (2023).

332. Wang, B., Li, D., Fiselier, A., Kovalchuk, I. & Kovalchuk, O. High-CBD cannabis extracts inhibit the expression of proinflammatory factors via miRNA-mediated silencing in human small intestinal epithelial cells. *Heliyon* **9**, e18817 (2023).

333. Keen, L., Pereira, D. & Latimer, W. Self-reported lifetime marijuana use and interleukin-6 levels in middle-aged African Americans. *Drug and Alcohol Dependence* **140**, 156–160 (2014).

334. Suryanarayanan, A. *et al.* Role of interleukin-10 (IL-10) in regulation of GABAergic transmission and acute response to ethanol. *Neuropharmacology* **107**, 181–188 (2016).

335. Gao, B. & Xiang, X. Interleukin-22 from bench to bedside: a promising drug for epithelial repair. *Cell Mol Immunol* **16**, 666–667 (2019).

336. Yue, R. *et al.* Promoting intestinal antimicrobial defense and microbiome symbiosis contributes to IL-22-mediated protection against alcoholic hepatitis in mice. *Front. Immunol.* **14**, (2023).

337. Becker, W. *et al.* Activation of Cannabinoid Receptor 2 Prevents Colitis-Associated Colon Cancer through Myeloid Cell De-activation Upstream of IL-22 Production. *iScience* **23**, (2020).

338. Volpato, S. *et al.* Relationship of Alcohol Intake With Inflammatory Markers and Plasminogen Activator Inhibitior-1 in Well-Functioning Older Adults. *Circulation* **109**, 607–612 (2004).

339. Alshaarawy, O. & Anthony, J. C. CANNABIS SMOKING AND SERUM C-REACTIVE PROTEIN: A QUANTILE REGRESSIONS APPROACH BASED ON NHANES 2005–2010. *Drug Alcohol Depend* **0**, 203–207 (2015).

340. Morgan, E., Hudson, H., D'Aquila, R. & Mustanski, B. Plasma C-reactive protein is lower among marijuana using HIV-negative individuals but not among persons living with HIV. *Sci Rep* **11**, 4816 (2021).

341. Grodin, E. N., McManus, K. R. & Ray, L. A. Examining the moderating role of cannabis use on the relationship between alcohol consumption and inflammation in individuals with alcohol use disorder. *Addict Biol* **29**, e13431 (2024).

342. Hasegawa-Ishii, S., Inaba, M. & Shimada, A. Widespread time-dependent changes in tissue cytokine concentrations in brain regions during the acute phase of endotoxemia in mice. *NeuroToxicology* **76**, 67–74 (2020).

343. Tomioka, K. *et al.* Time course of serum cytokine level changes within 72 h after onset in children with acute encephalopathy and febrile seizures. *BMC Neurol* **23**, 7 (2023).

344. Kowalski, R. G. *et al.* Circulating Interleukin-6 Levels and Timing of Acute Ischemic Stroke Onset. *Ann Clin Transl Neurol* **12**, 1926–1931 (2025).

345. Garbarino, S., Lanteri, P., Bragazzi, N. L., Magnavita, N. & Scoditti, E. Role of sleep deprivation in immune-related disease risk and outcomes. *Commun Biol* **4**, 1304 (2021).

346. Poller, W. C. *et al.* Brain motor and fear circuits regulate leukocytes during acute stress. *Nature* **607**, 578–584 (2022).

347. Sun, Y., Qu, Y. & Zhu, J. The Relationship Between Inflammation and Post-traumatic Stress Disorder. *Front. Psychiatry* **12**, (2021).

348. Andersen, L., Corazon, S. S. & Stigsdotter, U. K. Nature Exposure and Its Effects on Immune System Functioning: A Systematic Review. *Int J Environ Res Public Health* **18**, 1416 (2021).

349. Volpato, S. *et al.* Relationship of Alcohol Intake With Inflammatory Markers and Plasminogen Activator Inhibitior-1 in Well-Functioning Older Adults. *Circulation* **109**, 607–612 (2004).

350. Gulhar, R., Ashraf, M. A. & Jialal, I. Physiology, Acute Phase Reactants. in *StatPearls* (StatPearls Publishing, Treasure Island (FL), 2025).

351. Hillmer, A. T., Nadim, H., Devine, L., Jatlow, P. & O'Malley, S. S. Acute alcohol consumption alters the peripheral cytokines IL-8 and TNF-α. *Alcohol* **85**, 95–99 (2020).

352. Raum, E., Gebhardt, K., Buchner, M., Schiltenwolf, M. & Brenner, H. Long-term and short-term alcohol consumption and levels of C-reactive protein. *Int J Cardiol* **121**, 224–226 (2007).

353. Costello, E. J., Copeland, W. E., Shanahan, L., Worthman, C. M. & Angold, A. C-Reactive Protein and substance use disorders in adolescence and early adulthood: A Prospective Analysis. *Drug Alcohol Depend* **133**, 712–717 (2013).

354. Albert, M. A., Glynn, R. J. & Ridker, P. M. Alcohol Consumption and Plasma Concentration of C-Reactive Protein. *Circulation* **107**, 443–447 (2003).

355. Pepys, M. B. & Hirschfield, G. M. C-reactive protein: a critical update. *J Clin Invest* **111**, 1805–1812 (2003).

356. Fachi, J. L. *et al.* Deficiency of IL-22–binding protein enhances the ability of the gut microbiota to protect against enteric pathogens. *Proceedings of the National Academy of Sciences* **121**, e2321836121 (2024).

357. Cineus, R. *et al.* The IL-22–oncostatin M axis promotes intestinal inflammation and tumorigenesis. *Nat Immunol* **26**, 837–853 (2025).

358. Li, L.-J., Gong, C., Zhao, M.-H. & Feng, B.-S. Role of interleukin-22 in inflammatory bowel disease. *World Journal of Gastroenterology* **20**, 18177–18188 (2014).

359. Mangnus, L., van Steenbergen, H. W., Nieuwenhuis, W. P., Reijnierse, M. & van der Helm-van Mil, A. H. M. Moderate use of alcohol is associated with lower levels of C reactive protein but not with less severe joint inflammation: a cross-sectional study in early RA and healthy volunteers. *RMD Open* **4**, e000577 (2018).

360. Bakhru, A. & Erlinger, T. P. Smoking Cessation and Cardiovascular Disease Risk Factors: Results from the Third National Health and Nutrition Examination Survey. *PLOS Medicine* **2**, e160 (2005).

361. Malfait, A. M. *et al.* The nonpsychoactive cannabis constituent cannabidiol is an oral anti-arthritic therapeutic in murine collagen-induced arthritis. *Proceedings of the National Academy of Sciences* **97**, 9561–9566 (2000).

362. Gallily, R., Yekhtin, Z. & Hanus, L. O. Overcoming the Bell-Shaped Dose-Response of Cannabidiol by Using Cannabis Extract Enriched in Cannabidiol. *Pharmacology & Pharmacy* **6**, 75–85 (2015).

363. Muscatell, K. A., Brosso, S. N. & Humphreys, K. L. Socioeconomic status and inflammation: a meta-analysis. *Mol Psychiatry* **25**, 2189–2199 (2020).

364. Adams, C., Conigrave, J. H., Lewohl, J., Haber, P. & Morley, K. C. Alcohol use disorder and circulating cytokines: A systematic review and meta-analysis. *Brain, Behavior, and Immunity* **89**, 501–512 (2020).

365. Bai, D. *et al. Eubacterium coprostanoligenes* alleviates chemotherapy-induced intestinal mucositis by enhancing intestinal mucus barrier. *Acta Pharmaceutica Sinica B* **14**, 1677–1692 (2024).

366. Jovanovic, J., Ornelis, V. F. M., Madder, A. & Rajkovic, A. Bacillus cereus food intoxication and toxicoinfection. *Comprehensive Reviews in Food Science and Food Safety* **20**, 3719–3761 (2021).

367. Zha, H. *et al.* Characteristics of the gut microbiota in Bifidobacterium catenulatum LI10 pretreated rats with lower levels of D-galactosamine-induced liver damage. *J. Appl. Microbiol.* **133**, 375–384 (2022).

368. Ma, Y. *et al.* Distinguishing feature of gut microbiota in Tibetan highland coronary artery disease patients and its link with diet. *Sci Rep* **11**, 18486 (2021).

369. Mizoguchi, A. Healing of Intestinal Inflammation by IL-22. *Inflamm Bowel Dis* **18**, 1777–1784 (2012).

370. Yu, C. *et al.* Interleukin-22 protects from endotoxemia by inducing suppressive F4/80+Ly6GhiLy6Chi cells population. *BMC Immunology* **23**, 45 (2022).

371. Patnaude, L. *et al.* Mechanisms and regulation of IL-22-mediated intestinal epithelial homeostasis and repair. *Life Sciences* **271**, 119195 (2021).

372. Jones, J., Reinke, S. N., Ali, A., Palmer, D. J. & Christophersen, C. T. Fecal sample collection methods and time of day impact microbiome composition and short chain fatty acid concentrations. *Sci Rep* **11**, 13964 (2021).

373. Hammer, A. M. *et al.* Interleukin-22 Prevents Microbial Dysbiosis and Promotes Intestinal Barrier Regeneration Following Acute Injury. *Shock* **48**, 657–665 (2017).