THESIS

# DIET AND MICROBIOME INTERACTIONS: ASSOCIATIONS IN POSTTRAUMATIC STRESS DISORDER (D-MAPS)

Submitted By

Thomas M. Aquilino

Department of Food Science and Human Nutrition

In partial fulfilment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Spring 2026

Master's Committee:

Advisor: Tiffany L. Weir
Brittney Sly
Bradley Conner

ABSTRACT

DIET AND MICROBIOME INTERACTIONS: ASSOCIATIONS IN POSTTRAUMATIC STRESS DISORDER (D-MAPS)

Posttraumatic Stress Disorder (PTSD) is a mental health disorder that develops after an individual experiences or witnesses a traumatic event. People with PTSD often have higher levels of inflammation potentially mediated by the gut microbiome. Current research suggests that increased gut microbial diversity reduces inflammation and inflammation-associated conditions. Specifically, the "Old Friends" hypothesis suggests that humans co-evolved with an extremely diverse microbial ecosystem that interacts with our immune system to protect against pathogenic microbes and inflammatory conditions through direct effects in the gut, as well as systemically, via the Gut-Brain-Immune axis. These microbes are influenced by our diets and lifestyles, and over time, the amount and diversity of plant-based foods in human diets have decreased. As a result, we have lost some of these "old friends" from the gut microbiota. Here, I review literature related to the "old friends" hypothesis and the interactions between the gut and brain (Chapter 1), describe a parallel arm, randomized controlled clinical intervention study protocol designed as a pilot study to test the impact of increasing plant diet diversity on gut microbial diversity, PTSD symptoms, and inflammation (Chapter 2), and describe the microbial communities of test beverages for the study (Chapter 3). Results for Chapter 2 have yet to be determined as the clinical study is still under way. Results from Chapter 3 revealed higher microbial diversity in the high plant diversity treatment beverage (30 plants) compared to the low plant diversity treatment beverage (3 plants). Both treatment beverage microbiomes were stable under refrigerated conditions (4°C) for four weeks.

The long-term objective is to test the hypothesis that daily consumption of a high plant diversity beverage will increase microbial diversity in the gut-microbiome of people with diagnosed PTSD, improve PTSD symptoms, and lower levels of inflammation-associated biomarkers. This work lays the foundation for pursuing this hypothesis.

# ACKNOWLEDGEMENTS

My academic journey has been shaped by several good people who have influenced my education to this point, even by simply lending me their time.

I would like to express my deepest gratitude to Dr. Tiffany Weir for accepting me into Colorado State University, giving me a position in her clinical research laboratory, guiding my understanding of academic research, allowing me to fail in the process, and giving me grace to learn from those failures.

I would like to thank all of the professors who have taught and instructed my graduate classes. Every one of them has helped me to reacclimate to an academic environment and faithfully answered every question I had so I could gain clarity in their lessons.

To the friends I have made during my time at CSU, I am grateful that our paths have crossed, even if it was only briefly. I have been in the best company throughout my time here. I hope for the best for each one of you and hope that I might return the favor in the future.

To my undergraduate mentor, Dr. Cesar Marquez, I am grateful for setting a high standard for my academic and professional career. The simple act of talking over coffee in your office allowed me to integrate my imagination into my education and your mentorship steered my interests in academia, leading me to this point.

To my family, I am forever grateful. Each one of you helped to raise me with the values of honesty, compassion, respect, and responsibility which govern my actions every day.

To my daughter, Violet, you are my reason to improve every day. I would otherwise fail my duty as a father if I do not act as a man should to protect you, provide for you, and to be your guiderail as you make your own way through life. You give me strength to fight the monsters, and the happiness to laugh in this world.

TABLE OF CONTENTS

ABSTRACT……ii

ACKNOWLEDGEMENTS……iii

Chapter 1: The Loss of Old Friends and The Rise of Inflammation Associated Mental Health Disorders…….....1

1.1. Introduction.....1

1.2. The "Old Friends" Hypothesis…….….2

1.3. Microbiota and Immune Development…….4

1.4. Microbiota-Gut-Brain Axis…….……..5

1.4.1. Short Chain Fatty Acids.....7

1.4.2. Microbial Hormone Production……..…….....10

1.5. Aberrant MGBA Signaling and Inflammation……..…….….12

1.6. Microbiome and Mental Health…….….14

1.7. Dietary Plant Diversity as A Source of Microbial Diversity…….……..15

1.8. Recovering "Old Friends"……..……..…16

Chapter 2: Diet and Microbiome Associations in Posttraumatic Stress Disorder (D-MAPS) Study Protocol……..17

2.1. Introduction…….17

2.2. Methods……..……..……19

2.2.1. Study Design……...……...……..19

2.2.2. Primary Endpoints……...……20

2.2.3. Secondary endpoints……...……...……..21

2.3. Method for Randomization……..……...….21

2.4. Blinding Procedures…….……...……21

2.5. Treatment Beverages……..……...……..22

2.6. Location of Study Visits, Data Collection, and Personnel……..……..……..23
2.7. Sample/Specimen Collection……..……...…….23
2.8. Recruitment and Enrollment……...……24
2.8.1. Screening Call……..……24
2.8.2. Eligibility Criteria……...…….25
2.9. Subject Participation……...……26
2.9.1. Informed Consent Process and Timing of Obtaining of Consent……26
2.9.2. Withdrawal/Termination Criteria…….26
2.9.3. Costs to Subjects, Individual Data Information, Payment, and Injury Protocol……..27
2.9.4. Risk/Benefit Assessment……...…28
2.9.5. Assessment of Subject Safety and Development of Data and Safety Monitoring Plan…...29
2.10. Data collection and procedures to protect Subject confidentiality…….…..30
2.11. Study Procedures…….……..32
2.11.1. Visit 1…….……32
2.11.1a Vegetable Standardization……..……...…34
2.11.2. Visit 2……..……...……35
2.11.3. Visit 3……..……...35
2.11.4. Visit 4……..……...…….35
2.12. Results…….……..……36
Chapter 3: Microbial Stability of Plant-based Beverages……..37
Abstract……...……37
3.1. Introduction……...……..38
3.2. Methods……..…….40
3.2.1. Plant-based beverage preparation and sampling…….……40
3.2.2. Sequencing Library Preparation…….…….……40
3.2.3. Sequence Data Analysis…….……..……..……..41

3.3. Results……...……..43
3.3.1. Microbial stability over time ……..…….43
3.3.2. Differences in microbial communities between high and low diversity beverages…….…46
3.3.3. Differences in microbial communities between treatment beverages…….47
3.3.4. Differential abundance within treatment beverages …….49
3.4. Discussion……...……50
3.5. Limitations… ……...……..51
3.6. Future Directions ……...……52
References…….…….……...……..54
Appendix……....62

# CHAPTER 1: THE LOSS OF OLD FRIENDS AND THE RISE OF INFLAMMATION ASSOCIATED MENTAL HEALTH DISORDERS

## *1.1. Introduction*

No matter how hostile an environment, microbial communities can be found thriving (Shu and Huang 2022). The human ecosystem is no exception; the gut microbiome, containing trillions of organisms that occupy the gastrointestinal (GI) system, has co-evolved with humans (Moeller et al. 2016). The gut microbiome is comprised of bacteria, fungi, archaea, and viruses, although the bacterial component has been the most intensively studied (Matijašić et al. 2020). These microorganisms colonize the GI tract during birth and are further shaped by dynamic environmental factors of each individual's lifestyle, including diet, exercise, degree of societal industrialization, proximity to animals, and cultural and sanitation practices.(Rook and Lowry 2008) These microorganisms produce bioactive compounds from indigestible dietary ingredients, mediate host immune interactions, and provide protection from pathogenic organisms to profoundly impact human health.(Rook and Lowry 2008; Turnbaugh and Gordon 2009).

One area of human health that is impacted by the gut microbiome is mental health and wellbeing (Cryan et al. 2019). Over one billion people worldwide live with at least one mental health condition (The Lancet, 2022), including almost 20% of adults in the US (~52 million) who report anxiety and depression (Bureau, 2021). Recently, links between the gut microbiota and mental health have been revealed through studies of the microbiome-gut-brain axis (MGBA) and understanding these links could open new avenues for treating or preventing mental health disorders (Tait and Sayuk 2021).

In this chapter, the association of gut microbial diversity with development of chronic inflammation, leading to or resulting from aberrant signaling of the MGBA, will be explored for its potential to cause or exacerbate mental health issues. In addition, a dietary strategy to improve mental health outcomes through enhancing gut microbiota diversity, which includes dietary enrichment of environmental microbes, will be discussed.

### *1.2. The "Old Friends" Hypothesis*

As humans progressed into industrialized societies, key exposure points with microbial species have been greatly restricted (Parajuli et al. 2018). While this has largely been to our benefit, reducing the burden of diseases like dysentery and cholera, decreased exposure to microbes is also correlated with the rising rates of autoimmune and chronic inflammatory diseases including asthma, allergies, inflammatory bowel disease, and inflammation associated mental disorders (Langgartner et al. 2019). Most denizens in developed cities have reduced their exposure to diverse microorganisms as they do not trek through the wilderness, hunt and butcher their own meat, or even tend gardens, as was common practice prior to industrialization (Parajuli et al. 2018). Imagine how many microbial interactions our ancestors encountered while foraging food or hunting wild game. Studies in modern hunter/gatherer populations have shown that the microbiomes of these individuals contain higher microbial diversity (Turroni et al. 2016; Smits et al. 2017). As well, their gut microbiomes harbor microbes that have disappeared from westernized populations (Smits et al. 2017).

Our gut microbiota is influenced by factors encountered in everyday life including what we eat, drink, and interact with in our environments (Flandroy et al. 2018). Numerous factors associated with modern life- such as increased intake of ultra-processed foods, reduced intake of

fiber rich plant-based foods, reduced rate and duration of breastfeeding, and less time outdoors have resulted in "microbial extinctions" that are compounded across generations (Mueller et al. 2015). For example, infants delivered via Cesarean section (C-section) do not show colonization of several common microbes like *Lactobacillus* and *Prevotella,* yet these infants display markedly increased levels of the intestinal pathogen *Clostridium difficile* (Mueller et al. 2015). In addition, the authors noted that infants who were exposed to antibiotics in the womb displayed significantly lower abundance of keystone neonatal gut bacteria, such as *Bifidobacterium* and *Lactobacilli* (Mueller et al. 2015; Bjorksten 2000).

Advances in sanitation have further reduced our microbial exposures (Freeman et al. 2017). Store bought produce is usually treated to prevent pests and contamination, and most packaged food and beverages have been pasteurized, sterilized, and processed to prevent microbial exposure (Chiozzi et al. 2022). Thus, it is unsurprising that modern lifestyles limit our microbial exposures and disrupt the balance and proper functioning of our gut microbiota, leading to development of chronic inflammation and stress-related diseases, including chronic intestinal diseases and psychological conditions (Redondo-Useros et al. 2020).

Two complementary hypotheses have been proposed to highlight the role of human microbiota as a driving factor in the development of these chronic, inflammation-associated diseases. The "Hygiene Hypothesis" posits that excessive food safety and personal hygiene practices, particularly in early life, cause aberrant training of the immune system resulting in increased incidence of asthma and allergies (Langgartner et al. 2019). The "Old Friends Hypothesis" refines this idea by suggesting that humans have co-evolved with certain microbes that are important in modulating host responses to their environment, including immune and inflammatory responses (Rook and Lowry 2008). The loss of these co-evolved microbes is

associated with increased inflammation leading to higher incidence of autoimmune and chronic diseases. For example, children with allergies tend to have fewer gut-associated *Lactobacilli* (Bjorksten 2000), and a clinical study suggests that administering high doses of *Lactobacilli* could help prevent atopic eczema in children who are genetically predisposed, although the conclusions of this trial have been criticized due to an insufficient body of evidence to support their claims as well as questions pertaining to selection bias (Reading 2001; Kalliomäki et al. 2003).

### *1.3. Microbiota and Immune Development*

How the infant gut microbiome develops from birth through the first few years of life, along with subsequent microbial exposures, are thought to have substantial consequences for inflammatory and autoimmune implications throughout the lifespan (Stewart et al. 2018). Up to 70% of the body's immune cells reside within gut-associated lymphoid tissue, or GALT (Mayer 2011). During early life development, microbiota play a key role in maturing the immune system and enteric nervous system (ENS) signaling (Stewart et al. 2018). This is thought to be mediated to a large extent through short-chain fatty acids (SCFAs), which provide fuel and act as signaling molecules for developing immune cells (Corrêa-Oliveira et al. 2016). The gut microbiota also produces hormones and other neuroactive compounds that affect digestion and gut motility (Strandwitz 2018). Finally, dendritic cells check gut contents for allergens and pathogens and help activate T cells that launch immune responses or quell immune reactivity against benign signals, establishing immune tolerance (KELSALL and LEON 2005). Aberrant "training" of the immune system can impair immunosuppressive T regulatory (Treg) cell development, as well as

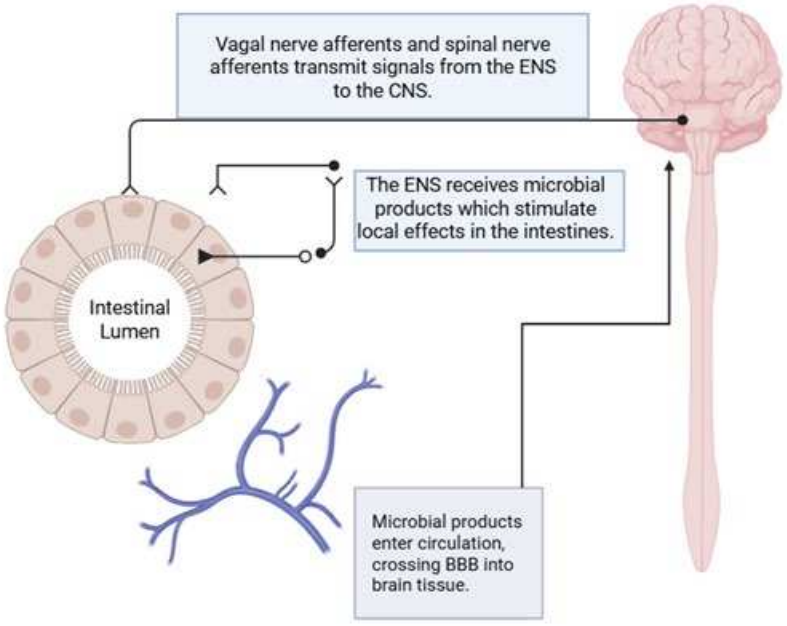
typically immunogenic T-helper 1 (Th1) and T-helper 2 (Th2) immune cells (Bjorksten 2000), resulting in increased inflammatory and autoimmune diseases.

A European study investigating the development of wheeze, rhinitis, and fever among 983 European infants with a genetic predisposition for developing asthma found that children raised in a rural environment, specifically those exposed to animal sheds and dogs, had a lower incidence of wheeze in the first year of life and lower rates of asthma diagnosis at a 6 year follow-up (Loss et al. 2016). While development of these conditions is multifactorial, environmental microbial exposures were hypothesized as priming the infants' immune systems against viral and bacterial infections inherent to rhinitis and viral wheeze.

### *1.4. Microbiota-Gut-Brain Axis*

"Old friends" inhabiting the gut microbiome communicate through a microbial-gut-brain-axis (MGBA) via metabolite, hormone, and neuroactive compounds (Tait and Sayuk 2021). Although early work in this area focused on inflammation-driven allergies, autoimmune, and other chronic diseases, the identification of the MGBA has further highlighted the importance of microbial exposure and its consequences on human health. The MGBA consists of the central nervous system (CNS), the autonomic nervous system (ANS), the gastrointestinal tract, and gut microbiota (Tait and Sayuk 2021). Cross talk within the MGBA occurs primarily through stimulation of part of the parasympathetic nervous system known as the vagus nerve (Bonaz et al. 2018). The vagus nerve connects the brainstem to the abdomen and links the brain (CNS) to the enteric nervous system (ENS), a subsection of the ANS that spans the gastrointestinal tract. The ENS is comprised of two major layers: the myenteric and submucosal nerve plexuses

(Benarroch 2007). The myenteric nerve plexus forms around the muscle layer of the intestines where most of the motor neurons responsible for GI motility reside (Benarroch 2007). The submucosal nerve plexus, situated closer to the intestinal lumen, operates along the border of the intestinal mucosa and muscles lining the intestine and is thought to interact with microbial signals produced in the intestinal lumen (Vicentini et al. 2021). Enteroendocrine cells (EECs) act as a connection point, passing microbial signals between the gut and the CNS via enteric and vagus nerve signaling (Ye et al. 2020). Microbes interface through the ENS to stimulate muscle contraction, relaxation, neurogenesis, and stimulate the release of gastric secretions as well as signaling hormones. Below, specific microbial metabolites important in MGBA signaling are highlighted.




(Fig 1.) Microbial metabolites capable of crossing the BBB travel through circulation, while neuroactive compounds are received by enteroendocrine cells where they interact with neural afferents stimulating vagal signaling to the CNS, resulting in immuno- and inflammatory modulation in the Brain. The enteric nervous system (ENS) receives and sends signals within the intestine as well as outside through the autonomic nervous system (ANS) subsection of the central nervous system (CNS). These interconnected nerves transmit signals throughout host tissues influencing motor, hormone, and secretory functions in the intestinal tract. In the brain, these signals influence processes related to hunger, satiety, pain, and inflammation.

### *1.4.1 Short Chain Fatty Acids*

Microbial metabolites, such as short chain fatty acids (SCFAs) act as signaling molecules along the MGBA, modify immune function, and regulate inflammatory processes (Tait and Sayuk 2021). SFCAs, primarily acetate, propionate, and butyrate, are produced from microbial

fermentation of dietary fiber and resistant starches. Acetate crosses the blood-brain barrier to directly interact with the hypothalamus, affecting appetite regulation and energy homeostasis (Frost et al. 2014) and can also be utilized as a substrate for cholesterol and fatty acid production, as well as acetyl-CoA (Bose et al. 2019). Propionate can regulate gluconeogenesis in human hepatocytes as well as provide a substrate for gluconeogenesis in intestinal cells (Yoshida et al. 2019; De Vadder et al. 2014). Butyrate largely remains in the intestines and is the primary fuel source for intestinal colonocytes (Donohoe et al. 2011), performing a supportive function in gut barrier maintenance (Kelly et al. 2015). Although butyrate largely remains in the intestines, it has modulatory effects on immune and inflammatory activity by stimulating the differentiation of dendritic, T reg, and microglial cells (Zhu et al. 2021), highlighting an important immunoregulatory function of SCFAs.

Butyrate is also a signaling molecule for glial cells within the enteric nervous system (Defries and Beltran 2020) and can circulate through the blood stream to cross the blood brain barrier (BBB), affecting microglial cells in the CNS (Oldendorf 1973). Microglial cells are primary immune responders in the brain and thus form an important foundation as progenitors of neuroinflammation. SCFA activity directly reduced the secretion of the neuroinflammatory cytokine TNF-α in a mouse model (Ezzine et al. 2022). SCFAs have also been reported to reduce NF-kB pathway activation, a proinflammatory pathway that is often overactive in disorders associated with hyper-vigilance like PTSD (Zhu et al. 2018). This suppression occurs via a reduction in histone deacetylase activity in microglial cells after intercellular diffusion (Zhu et al. 2021). Butyrate's histone deacetylase activity also induces the differentiation of nascent T-cells into T-regulatory (Tr1) cells and upregulating production of anti-inflammatory IL-10 (Kaisar et al. 2017). A triple blind, randomized, controlled trial investigating SCFA supplementation and

psychosocial stress in males revealed SCFAs effectively attenuated cortisol responses to acute psychosocial stress (Dalile et al. 2020).

### *1.4.2 Microbial hormone and neuroactive compound production*

Microbially-derived hormones like serotonin and neuroactive compounds like dopamine and gamma-amino-butyric acid (GABA) signal through enteroendocrine cells within the Meissner's plexus, a branch of the submucosal nerve plexus as well as the myenteric plexus of the ENS (Dicks 2022).

GABA production is common among Bacteroides strains, including lactobacillus species, and is readily produced under pH dependent conditions (Otaru et al. 2021). GABA is known to act as an inhibitory neurotransmitter and its production can protect against acidic damage in the intestinal lumen (Hyland and Cryan 2010; Otaru et al. 2021). Although it is not yet understood how much GABA produced in the GI tract is consumed by the habitant microbiota to maintain homeostasis, one study found that relative abundance of Bacteroides species, a prominent GABA producer, was negatively associated with brain specific magnetic resonance imaging (MRI) signatures associated with depression in a cohort of 23 participants with diagnosed depression or bipolar II disorder (Strandwitz et al. 2019).

Dopamine, a catecholamine similar to norepinephrine, is an excitatory neurotransmitter with regulatory implications in both the central nervous system for cognitive abilities and mood, as well as in the peripheral nervous system, especially for gastric secretions and motility (Asano et al. 2012). Although short-lived in biological environments, dopamine has been shown to be produced by several Bacteroides strains, including lactobacillus, Enterococcus, and Bacillus species, given access to its precursor L-Dopa (Hamamah et al. 2022).

Microbes can synthesize the amino acid tryptophan, or facilitate its release from dietary peptides, increasing the production of intestinal serotonin (5-HT). Around 90% of serotonin in the body is found within the intestine (Koopman et al. 2021). Serotonin within the intestine functions to maintain GI motility and modulate inflammation. It can also influence the proliferation of certain microbial taxa within the gut microbiome (Hwang and Oh 2025). In this way, serotonin both directly and indirectly affects the gut microbiome. However, higher concentrations of intestinally derived serotonin have been shown to stimulate the proliferation of inflammatory cytokines like IL-1B and immune cells while lower concentrations inhibit pro-inflammatory TNF-α, highlighting a regulatory role on the inflammatory process that can have consequences on the CNS (Herr et al. 2017).

Interactions between microbial metabolites and enteric neural cells can influence neurotransmitter production and signaling pathways, including communication with the hypothalamus and other brain regions (Ahmed et al. 2022). In this way, the MGBA comprises a concert of effector signaling that regulates vital gut functioning such as GI motility, secretory function, and epithelial barrier integrity and can dictate patterns of inflammatory signaling that influence mood and behavior (Wood 2011).

### *1.5. Aberrant MGBA Signaling and Inflammation*

Inflammatory responses can result from signaling cascades triggered by a stressful event that activates the fight or flight response (Frank et al. 2015). Take the example of coming face to face with a grizzly bear. Visual receptors in our eyes identify the threat, and the message is sent to the brain, specifically the amygdala, which initiates a stress response through noradrenaline (norepinephrine) release from the locus coeruleus (Kim et al. 2020; McCall et al. 2017). Noradrenaline signaling to the hypothalamic-pituitary adrenal axis (HPA axis) stimulates release of corticotropin releasing hormone (Herman et al. 2016). The pituitary gland then secretes adrenocorticotropic hormone into the bloodstream where it stimulates release of cortisol from adrenal glands and adrenaline (epinephrine), a key hormone in the fight-or-flight response system (Kageyama et al. 2021). The resulting effect is an increase in heart rate and blood circulation, but β-adrenergic signaling is also received by cell surface receptors on immune cells like macrophages and neutrophils to stimulate proinflammatory cytokine signaling, mainly through Interleukin-6 (IL-6) (Li et al. 2015).

Elevated levels of cortisol suppress the neurotransmitters, dopamine and serotonin, which can negatively alter mood, while cortisol also enhances vigilance to optimize probability of survival in a dangerous scenario (Baumeister et al. 2014). Modulation of endocrine homeostasis alters the stasis of the human body as a connected system disrupting immune cell synthesis and maturation as well as signaling related to psychological stasis (James et al. 2023). Chronically elevated levels of cortisol dampen dopamine and serotonin signaling to such a point that it has been implicated in the progression of psychological and mood disorders including symptoms of depression, anxiety, obsessive compulsive disorder (OCD), and posttraumatic stress disorder (PTSD)(Baumeister et al. 2014).

Moreover, stressful events such as life trauma and combat experiences might instill an adapted behavior to predict and avoid future stressful events by sustaining this elevated state of vigilance, as if the grizzly bear were still nearby (Radley et al. 2015). This adapted behavior is thought to arise from changes in brain regions specific to stress response, namely the amygdala, hippocampus, and prefrontal cortex with alterations to glucocorticoid receptor density in response to HPA axis hyperactivity (Alt et al. 2010; Bremner 2002). The facilitation of This adapted behavior is associated with elevated levels of inflammatory biomarkers like TNF-α, IL-6, IL-1β, and C-reactive protein (CRP) in the progression of psychological disorders such as posttraumatic stress disorder (PTSD) in which those diagnosed with PTSD can exhibit persistent stress responses similar to those experienced at the moment of a witnessed traumatic event (Michopoulos et al. 2015).

Conflicting research suggests that chronic stress exerts an excitatory effect on the nfkB pathway (Liu et al. 2017; Feng et al. 2019), leading to overstimulation of inflammatory pathways that may result in a sustained immune response, including elevated cytokine and chemokine production. A persistently primed immune response could cause continued tissue damage and aberrant immune cell development that may overreact to normally commensal microbial species (Gottschalk et al. 2016).

While there is limited data from human studies exploring the implications of gut microbiota on stress responses, animal studies have suggested that resilience to stressors can be associated with more abundant beneficial taxa, such as *Akkermansia* and *Lactobacillus*, and fecal transplant from stress-resilient mice into naïve mice resulted in a transfer of the stress resilience phenotype(He et al. 2024). A suggested mechanism of action for the observed resilience to

stressors is regulation of microglial activation in the hippocampus, which led to fewer behaviors associated with depression when compared to the stress-sensitive mouse cohort.

Continued research has revealed the potential roles for interventions focused on the gut microbiome as therapeutic targets for inflammation-associated mental disorders, especially PTSD, where aberrant inflammatory signaling appears to overlap with the loss of microbial training of the immune system (Ke et al. 2023). Studies have shown microbial products impact a wide variety of implications on human health depending on microbial composition of the gut microbiome, host diet and lifestyle factors, and health status of the host (Singh et al. 2017; Martinez et al. 2021). As discussed, microbially derived SCFAs have been shown to support intestinal cell barrier integrity and immune cell development, while bacterial lipopolysaccharides (LPSs) can produced  a range of proinflammatory effects on the host system, which been linked to cognitive impairment and progression of depressive -like symptoms in murine models (Zhao et al. 2019).

### *1.6. Microbiome and Mental Health*

Emerging research highlights a strong connection between the gut microbiome and mental health, with growing evidence suggesting that microbial communities in the gastrointestinal tract can influence brain function and emotional well-being. Microbial metabolites interact with the gut-brain axis, affecting neurotransmitter synthesis, neuroinflammation, and stress responses. Individuals with major depressive disorder (MDD) often show altered gut microbiota composition (Cao et al. 2025; Prandovszky et al. 2025) In addition, a systematic review published in *BMC Psychiatry* found that individuals with depression and anxiety often exhibit reduced microbial diversity and an increase in pro-inflammatory bacteria, alongside a decrease in beneficial SCFA-producing microbes (Cao et al.

2025). In children, links between early-life gut microbiome composition showed changes in brain connectivity and increased risk of internalizing symptoms such as anxiety and depression in children (Querdasi et al. 2025). These findings support the idea that gut dysbiosis may contribute to mental health disorders and that microbiome-targeted therapies—including dietary interventions, could offer promising options for complementary therapies.

### *1.7. Dietary Plant Diversity as a Source of Microbial Diversity*

Plant foods inherently shape gut microbial diversity in animals, with higher intake of plant diversity generally correlating with increased gut microbial diversity and beneficial changes in microbial composition (Santhiravel et al. 2022; Beam et al. 2021). Plants also provide prebiotic nutrients in the form of fiber allowing for more microbial taxa to proliferate (Beam et al. 2021). Moreover, different plants harbor various microbial niches at different physical locations of the plant resulting in different microbiota presence across the roots, stem, and leaves of each plant (Schmid et al. 2021).

In a six-week- randomized-controlled dietary intervention consisting of a high diversity plant diet (≥30) or low diversity plant diet (<15), consuming a higher diversity of plant foods was associated with an alteration of the gut microbiome favoring increased microbial metabolite production as well as an alleviation of renal acid load in a cohort of 25 adults with chronic kidney disease (Stanford et al. 2025). This finding supports the hypothesis that increasing diversity of plant intake may incur increased exposure to diverse microbial species, and this increased microbial diversity is associated with rescue of metabolic function in a disease state.

Plant foods comprise a major exposure point to microbial interactions. Raw plant foods directly introduce microorganisms, while the fibers contained within plant foods provide a fuel source for gut microbiota to consume, generating the metabolic byproducts that can be harvested by GI absorption as nutrients or stimulate GI secretions and motility (Beam et al. 2021). The combination of fiber from plant foods as well as abundance of symbiotic SCFA producers can have a modulatory effect on neuronal signaling throughout the body and potentially regulate immune function, mood, and emotional responses (Silva et al. 2020). Therefore, plants are shown to be a suitable vehicle for the reintroduction of these microbial 'old friends" into the gut microbiome.

### 1.8. *Recovering "Old Friends"*

In the process of optimizing health outcomes, advancements in sanitation and hygiene have revealed vital roles for microbial interactions in the immune and inflammatory functions in human and other animal models as well as the consequences of removing these microbial "old friends.". Targeting the diversity of the human gut microbiome is a potential therapeutic to assist in slowing the progression, and potentially reversing some of the major risk factors for psychological disorders and diseases associated with chronic inflammation. Utilizing plants as a natural source of pre and probiotics to increase the diversity of microbiota may increase the overall stability of the gut microbiome and may have restorative effects on immunomodulation and reducing stress responses. Research in this field is necessary to elucidate the functions that microbial taxa perform within the human gut microbiome, and how these functions affect risk factors for inflammation-associated disorders and diseases.

# CHAPTER 2: DIET AND MICROBIOME ASSOCIATIONS IN POSTTRAUMATIC STRESS DISORDER (D-MAPS) STUDY PROTOCOL

## *2.1. Introduction*

Industrialized societies have seen a rise in stress-related psychiatric disorders, including anxiety disorders, mood disorders, and trauma-related disorders such as post-traumatic stress disorder (PTSD). It is thought that one of the major drivers of psychiatric disorders is neuroinflammation subsequent to chronic systemic inflammation. A contributing factor may be altered exposure to microbial species that have co-evolved with humans and influenced the development and maturation of the immune system. The Hygiene Hypothesis, or "Old Friends" hypothesis states that the marked rise in inflammation-associated disorders are, at least in part, due to reduced exposure to diverse microbial species with which humans have co-evolved. Recent studies have provided evidence that people who report consuming higher numbers of plant food types, and a higher frequency of fruits and vegetables, develop a more diverse gut microbiome which is associated with reduced inflammation and better health outcomes.

While the original plan was to have this study completed at the time of this document, unforeseen circumstances delayed the study. Since the study is currently underway, this chapter describing the protocols used are written as they will be executed throughout the study.

We will evaluate whether daily consumption of a drink consisting of an extremely high diversity of plants (30 plant species) for four weeks impacts the diversity of the gut microbiome, biological signatures of inflammation, quality of life and sleep quality, and PTSD symptoms among persons with a diagnosis of PTSD. We hypothesize that four weeks of daily consumption

of the high plant diversity beverage will increase gut microbiome α-diversity, reduce markers of systemic inflammation, and improve PTSD symptom severity relative to daily consumption of a beverage containing only three plant species. Overall, this study will provide a better understanding of whether increasing F&V diversity has additional benefits on measured outcomes compared to the amount of F&V consumption. We have designed a pilot, randomized controlled parallel arm diet intervention to address the following objectives:

*Objective 1:* To determine whether consuming a higher number of plant types, thereby increasing exposure to diverse plant-associated microbes, increases gut microbial diversity. Specifically, we will use fecal samples from individuals before and after 4-week consumption of a 4 oz beverage made with high (30 different vegetables) and low botanical diversity (three different vegetables) to assess taxonomic richness (CHAO) and diversity (Shannon) using 16s rRNA and metagenomic sequencing approaches.

*Objective 2:* To determine how differences in plant diversity consumption influence inflammation and immune signatures, specifically plasma hsCRP levels and number of circulating T-regulatory cells. hsCRP will be assayed using ELISA and T-cells and other immune cells will be profiled from collected peripheral blood mononuclear cells (PBMCs) via flow cytometry.

*Objective 3:* To determine whether gut microbial diversity and inflammatory profiles correlate with PTSD symptom severity. PTSD symptoms will be evaluated at first, midpoint, and last visits using the PCL-5 assessment and changes with treatment as well as correlating with other primary outcome measures will be determined.

This is a pilot study as we have no data with this intervention in a human population with which to accurately calculate a sample size powered to 80%. This data will serve as the basis of power calculations for future studies.

## *2.2. METHODS*

### *2.2.1. Study Design*

DMAPS study design is a blinded, randomized, parallel-arm, intervention trial conducted through Colorado State University's Food and Nutrition Clinical Research Laboratory (FNCRL) in the Department of Food Science and Human Nutrition.

The intervention consists of a four-week treatment period where the participants consume either a 4 oz beverage consisting of 30 different blended fruits and vegetables (high diversity F&V treatment; (see appendix for the type and weights of plants included) or a similar control beverage consisting of 4 oz of blended Power Greens mix (low diversity F&V treatment; Earthbound Farms Organic- containing baby kale, chard, and spinach). Both beverages will be prepared fresh every two weeks and packaged in sterile mylar foil 150 mL pouches with plastic spouts. The beverage contents were developed and standardized by study co-PI and the Clinical and Translational Research Centers (CTRC) Nutrition Services and will be prepared by trained Nutrition and Food Science students in the Gifford Building Metabolic Kitchen. Participants will receive their beverages as two-week allocations and will return to the clinic for a mid-point visit to receive the full beverage allocation.

During the intervention, participants will be free-living and adhere to their typical diets, with one exception. All participants will be instructed to consume the USDA Daily recommended servings of fruits and vegetables. The FNCRL is collaborating with a registered dietician nutritionist who will oversee Dietetics graduate students to provide instructions to personnel on completion of diet records and intake recommendations to standardize daily F&V serving intakes. Information materials will be provided to participants with guidelines for standardizing vegetable intake to the recommended 2.5 cups per day including 0.5 cups allotted in the 4oz treatment pouches). Further information materials will address guidelines for portion sizing in participants normal diets, acceptable forms of processed vegetables, examples of vegetables, and examples of foods that do not qualify as vegetables for the purposes of this study. The participants will be asked to provide 2-day diet records every two weeks throughout the study. They will also complete daily bowel movement records using the Bristol Stool Scale (BSS) and collect 3 fecal samples (baseline, mid-point and final) that will be returned to the clinic at scheduled visits. Blood samples and questionnaire data will be collected at the beginning and end of the study to measure baseline circulating inflammatory biomarkers and symptom severity of PTSD.

#### *2.2.2. Primary Endpoints*

i. Changes to PTSD severity: Measured by scores on a standard PTSD survey called PCL-5, a 20-item self-report measure that assesses the 20 DSM-5 symptoms of PTSD. Scores range from 0-80 with higher scores associated with worse outcomes.

ii. Microbiome Diversity: Changes in microbiome alpha diversity as assessed by 16sRNA and metagenomic sequencing approaches.

iii. Changes in inflammation markers: Changes in hsCRP levels and number of circulating T-regulatory cells.

#### *2.2.3. Secondary Endpoints*

i. Seated Blood Pressure

ii. Other self-assessment measurements of gut (GSRS, BSS) and mental health (RAND QOL, IDS-SR, PSS, PANAS, GAD-7, PSQI).

iii. Anthropometrics: Waist circumference, hip circumference, Body Mass Index (BMI).

### *2.3. Method for randomization*.

Randomization will be done in the Excel program for 50 potential subjects prior to the start of the enrollment. Upon enrollment into the study, each subject will be assigned either treatment A or B according to the randomization chart.

### *2.4. Blinding Procedures*

The study is a double-blind parallel arm intervention. The beverages will be labeled by food science students who are not directly involved in the study and they will provide the FNCRL's clinical lab coordinator with the blinding code in a sealed envelope. Our lab coordinator will not open the sealed envelope until all primary analyses are completed (Mixed Effects ANOVA per Intent to Treat on all primary and secondary outcomes). In a very unlikely case of a severe adverse event (which is an event that requires subject hospitalization), the medical personnel will be provided information about the A/B interventions by non-study personnel that is given access to the sealed envelope.

### *2.5. Treatment Beverages*

A recipe for the high diversity treatment beverage was compiled into a standardized recipe by co-PI and the Clinical and Translational Research Centers (CTRC) Nutrition Services and will be prepared by trained food science students in the Gifford Building Metabolic Kitchen. Standardized Recipe ingredients will be procured from local groceries stores in the Fort Collins/Denver area (See Appendix A). Participants will receive their beverages as two-week allocations and will need to return to the clinic for a mid-point visit to receive the full beverage allocation.

Vegetables selected for the high diversity treatment beverage represent an overall diversity of plant types and were not chosen for any specific ingredient. The high diversity treatment recipe consists of 15g of each of the following ingredients: Arugula, Belgian endive, bok choy, broccoli rabe, Brussell's sprouts, celery root, chayote squash, dill, Easter egg radish, ginger root, green onion, Italian parsley, kale greens, leek, beets, mint, nopales cactus pad, parsley, parsnip, poblano pepper, daikon radish, red chard, rhubarb, serrano pepper, sorrel, spinach, tomatillo, turmeric, watercress, yellow chili pepper, and lemon juice.

Substitutions for ingredients were allowed as long as the substituted ingredients exist within the same family as the lacking ingredient. 15g portions were combined in a blender with water and pureed until homogenous.

The low diversity beverage recipe consists of a blended Power Greens mix (baby kale, chard, and spinach as well as lemon juice. The ingredients were combined in a blender with water and blended until homogenous.

### *2.6. Location of Study Visits, Data Collection and Personnel*

All research will be conducted at the Food and Nutrition Clinical Research Laboratory (FNCRL) in the Gifford Building at Colorado State University.

### *2.7. Sample / Specimen Collection*

Samples will be labeled with a random three-digit number that corresponds to the specific participant. Study samples will be stored according to appropriate conditions for their optimized analysis (ie. frozen at -80C, -20C, refrigerated, or lyophilized). After the study analyses are completed, samples will be stored for no more than 3 years unless the participant has indicated the samples may be kept for future analyses in the consent form. If this is the case, they will be kept indefinitely.

Blood will be collected on site. A member of the research team trained in phlebotomy will place a small needle in an antecubital vein to collect a blood sample (~2 tablespoons or 30 mL) for measurements of overall health (comprehensive metabolic panels) and inflammation (hsCRP, PMBC analysis of immune cell populations).

Stool samples will be collected at home within 24 hours of the study visit. Participants will be provided with a stool collection kit and directions for collection and storage. Stool samples will be used to assess gut microbiota changes.

Participants will answer questions about food consumed on 2 separate days (one weekday and one weekend day). This will be completed at home using the online Automated Self-Administered 24-hour recall (ASA-24).

### *2.8. Recruitment and Enrollment*

We expect to screen approximately 100 individuals based on the number of participants screened in previous similar studies. We are planning to enroll ~50 participants with an anticipated attrition rate of 20%. We expect ~ 40 individuals to complete the study, which will give us sufficient power for determining differences in the primary endpoints. We have previously determined that an n=20/group was sufficient to detect changes in microbiota richness (CHAO) at alpha level= 0.05 with 80% power in a healthy adult population. However, we have no specific data in this participant population or with the intervention beverage we are providing; therefore, this is considered a pilot study that will be used to appropriately power future study outcomes.

Forty participants in the Fort Collins, Colorado area will be recruited through CSU Health network, CSU Adult Learner and Veteran's Services (ALVS), Larimer County Behavioral Health Services Longview Campus, Summit Stone Health Partners, and the Connections Adult Services from the Health District of Northern Larimer County, as well as through social media and physical flyers.

Interested individuals will be screened through an online Qualtrix survey and those who meet eligibility criteria are then scheduled for an in-person screening visit to confirm eligibility and consent to participate in the study.

#### *2.8.1. Screening call*

Prospective participants will be contacted via phone call where initial eligibility will be assessed by general screening criteria. Provisionally eligible and interested individuals will be emailed a copy of the consent form and scheduled for an in-person clinic visit (Visit 1).

Individuals will be enrolled in this study based on the following inclusion and exclusion criteria in Table 1. Participants will be between the ages of 18-60 years of age, adhere to an omnivorous diet, have a BMI between 18-29 $Kg/m^2$, and have a diagnosis of posttraumatic stress disorder or score of at least 31 on the PCL-5 (PTSD Checklist for DSM-5). Participants will be excluded if they have undergone antibiotic treatment within the past 2 months prior to the start of the study, if their BMI is less than 18 or over 29.9, if they adhere to a vegan or vegetarian diet, have any food allergies, unstable medication regimen, have a diagnosis of diseases such as cancer, diabetes, or autoimmune disease, or if they are pregnant or breast feeding at the start of the study. Specific medication use (other than antibiotics) would not necessarily disqualify potential participation if the participant has followed a consistent medication regimen for at least two months prior to the beginning of the study. Any medication changes or antibiotic use during the study would result in dismissal from participation.

### *2.8.2. Eligibility Criteria*

| Inclusion Criteria | Exclusion Criteria |
|---|---|
| Between the ages of 18-60 | Younger than 18 or older than 60 years of age |
| Omnivorous diet | Vegan or vegetarian diet |
| BMI between 18-29. $Kg/m^2$ | BMI less than 18 or over 29.9 |
| Diagnosis of posttraumatic stress disorder or a score of at least 31 on the PCL-5 | |
| | if potential participants have undergone antibiotic treatment within the past 2 months prior to the start of the study |
| | food allergies |
| | unstable medication regimen |
| | diagnosis of diseases such as cancer, diabetes, or autoimmune disease |
| | pregnant or breast feeding at the start of the study |
| | Any medication changes or antibiotic use during the study would result in dismissal from participation |

### *2.9. Subject Participation*

#### *2.9.1. Informed consent process and timing of obtaining of consent*

Informed consent is required prior to any data or sample collection. The "Consent Form," including detailed and comprehensive information about the study, is emailed to participants prior to their first scheduled visit where it is explained in detail by trained clinical personnel. Potential subjects are given time to review the form privately, and all questions are answered to completely clarify the study conditions before the subject signs the form. An additional copy of the consent form is also given to the subject to keep. Only individuals who are deemed able to understand and provide consent for themselves are enrolled in the study. This is assessed by study personnel at the time of screening and consent. All participants will be informed that consent can be withdrawn at any time, and they are free to end their participation in the study.

#### *2.9.2. Withdrawal/Termination Criteria*

Potential subjects are instructed on the first clinic visit that they can withdraw from the study any time at their own consideration. On the investigators' side, any subject that has a severe adverse event during the study (which is qualified as requiring medical attention or psychological referral) should be advised to withdraw from the study. Additionally, study participants will be withdrawn from the study if their medical and health status change over the course of the study such that they no longer meet inclusion and exclusion criteria (e.g. suicidal or homicidal ideation, pregnancy, initiation of antibiotics, engage in activities that interfere with the study endpoints, or if they develop allergies or contraindication to study intervention capsules or procedures).

If a mental health emergency should occur during study visits, data collection will be immediately terminated and that participant will be removed from the study and directed to the research team Clinical Psychologist if they indicate suicidality or homicidality. All participants will be provided with a handout that contains information regarding contact to the VA crisis line (988 #1) if the participant is a veteran, or the SAMHSA line (1-800-662-HELP (4357) if the participant is a civilian.

### *2.9.3. Costs to Subjects, individual data information, payment, and injury protocol*

There are no costs to subjects for participation in this study. Participants will be compensated $50 at the end of Visits 1 and 2 and an additional $100.00 at the end of the final visit. If the entire study is completed, that will equal a total of $200/participant.

Individual data will not be provided to the study participants. They will have access to the data analyzed upon publication through the clinicaltrials.gov website.

If a participant is injured because of participation in this study, they will be instructed to contact the Principal Investigator at the number listed in the "What If I Have Questions" section of the Consent form. The Colorado Governmental Immunity Act determines and may limit Colorado State University's legal responsibility if an injury happens because of this study. Should a participant need medical aid, their health insurance will be responsible for the costs. No compensation for injury will be provided to participants in this study.

### *2.9.4. Risk/benefit assessment:*

The risks to participation in this study are minimal and include psychological discomfort with collecting stool and physical discomfort from blood draws.

*Physical Risk*: The blood draws are only conducted by well-trained individuals and participants are given the opportunity to opt out of the blood draw if a vein is not found on the second attempt. Another possible physical risk could be increased gastrointestinal discomfort. Some people experience temporary increases in these symptoms after increasing vegetable intake. Potential allergic reactions to treatment or placebo ingredients may also occur; however, we are minimizing this risk by excluding participants with known allergies to any ingredient. There is a small chance for allergic reaction to unique vegetable ingredients that had not been previously consumed.

*Psychological risk*: To minimize the psychological discomfort of stool collection, it will occur in the participant's home and we provide a touch-free collection kit and discreet containers for transporting the stool from home to clinic. As mentioned in the physical risk section, there is a possibility of increased gas or bloating which could cause a level of psychological distress.

We do not expect any of the study procedures to trigger psychological distress, however, we will be administering surveys that ask about mood status, suicidal thoughts, and behavior responses to stress events. We will also be drawing blood, which is low risk, but may cause psychological distress in certain individuals. All study personnel will be instructed to monitor psychological stress levels and stop any procedure that is causing distress. A licensed psychologist in the State of Colorado and a member of our research team will review answers to questions about suicidality that indicates risk or any other information reported to study personnel that indicates suicidality or homicidality and will take necessary steps to protect the participant and others in accordance with Colorado State Law. We will also provide a flyer with crisis hotline phone numbers: VA crisis line (988 #1) for veterans, and SAMHSA line (1-800-662-HELP (4357) if the participant is a civilian. Additionally, the flyer will include information

regarding Colorado State University services for crisis intervention offered at the CSU Health & Medical Center.

We also collect emergency contact information in the consent form and assist in contacting these individuals in the event a significant stress event occurs. The diet quality of all participants in this study may improve and the improved diet quality may be associated with benefits such as improved gastrointestinal health or decreased inflammation due to the daily consumption of these vegetable drinks.

### *2.9.5. Assessment of Subject Safety and Development of Data and Safety Monitoring Plan*

Participants in this study will only be exposed to minimal risks. The intervention product has been developed in collaboration with CTRC Nutrition Center and will be prepared by students with ServSafe training certification and other food safety training and participants will be provided with explicit instructions on storage and use of the beverages (ie. maintain in refrigeration, consume within 2 hours of opening packages). Despite minimal participant risk, we anticipate potential for minor study related adverse events (AE). In the event of more severe adverse events, the participants will be asked to immediately report them to study personnel. Depending on the nature of the AE, they may be removed from the study or referred to a physician.

All observed or reported adverse events (AE) will be recorded at each study visit and reported to study personnel and the University Institutional Review Board. A serious AE will result in termination of participation, regardless of treatment causality. A serious AE is defined as an AE resulting in medical intervention, hospitalization, death or disability. All serious adverse

events must be reported to the sponsor within 24 hours of site notification. Investigators will follow CSU IRB reporting requirements for "reportable new information" using HRP 214-Form Reportable New Information.

### *2.10. Data collection and procedures to protect subject confidentiality*

The study data will be kept in separate locked locations. Samples will be labeled with a random three-digit number that corresponds to a specific participant. Participants identifying information including the informed consent documents will be kept separate from the obtained data. Only approved study staff will have access to the records.

All of the collected materials are obtained for research purposes only, and data are kept in strict confidence. No information will be given to anyone without permission from the subject. Confidentiality is assured by use of numerical coding for all data collected, and this information will be secured on a password protected computer or a locked file cabinet in the Principal Investigators' laboratory. Personnel who conduct the study and will have access to the data prior to de-identification and anyone else assisting with data entry or analysis will only be able to access de-identified data. De-identified data may be accessed and analyzed by graduate students that are not named on the protocol and will also be available to the project sponsors.

Clinical and laboratory data will be collected and stored electronically using Research Electronic Data Capture (REDCap; https://www.project-redcap.org/), an encrypted, secured, HIPAA compliant, and robust web-based software that meets all requirements for secure data management. Data will be double entered on a weekly basis by two authorized and trained individuals, as a measure of quality control, and a tracking sheet will be kept.

The data produced from this study will primarily consist of raw physiologic and biochemical data. When applicable, data will be presented using the International System of Units as defined by the National Institute of Standards and Technology. Other data will be provided with a detailed description of units of measure and how they were derived, along with other study information.

All data will be deposited into ClinicalTrials.gov, which is an NIH-approved repository. Data will be deposited prior to publication. Additionally, if gut microbiome sequence data is obtained (not currently part of the funded study), it will be uploaded along with the appropriate metadata to the Short Read Archive (http://www.ncbi.nlm.nih.gov/sra), hosted by the National Center for Biotechnology Information or the European Molecular Biology Laboratory-European Nucleotide Archive (EMBL-ENA; https://www.ebi.ac.uk/ena/browser/home). These archives make data widely available for use by the public.

The research community will be able to access scientific data through ClinicalTrials.gov, which is where the clinical trial will be registered prior to study onset. The associated NCT number will be included in scientific presentations, conference proceedings, and manuscripts. ClinicalTrials.gov is publicly available to anyone globally.

All research participants will be consented for broad sharing and re-use of de-identified individual-level data. Researchers will not need to request access to the data as it will be freely available for access on ClinicalTrials.gov. All participant identifiers will be removed from study data and maintained in a secure, separate file that will not be included in the repository. Identifiable data will be destroyed once finalized data are made available for public use.

No individual results will be provided to participants, unless there are concerning results (i.e. comprehensive metabolic panels). This data will then be provided to the participants, and they will be asked to share them with their personal physician.

### *2.11. Study Procedures:*

#### *2.11.1 Visit 1*

Study personnel will explain the purpose of the study and procedures and will obtain written consent from interested individuals.

Seated blood pressure will be taken followed by anthropometrics, including height and weight (to assess BMI), waist circumference, and hip circumference.

The PCL-5 will be administered to provide provisional confirmation of PTSD. The PTSD Checklist for DSM-5 (PCL-5) is the diagnostic criteria used by health care professionals to officially diagnose a patient with posttraumatic stress disorder (PTSD) (Ferrie et al. 2023). A score of >2 on 1 B item (questions 1-5), 1 C item (questions 6-7), 2 D items (questions 8-14), 2 E items (questions 15-20) or a total score between 31-33 is indicative of probable PTSD.

The PHQ-9 will also be administered to determine any risk of suicidal thoughts, which may indicate exclusion based on the clinical psychologist's evaluation of affirmative answers to question #9. The Patient Health Questionnaire (PHQ-9) is a 9-item questionnaire designed to capture self-reported symptomology and severity of depression and has been validated in medical, general population, and psychiatric samples (Beard et al. 2016).

Individuals meeting eligibility criteria will be administered additional questionnaires and will also provide a blood sample. Surveys related to gastrointestinal status include the Gastrointestinal Symptom Rating Scale (GSRS). The GSRS is a 15 item likert-type questionnaire

designed to capture symptom severity or absence of GI distress, including abdominal pain, indigestion, reflux, diarrhea, and constipation (Kulich et al. 2008). The Bristol Stool Scale (BSS) is a 7-point grading scale for self-assessment of bowel movement type based on consistency that has been validated as a reliable system for data collection (Blake et al. 2016). Quality of Life Assessment (RAND QOL SF-36) (Brazier et al. 1992) is a 36-item self-reported questionnaire design to assess health status across eight multi-item dimensions within three health domains, namely functionality, well-being, and general health. The eight dimensions assessed include physical functioning, social functioning, physical limitations to role functioning, emotional limitations to role functioning, mental health, vitality, pain, and general feelings of perceived changes in overall health. 7-Item Generalized Anxiety Disorder Questionnaire (GAD-7) (Löwe et al. 2008) is a well validated self-reported survey of anxiety symptomology in both professional and general population settings. Positive and Negative Affect Scale (PANAS) (Hovmand et al. 2023) is a 20-item questionnaire that is designed to capture participants' self-assessed experiences with a list of emotional descriptors such as "interested," "enthusiastic," or "afraid" using a 1-5 likert scale with 1 indicating "very slightly or not at all," and 5 indicating "extremely." The perceived Stress Scale (PSS) (Ezzati et al. 2014) is a well validated measure of subjective perceptions of stress that has been in use in various forms since 1983. DMAPS will utilize the PSS-10, a 10-item questionnaire to capture participants subjective estimate of their stress and mental health status. Pittsburgh Sleep Quality Index (PSQI) (Mollayeva et al. 2016) is a well validated tool for assessing one's overall sleep quality with a sensitivity of 89.6% and a specificity of 86.5%. However, the PSQI has noted limitations regarding issues of self-awareness on sleep quality. Also, there is no consensus on what defines optimal sleep quality, potentially resulting in confounding differences in perceived sleep quality among individuals.

7-Day Physical Activity Recall (PAR) is a guided survey capturing physical activity and hours of sleep within a 1-week reference period. Limitations include a lack of detail regarding quality of sleep and perception of physical exertion when categorizing bouts of physical activity logged.

Participants will be provided with a Bristol Stool Scale (BSS) diary, they will then be instructed on completion of the 2-day diet records including instruction on assessing portion sizes, and given a stool collection container and scheduled for Visit 2, which should occur ~1 week after the first visit. Compensation for eligible participants that complete this visit is $50. *Total time of this visit is approximately 1.5-2 hours.*

#### *2.11.1a Vegetable Standardization*

The FNCRL has developed an informational graphic (See Appendix) with our collaborating registered dietician to educate participants on how to standardize vegetable intake as well as what foods do or do not count as servings of vegetables to avoid confounding results due to potential increases in vegetable intake from "none at all" at baseline. Each participant will be given informational material as well as a scripted explanation about standardizing vegetable intake to meet the recommended daily intake for vegetables during the duration of the study.

#### *2.11.2 Visit 2*

Participants will come to the clinic with a self-collected stool sample, at least 7 days of bowel movements recorded, and a 2-day diet record. They will then be provided with an additional stool sample collection kit, BSS diary, treatment compliance record, adverse event forms, and a 2-week supply of intervention beverages (A or B). They will be scheduled for a mid-point check-in visit (Visit 3) and compensated $50.

### *2.11.3 Visit 3*

Participants will return their stool sample, 2-day diet records (completed online at home), the treatment compliance log and any unconsumed beverage packages, their BSS records and report any adverse events. They will be provided with their new allocation of beverages, as well as additional adverse events forms, stool and compliance diaries and a container for their final stool collection. They will be scheduled for their final visit (Visit 4).

### *2.11.4 Visit 4*

Participants will come to the clinic with a self-collected stool sample, BSS records, 2-day diet records (completed online at home), compliance log and any unused intervention beverages and any completed adverse event forms. At the clinic visit, they will complete all of the study questionnaires. A follow up assessment of anthropometrics, seated blood pressure, and a venous blood sample will also be performed/collected. They will be provided with $100 compensation.

## *2.12. Results*

Due to the study still being conducted, the results section will not contain data from study participants. Instead, results from analysis of the alpha diversity, beta diversity, and shelf stability for the two treatment beverages used in the study will be provided.

# CHAPTER 3: MICROBIAL STABILITY OF PLANT-BASED BEVERAGES

ABSTRACT

The “Old Friends Hypothesis” asserts that modern lifestyle factors including excessive hygiene practices and poor diet have contributed to a loss of interactions with microbial species that humans have relied on through our evolution for nutrient harvesting and immune function. These losses in microbial exposures have led to an increased prevalence of immune system dysregulation and therefore chronic inflammation and disease.

A potential remedy to reintroduce these microbial exposures is consumption of minimally processed plant-based foods. We hypothesize that increasing the diversity of consumed plants will result in increased exposure to microorganisms.

We used 16s rRNA sequencing to assess the stability and microbial communities of two vegetable-based beverages, one that had 30 different plants (high diversity) and another that contained only three different plant types (low diversity).

We found no significant difference in CHAO1 richness estimates or Pielou’s evenness index within either treatment over a four-week period. These results suggest that both beverages had microbiomes that were stable over time. Interestingly, the low diversity plant beverage had a higher Shannon’s Diversity than the high diversity beverage ($p=0.0038$). Additional studies are underway to see if these patterns will be replicated in different batches of these beverages. It also remains unknown what effects either beverage will have on the diversity of the human gut microbiome.

### *3.1. Introduction*

Diets rich in plant foods are reported to confer numerous health benefits including lower risk for mortality, cancer, and chronic diseases such as type II diabetes mellitus (Peña-Jorquera et al. 2023). These benefits potentially result from several factors including exposure to diverse phytochemicals (Saravanan and Parimelazhagan 2014), increased fiber intake (He et al. 2022), and lowering glycemic load through induction of incretin secretion and GLP-1 agonism (Zhou et al. 2008). However, it is currently not known whether ingesting a higher diversity of plants will also increase the diversity of mutualistic microorganisms introduced into the gut microbiome. We propose that consuming a diet rich in plant foods carries the additional benefit of diversifying the gut microbiome by introducing novel plant-associated microorganisms.

The "Old Friends Hypothesis" suggests that humans co-evolved with certain microorganisms present in their natural environment, and increased gut microbial diversity is often associated with health benefits to the host (Langgartner et al. 2019). The loss of these microbes due to modern lifestyles is associated with increased incidence of non-communicable chronic diseases, and their re-introduction may be beneficial for the host immune system and metabolic homeostasis (Langgartner et al. 2019).

Plants also co-evolved with microorganisms as they were cultivated in agricultural societies, and many functions of a healthy agricultural ecosystem rely on microbial activity. For example, microbial associations with plant roots can increase nutrient acquisition, as is the case with nitrogen-fixing rhizobia that is associated with legume roots (Chauhan et al. 2023). Other microbes can stimulate the production of phytochemicals and phytoalexins, components of the plant "immune system", providing protection against predators and pathogens (Darvill and Albersheim 1984).

Plant microbiomes are immensely diverse and vary by plant species as well as the different sections of the plants. In other words, the leaves of a plant contain different microbial taxa than the roots of that same plant (Beckers et al. 2017). A recent study reported that fruits and vegetables were the second largest dietary contributor, following dairy products, to food-associated microbes found in the human gut (Fackelmann et al. 2025). Therefore, the number and type of different bacterial taxa introduced into the human gut microbiome by plant-based foods may depend on consuming a variety of plant species and parts.

Our long-term objective is to investigate the hypothesis that increased consumption of diverse plant species and parts will increase the diversity of the gut microbiome, both through increasing exposure to plant-associated microbes and through providing diverse substrates to fuel the growth of commensal gut organisms. To explore this hypothesis, we developed a low plant diversity (3 types of leafy green vegetables) and a high plant diversity (30 plant types, including leaves, tubers, roots and modified stems) beverage to be tested in a human nutrition intervention study. However, to ensure these could be safely consumed by human research participants, we needed to establish the safety and stability of these beverages. Therefore, the objective of the current study is to 1) determine the microbial stability of these beverages over a four-week period, and 2) determine how the microbial communities differed between these beverages. To address these questions, we used 16s rRNA sequencing of longitudinally collected samples to assess the microbial dynamics of the test beverages.

### *3.2. Methods*

#### *3.2.1 Plant-based beverage preparation and sampling.*

Ingredients were procured from local grocery stores in the Fort Collins/Denver area. To prepare the high diversity vegetable beverage, 15g each of the following ingredients were minimally processed to remove dirt and other particulate matter while intending to preserve surface dwelling microbiota. The ingredients were combined in a kitchen blender and blended with a final volume of 2.35L and pureed until homogenous: mint, oregano, collard greens, leek, rainbow chard, anise-fennel bulb, spinach, green kale, chives, basil, dill, Italian parsley, dandelion greens, poblano pepper, parsnip, cilantro, brussels sprouts, celery root, broccoli, red cabbage, celery, tomatillo, radicchio, turmeric root, daikon, ginger, golden beet, red radish, and rutabaga.

The low diversity beverage recipe consists of a 450g clamshell container of "Simple Truth Power Greens" mix (baby kale, arugula, and spinach) placed in a blender and filled with water to a total volume of 2.35L and blended until homogenous.

Each batch was divided into six replicate samples, packaged in sanitized Mason jars and refrigerated at 4°C for four weeks. During this time, a subsample was collected each week from every replicate jar, for a total of 48 samples (6 low diversity week 1, 6 low diversity week 2, 6 low diversity week 3, 6 low diversity week 4, 6 high diversity week 1, 6 high diversity week 2, 6 high diversity week 3, 6 high diversity week 4) and stored at -80°C until extracted for DNA.

#### *3.2.2 Sequencing Library Preparation.*

DNA extraction from the collected subsamples was completed using the FastDNA® Kit (MP Biomedicals, #116540400) per manufacturer's protocol. Amplification of the V4 region of the 16S rRNA gene was carried out following the Earth Microbiome Project protocol and using

the 515F-806R primer set (Caporaso et al. 2012) containing a unique 12bp error-correcting barcode included on the forward primer. Cycling and sequencing conditions were as previously described (Lee et al. 2020).

Briefly, samples were purified using AmPure beads and pooled in equimolar ratios before being quantified on a Tape Station. DNA extraction controls, no template PCR controls, and a mock community (Zymo Company) were included on each sequencing plate. Sequencing was completed at the Colorado State University Next Generation Sequencing Core Facility.

### *3.2.3 Sequence Data Analysis.*

Sequence reads were imported into QIIME2 (version 2024.2) for quality control (Bolyen et al. 2019). Briefly, the sequence reads were demultiplexed and concatenated. Utilizing a Phred score cutoff of 30, sequences were examined for quality filtering. Upon examining the demultiplexed data, reverse reads did not meet quality filtering parameters. Data processing proceeded with single-end forward reads, which were truncated to 103 base pairs. All reads were binned into ASVs using the DADA2 pipeline (Callahan et al. 2016). Taxonomic assignments were made using Silva version 138 (Pruesse et al. 2007). Mitochondrial and chloroplast sequences were filtered from samples. Resulting feature tables, metadata, and taxonomy files were imported into MicrobiomeAnalyst (version 2.0) for marker data profiling (Chong et al. 2020; Lu et al. 2023). The minimum count filter was set at 4 and the low count filter set at 10% based on prevalence in samples. Data normalization was performed using total sum scaling. All downstream statistical analyses were performed at the feature-level where applicable, and at the highest taxonomic resolution otherwise. Chao1 index was used to estimate species richness which estimates the number of taxa in a sample. Pielou's evenness index was used to assess the abundancies of taxa distributed within a community based on frequency of occurrence. Diversity

was calculated using the Shannon-Wiener Index, which uses both the number and distribution of taxa to estimate the total community diversity. One-Way ANOVA was used to search for variance among the subsamples for each beverage, and a Dunnet's test was performed to determine whether the treatment beverage microbiomes differed among each week when compared with week 1 data.

Beta-diversity, the degree of dissimilarity, or distance between two communities, was determined by calculating Bray-Curtis distances and visualizing them on principle coordinates ordinations (PCoA). Distances were statistically analyzed using PERMANOVA with 999 permutations. Differentially abundant taxa were identified using Linear Discriminant Analysis Effect Size (LeFSe), which employs Kruskal-Wallis rank sum test to identify differentially abundant features and applies linear discriminant analysis (LDA) to evaluate the effect size of significant functional gene biomarkers within a sample microbiome.

### *3.3. Results*

#### *3.3.1 Microbial stability over time*

Figure 1. Alpha Diversity

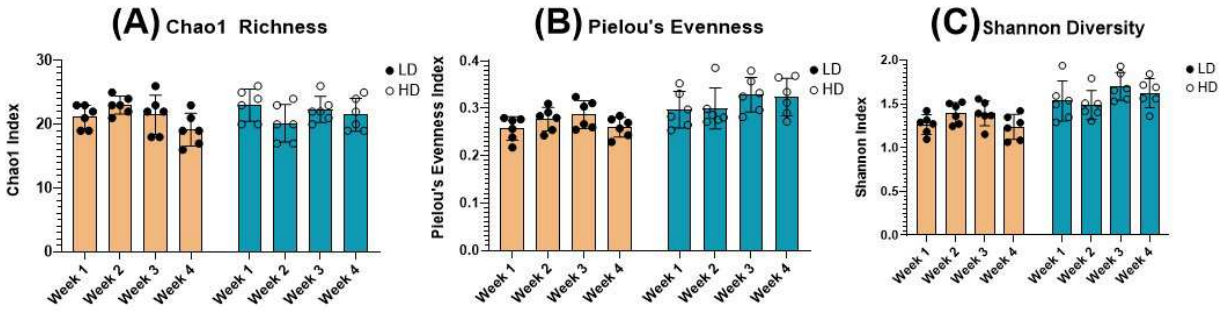



Figure 1. Alpha diversity indices for the high diversity vs low diversity treatments displaying changes in (A) Chao1 taxonomic richness of each treatment microbiome over the four-week period. (B) Pielou's evenness. (C) Shannon-Wiener index over a four-week period.

We assessed the stability of each drink preparation over a four-week time period. One-Way ANOVA followed by Dunnett's multiple comparisons test were employed to determine changes taxonomic richness, evenness, and Shannon-Wiener diversity within each treatment over the four-week time-period

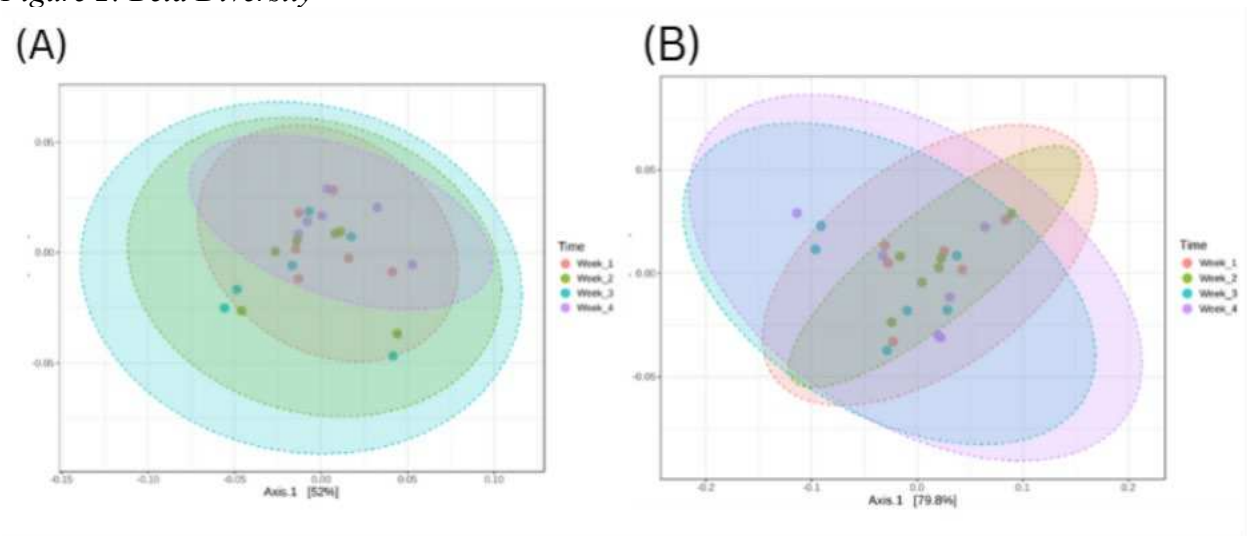
Chao1 richness indices were not statistically significant for either the low diversity or the high diversity beverage at any time over the four weeks of analysis (mean index = 21.21 for low diversity and mean index = 21.75 for the high diversity beverage).(Fig 1A)

One-Way ANOVA results showed Pielou's Evenness indices did not differ significantly across all for time points in either treatment beverage (low diversity p-value = 0.171; high diversity p-value = 0.409). A Dunnet's test revealed no week-to-week differences for either

treatment group compared with their respective week 1 samples. (low diversity week 2 p-value = 0.406, week 3 p-value = 0.138, week 4 p-value = 0.995; high diversity week 2 p-value = 0.999, week 3 p-value = 0.39, week 4 p-value = 0.519). (Fig 1b)

Results from One-Way ANOVA showed no significant difference in Shannon-Wiener alpha diversity between Week 1 and Week 4 for either of the two beverages (low diversity p-value = 0.7552; high diversity beverage p-value = 0.077). After employing a Dunnet's test, we found no significant difference from any week samples compared to the week 1 samples for either the low diversity treatment beverage (week 2 p –value = 0.510, week 3 p-value = 0.384, week 4 p-value = 0.5755) or the high diversity treatment beverage (week 2 p-value = 0.92, week 3 p-value = 0.111, week 4 p-value = 0.973).

*Figure 2: Beta Diversity*

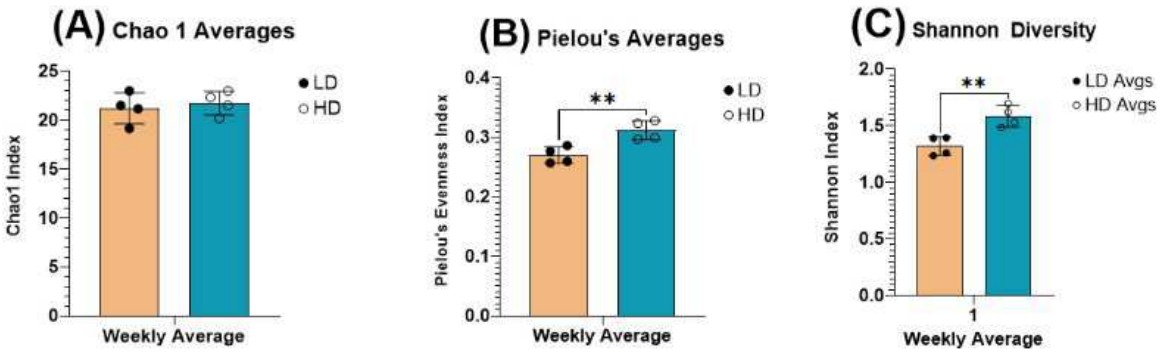



(Fig 2A) Low Diversity Treatment Beverage Beta Diversity PCoA ordination plot over four weeks.

A PCoA plot of Bray-Curtis distances demonstrated no significant longitudinal changes in community composition within either beverage preparation over the four weeks using PERMANOVA (low diversity: p= 0.340), (high diversity: p= 0.529). PERMDISP, which looks at the dispersion of communities within a group, also showed no significant differences within each beverage preparation (low diversity, p = 0.412; high diversity p = 0.766).

### *3.3.2 Differences in microbial communities between high and low diversity beverages*

Figure 3. Alpha Diversity between treatment groups

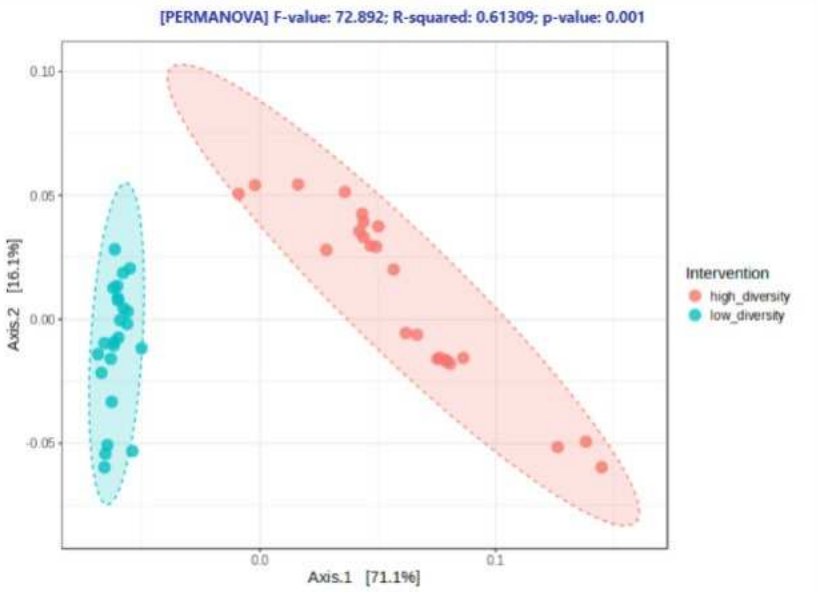



(Fig 3) (A)CHAO1 Index for the averages of the low and high diversity treatment beverages. (B) Pielou's Evenness index for the averages of the low and high diversity treatment beverages. (C) Shannon-Wiener Indices for the weekly averages.("*" indicates a p-value less than 0.05. "**" indicates a p-value less than 0.01. "***" indicates a p-value less than 0.001.)

We performed Students T Tests to determine if the average of the two treatment beverages were different from each other in Chao1, Pielou's evenness, or Shannon-Wiener indices. Differences were analyzed using a 2-tailed unpaired T-Test to compare the treatment microbiomes to each other. Results showed no significant difference in Chao1 richness (p-value = 0.683) between the treatment sample averages. There was a significant difference in Pielou's evenness indices (p-value = 0.008) between the low and high diversity treatment beverages, with the high diversity treatment microbiome having significantly higher evenness than the low diversity microbiome. Shannon-Wiener Diversity index for the high diversity treatment microbiome was significantly higher than the low diversity treatment microbiome (p-value = 0.006).

### *3.3.3 Differences in microbial communities between treatment beverages*

Figure 4. Beta Diversity between low diversity and high diversity treatment microbiomes.



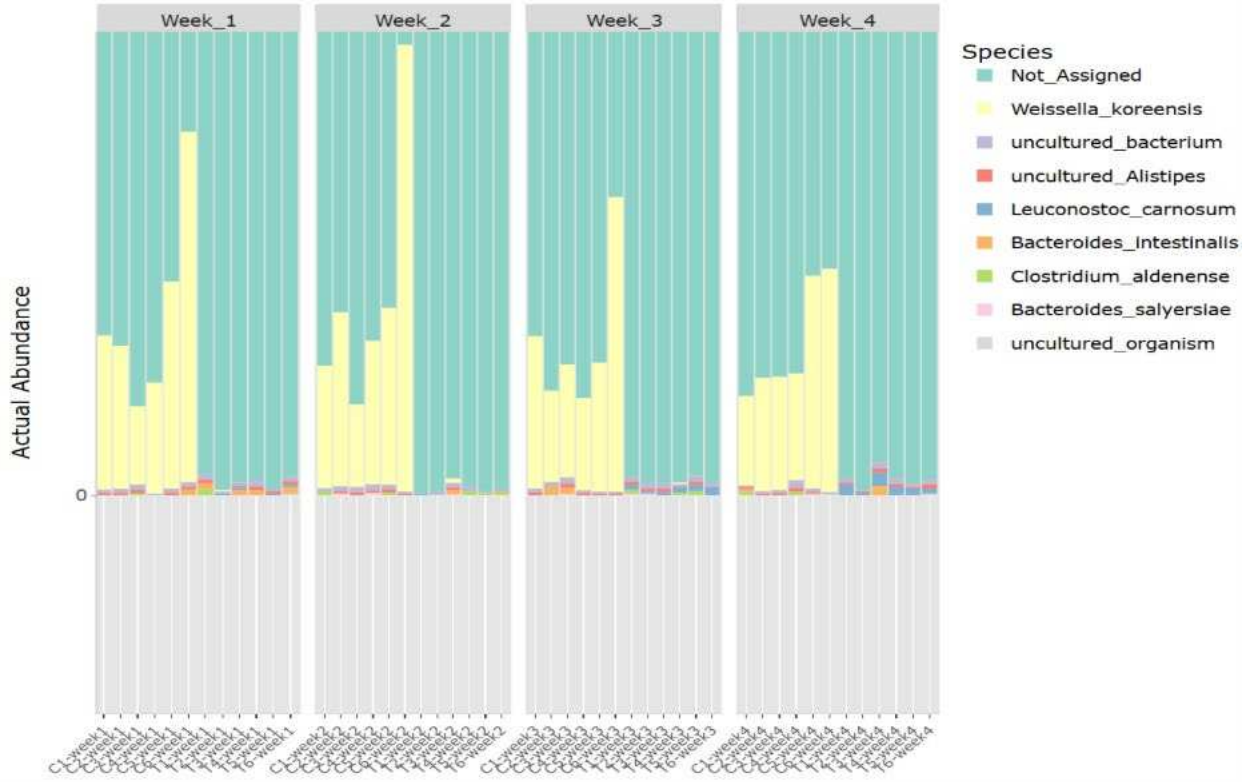

(Fig 4) High vs Low Beta Diversity ordination plot.

PERMANOVA and PERMDISP were conducted on feature-level beta diversity of both the low diversity treatment microbiome and high diversity over all four weeks of sample data. Results revealed a significant difference in Bray-Curtis distances between the two beverages (p-value = 0.001), confirming that they do significantly differ in microbial composition.

There was no significant difference observed between week 1 and week 4 (p-value = 0.36). (Fig 2B) Both treatment samples remained stable across all four weeks of sampling in every replicate as there was no significant difference in PCoA scores in the high diversity treatment beverage between week 1 and week 4 (p-value = 0.779). PERMDISP analysis of low

diversity treatment group revealed no significant difference in dispersion among either low diversity (p-value = 0.529) or high diversity (p-value = 0.766) over all four weeks.

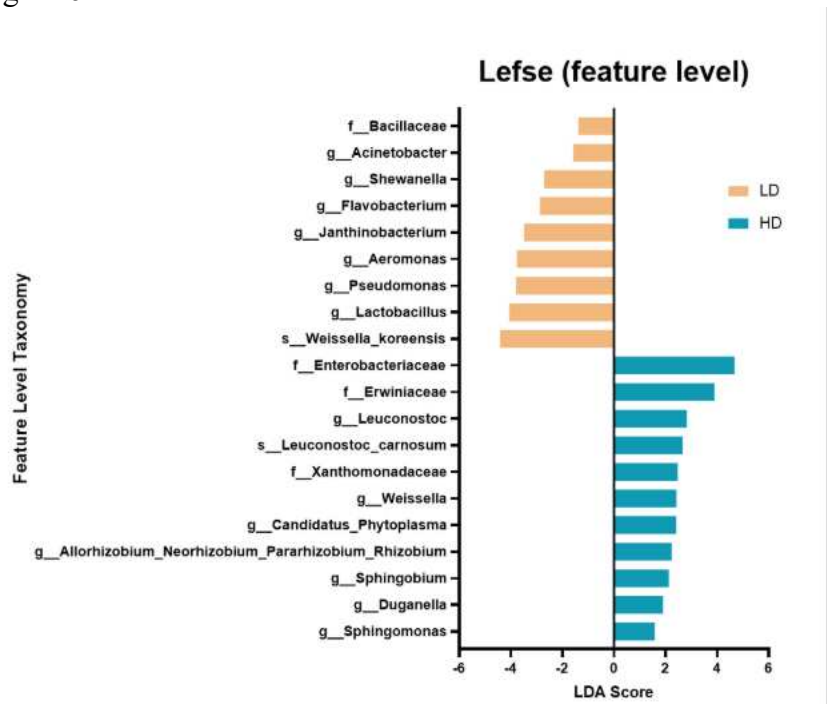
Figure 5. Taxabarplot (Species Level)




(Fig. 5) Relative abundance of identifiable species level taxonomic groups present in each sample of both the low diversity microbiome (C1-Week1 through C6-Week4) and the high diversity microbiome (T1-Week 1 through T6-Week 4).

### *3.3.4 Differential abundance within treatment beverages*

Figure 6. Lefse Feature Level




(Fig 6) LeFSe analysis differentially abundant taxa at the feature level characterizing the two distinct beverage preparations.

LeFSe was used to determine differentially abundant taxa associated with each of the beverages. Seven major genera, including *Acinetobacter, Shewanella, Aeromonas, Pseudomonas, Flavobacterium, Janthinobacterium*, and *Lactobacillus* were significantly higher in the low diversity beverage. In addition, one differentially abundant feature level ASV in the low diversity beverage was identified as *Weisella koreensis*, a primary fermentation organism in Korean kimchi (Park et al. 2010), constituting a large portion of the identified taxa within every week of sampling (LDA = 4.41).

The high diversity samples had eleven major differentially abundant taxa, including seven that identified to the genus-level: gen *Rhizobium, Weisella, Sphingobium, Duganella,*

*Leuconostoc, Sphingomonas*, and *Candidatus Phytoplasma;* three that could only be identified to the family level: Xanthmonadaceae, Erwiniceae, and Enterobacteraceae. Only one could be identified at the species-level, *Leuconostoc carnosum*, a species often associated with vaccuum packaged meat products but also found in fermented vegetables (Baka et al. 2014).

### *3.4. Discussion*

Before utilizing these beverages in a human dietary intervention study, it was important to evaluate the microbial communities to determine their safety and stability. We found the presence of the taxonomic family Enterobacteriaceae which contains species that are known as human pathogens, including Escherichia, Shigella, and Salmonella. However, it should be noted that 16s rRNA sequencing is insufficient to identify most bacteria to the species level and some bacteria, such as E. coli, cannot be distinguished from related genera, such as Shigella, using this approach. Further verification, such as fecal coliform testing and selective plating for toxin-producing enteric pathogens, will be needed to confirm absence of harmful bacteria.

There are several factors that could have contributed to the presence of these taxonomic families. Firstly, the low diversity vegetables were supplied by a "Power Greens" mix of spinach, chard, and baby kale from a local grocery store, however, it is unknown how many points of exposure it might have had through shipping and passerby customers.

Also, there were potential contaminations in at least one of the samples from bacteria exclusive to gut microbiome, including Bacteroides intestinalis and Bacteroides salyersiae. Therefore, it is unknown how many other bacteria were the result of contamination within this

singular sample data and lack of taxonomic resolution due to depth of analysis with the classification database used.

Given the multiple opportunities for sample contamination, the overall community compositions of the beverages remained stable over the four-week testing period. This is evidenced by both a lack of significant differences in alpha-diversity parameters at each time point compared to the Week 1 control and no significant clustering by week noted in Bray-Curts PCoA ordinations.

Finally, we found evidence that the high diversity beverage had significantly higher alpha diversity (Shannon-Wiener) than the low diversity treatment beverage. This is likely due to the variation in microbial composition among different plant parts, where leaf material has been shown to contain more diverse microbial taxa and therefore lower evenness, while the microbiome of plant roots often displays lower diversity of taxa but higher evenness among the taxa present (Beckers et al. 2017). While the low diversity treatment beverage contained only leaf material, the high diversity treatment beverage contained material from every part of each plants used (root, stem, and leaf).

## *3.5. Limitations*

It must be noted that these results are preliminary and there are several limitations to the study. First, the preliminary batch of the high diversity treatment beverage consisted of only thirty unique plant species. The clinical study will use a recipe including thirty unique plant species as well as a 5% dilution with lemon juice. As well, this data represents the microbial community of a single batch of each beverage preparation, tracked over time. The six replicate samples were split from these single large batches and are therefore replicated samples. However, this data is still useful as the beverages will be prepared in large batches and split into

individual packages for consumption. Our data shows limited variability in the distribution of microbes between individual packages within a batch but also demonstrates that these communities do not “evolve” individually over time as evidenced by no differences in PERMDISP. However, it is not known if these findings would be consistent across multiple batches made with ingredients sourced at different times. To address this, each test batch used in the human intervention trial will be subject to sequence analysis.

Another limitation to our study is the use of 16s rRNA sequencing. The resulting read length, in our case 104 bp, does not allow for detection of specific pathogens as noted above, but also is poor at classifying Microbes present on each vegetable might change depending on the location the vegetable was grown on, time of year, shipping conditions, as well as a plethora of other potential confounding factors. Therefore, it is unlikely that every batch made of the high diversity beverage would retain exactly reproduceable microbiomes. However, longitudinal analysis of multiple batches could potentially reveal patterns of microbial variation that allow for some valid predictions.

Lastly, these samples were maintained under refrigerated conditions that could have potentially interfered with the metabolic functioning of some microbial species more than others that could perhaps have more tolerance to colder temperatures. Therefore, this data is not generalizable to natural conditions for the microbial taxa present within the microbiomes of our treatment beverages.

## *3.6. Future Directions*

The available body of knowledge on the effects microbial interactions exert on human health is currently lacking. Specifically, our understanding of these interactions and human psychological health is a major gap in this body knowledge and requires much more research.

We are currently collecting data from participants adhering to a four-week double blind, parallel arm controlled clinical trial to analyze the effects of plant diversity on diversity of the gut microbiome. Our clinical study altered the recipe for the treatment beverages to include thirty plant species and a 5% dilution with lemon juice. While there is inherent risk of coming into contact with raw foods, the lemon juice dilution lowers pH below the threshold acidity in which Clostridium botulinum can thrive, offering a protective layer against illness caused by pathogenic bacteria.

Before conducting this study, we collected samples from a test batch of treatment beverages. We analyzed the microbiome profiles of two unique mixtures of vegetable drinks: a low diversity sample made of three plant sources and a higher diversity sample made from twenty-nine plant sources. We divided each sample into 6 replicates and stored them under refrigerated conditions for four weeks.

We used 16s rRNA sequencing to identify the microbial taxa present within each treatment microbiome and determined what changes occurred over each week relating to taxonomic richness, evenness, and overall diversity. Both treatment beverage microbiomes were found to be stable with no significant changes in any of the three metrics of diversity over a four week period suggesting that these treatment beverages are stable to measure changes to gut microbiome diversity in a clinical trial.

We are also collecting samples in triplicate from each batch of treatment beverages distributed during the clinical study to investigate how the low and high diversity treatment microbiomes compare across multiple iterations.

**References:**


1. Ahmed, Hany, Quentin Leyrolle, Ville Koistinen, et al. 2022. "Microbiota-Derived Metabolites as Drivers of Gut-Brain Communication." *Gut Microbes* 14 (1): 2102878-.
2. Baumeister, David, Alice Russell, Carmine M. Pariante, and Valeria Mondelli. 2014. "Inflammatory Biomarker Profiles of Mental D isorders and Their Relation to Clinical, Social and Lifestyle Factors." *Social Psychiatry and Psychiatric Epidemiology* (Berlin/Heidelberg) 49 (6): 841–49.
3. Alt, Simone R., Jonathan D. Turner, Melanie D. Klok, et al. 2010. "Differential Expression of Glucocorticoid Receptor Transcripts in Major Depressive Disorder Is Not Epigenetically Programmed." *Psychoneuroendocrinology* (Kidlington) 35 (4): 544–56.
4. Asano, Yasunari, Tetsuya Hiramoto, Ryo Nishino, et al. 2012. "Critical Role of Gut Microbiota in the Production of Biologically Active, Free Catecholamines in the Gut Lumen of Mice." *American Journal of Physiology: Gastrointestinal and Liver Physiology* (United States) 303 (11): G1288–95.
5. Baka, Maria, Estefanía Noriega, Laurence Mertens, Eva Van Derlinden, and Jan F.M. Van Impe. 2014. "Protective Role of Indigenous Leuconostoc Carnosum against Listeria Monocytogenes on Vacuum Packed Frankfurter Sausages at Suboptimal Temperatures." *Food Research International* (AMSTERDAM) 66: 197–206.
6. Beard, C, K.J Hsu, L.S Rifkin, A.B Busch, and T Björgvinsson. 2016. "Validation of the PHQ-9 in a Psychiatric Sample." *Journal of Affective Disorders* (Netherlands) 193: 267–73.
7. Benarroch, Eduardo E. 2007. "Enteric Nervous System: Functional Organization and Neurologic Implications." *Neurology* (United States) 69 (20): 1953–57.
8. Blake, M. R., J. M. Raker, and K. Whelan. 2016. "Validity and Reliability of the Bristol Stool Form Scale in Healthy Adults and Patients with Diarrhoea-predominant Irritable Bowel Syndrome." *Alimentary Pharmacology & Therapeutics* (HOBOKEN) 44 (7): 693–703.
9. Bonaz, Bruno, Thomas Bazin, and Sonia Pellissier. 2018. "The Vagus Nerve at the Interface of the Microbiota-Gut-Brain Axis." *Frontiers in Neuroscience* (Switzerland) 12: 49-.
10. Bose, Shree, Vijyendra Ramesh, and Jason W. Locasale. 2019. "Acetate Metabolism in Physiology, Cancer, and Beyond." *Trends in Cell Biology* (England) 29 (9): 695–703.
11. Brazier, J. E., R. Harper, N. M. Jones, et al. 1992. "Validating the SF-36 Health Survey Questionnaire: New Outcome Measure for Primary Care." *BMJ* (LONDON) 305 (6846): 160–64.

12. Bremner, J. Douglas. 2002. “Neuroimaging Studies in Post-Traumatic Stress Disorder.” *Current Psychiatry Reports* (United States) 4 (4): 254–63.

13. Bureau, US Census. n.d. “Population Under Age 18 Declined Last Decade.” Census.Gov. Accessed November 22, 2025. https://www.census.gov/library/stories/2021/08/united-states-adult-population-grew-faster-than-nations-total-population-from-2010-to-2020.html.

14. Caporaso, J Gregory, Christian L Lauber, William A Walters, et al. 2012. “Ultra-High-Throughput Microbial Community Analysis on the Illumina HiSeq and MiSeq Platforms.” *The ISME Journal* (London) 6 (8): 1621–24.

15. Chiozzi, Viola, Sofia Agriopoulou, and Theodoros Varzakas. 2022. “Advances, Applications, and Comparison of Thermal (Pasteurization, Sterilization, and Aseptic Packaging) against Non-Thermal (Ultrasounds, UV Radiation, Ozonation, High Hydrostatic Pressure) Technologies in Food Processing.” *Applied Sciences* (Basel) 12 (4): 2202-.

16. Chong, Jasmine, Peng Liu, Guangyan Zhou, and Jianguo Xia. 2020. “Using MicrobiomeAnalyst for Comprehensive Statistical, Functional, and Meta-Analysis of Microbiome Data.” *Nature Protocols* (London) 15 (3): 799–821.

17. Corrêa-Oliveira, Renan, José Luís Fachi, Aline Vieira, Fabio Takeo Sato, and Marco Aurélio R Vinolo. 2016. “Regulation of Immune Cell Function by Short-chain Fatty Acids.” *Clinical & Translational Immunology* (Australia) 5 (4): e73-n/a.

18. Cryan, John F., Kenneth J. O’Riordan, Caitlin S. M. Cowan, et al. 2019. “The Microbiota-Gut-Brain Axis.” *Physiological Reviews* (United States) 99 (4): 1877–2013.

19. Darvill, A.G, and P Albersheim. 1984. “Phytoalexins and Their Elicitors, a Defense against Microbial Infection in Plants.” *Annual Review of Plant Physiology* (Palo Alto, CA) 35 (1): 243–75.

20. Defries, Danielle, and Michelle Beltran. 2020. “Short Chain Fatty Acid Transporter/Receptor Expression and Signaling in Enteric Glial Cells.” *Current Developments in Nutrition* (Oxford) 4 (Supplement_2): nzaa057_017-nzaa057_017.

21. Dicks, Leon M. T. 2022. “Gut Bacteria and Neurotransmitters.” *Microorganisms (Basel)* (Switzerland) 10 (9): 1838-.

22. Ezzati, Ali, Julie Jiang, Mindy J. Katz, Martin J. Sliwinski, Molly E. Zimmerman, and Richard B. Lipton. 2014. “Validation of the Perceived Stress Scale in a Community Sample of Older Adults.” *International Journal of Geriatric Psychiatry* (Hove) 29 (6): 645–52.

23. Ferrie, Olivia, Thomas Richardson, Tanya Smart, and Colm Ellis-Nee. 2023. “A Validation of the PCL-5 Questionnaire for PTSD in Primary and Secondary Care.” *Psychological Trauma* (United States) 15 (5): 853–57.

24. Flandroy, Lucette, Theofilos Poutahidis, Gabriele Berg, et al. 2018. “The Impact of Human Activities and Lifestyles on the Interlinked Microbiota and Health of Humans and of Ecosystems.” *The Science of the Total Environment* (Netherlands) 627: 1018–38.

25. Frank, Matthew G., Michael D. Weber, Linda R. Watkins, and Steven F. Maier. 2015. “Stress Sounds the Alarmin: The Role of the Danger-Associated Molecular Pattern HMGB1 in Stress-Induced Neuroinflammatory Priming.” *Brain, Behavior, and Immunity* (Netherlands) 48: 1–7.

26. Freeman, Matthew C., Joshua V. Garn, Gloria D. Sclar, et al. 2017. “The Impact of Sanitation on Infectious Disease and Nutritional Status: A Systematic Review and Meta-Analysis.” *International Journal of Hygiene and Environmental Health* (Germany) 220 (6): 928–49.

27. “Global, Regional, and National Burden of 12 Mental Disorders in 204 Countries and Territories, 1990-2019: A Systematic Analysis for the Global Burden of Disease Study 2019.” 2022. *The Lancet. Psychiatry* 9 (2): 137–50.

28. Hamamah, Sevag, Armin Aghazarian, Anthony Nazaryan, Andras Hajnal, and Mihai Covasa. 2022. “Role of Microbiota-Gut-Brain Axis in Regulating Dopaminergic Signaling.” *Biomedicines* (Switzerland) 10 (2): 436-.

29. Herman, James P., Jessica M. McKlveen, Sriparna Ghosal, et al. 2016. “Regulation of the Hypothalamic-Pituitary-Adrenocortical Stress Response.” *Comprehensive Physiology* (Hoboken, NJ, USA), 603–21.

30. Hovmand, Oliver Rumle, Nina Reinholt, Anne Bryde Christensen, et al. 2023. “Affectivity in Danish Patients with Emotional Disorders: Assessing the Validity of the Positive and Negative Affect Schedule (PANAS).” *BMC Psychiatry* (England) 23 (1): 943–48.

31. Hyland, Niall P., and John F. Cryan. 2010. “A Gut Feeling about GABA: Focus on GABAB Receptors.” *Frontiers in Pharmacology* (Lausanne) 1: 124-.

32. James, Katharine Ann, Juliet Ilena Stromin, Nina Steenkamp, and Marc Irwin Combrinck. 2023. “Understanding the Relationships between Physiological and Psychosocial Stress, Cortisol and Cognition.” *Frontiers in Endocrinology (Lausanne)* (Switzerland) 14: 1085950-.

33. Kageyama, Kazunori, Yasumasa Iwasaki, and Makoto Daimon. 2021. “Hypothalamic Regulation of Corticotropin-Releasing Factor under Stress and Stress Resilience.” *International Journal of Molecular Sciences* (Switzerland) 22 (22): 12242-.

34. Kelly, Caleb J., Leon Zheng, Eric L. Campbell, et al. 2015. “Crosstalk between Microbiota-Derived Short-Chain Fatty Acids and Intestinal Epithelial HIF Augments Tissue Barrier Function.” *Cell Host & Microbe* (CAMBRIDGE) 17 (5): 662–71.

35. KELSALL, Brian L, and Francisco LEON. 2005. “Involvement of Intestinal Dendritic Cells in Oral Tolerance, Immunity to Pathogens, and Inflammatory Bowel Disease: Mucosal Immunity.” *Immunological Reviews* (Oxford) 206: 132–48.

36. Kim, T., N. Shen, J.-C. Hsiang, K.P. Johnson, and D. Kerschensteiner. 2020. “Dendritic and Parallel Processing of Visual Threats in the Retina Control Defensive Responses.” *Science Advances* (United States) 6 (47).

37. Koopman, Nienke, Drosos Katsavelis, Anne Ten Hove, Stanley Brul, Wouter de Jonge, and Jurgen Seppen. 2021. “The Multifaceted Role of Serotonin in Intestinal Homeostasis.” *International Journal of Molecular Sciences* (Switzerland) 22 (17): 9487-.

38. Kulich, Károly R, Ahmed Madisch, Franco Pacini, et al. 2008. “Reliability and Validity of the Gastrointestinal Symptom Rating Scale (GSRS) and Quality of Life in Reflux and Dyspepsia (QOLRAD) Questionnaire in Dyspepsia: A Six-Country Study.” *Health and Quality of Life Outcomes* (LONDON) 6 (1): 12–12.

39. Lee, Dustin M., Kayl E. Ecton, S. Raj J. Trikha, et al. 2020. “Microbial Metabolite Indole-3-Propionic Acid Supplementation Does Not Protect Mice from the Cardiometabolic Consequences of a Western Diet.” *American Journal of Physiology: Gastrointestinal and Liver Physiology* (United States) 319 (1): G51–62.

40. Löwe, Bernd, Oliver Decker, Stefanie Müller, et al. 2008. “Validation and Standardization of the Generalized Anxiety Disorder Screener (GAD-7) in the General Population.” *Medical Care* (PHILADELPHIA) 46 (3): 266–74.

41. Lu, Yao, Guangyan Zhou, Jessica Ewald, Zhiqiang Pang, Tanisha Shiri, and Jianguo Xia. 2023. “MicrobiomeAnalyst 2.0: Comprehensive Statistical, Functional and Integrative Analysis of Microbiome Data.” *Nucleic Acids Research* (England) 51 (W1): W310–18.

42. Matijašić, Mario, Tomislav Meštrović, Hana Čipčić Paljetak, Mihaela Perić, Anja Barešić, and Donatella Verbanac. 2020. “Gut Microbiota beyond Bacteria—Mycobiome, Virome, Archaeome, and Eukaryotic Parasites in IBD.” *International Journal of Molecular Sciences* (Switzerland) 21 (8): 2668-.

43. McCall, Jordan G, Edward R Siuda, Dionnet L Bhatti, et al. 2017. “Locus Coeruleus to Basolateral Amygdala Noradrenergic Projections Promote Anxiety-like Behavior.” *eLife* (England) 6.

44. Moeller, Andrew H., Alejandro Caro-Quintero, Deus Mjungu, et al. 2016. “Cospeciation of Gut Microbiota with Hominids.” *Science (American Association for the Advancement of Science)* (United States) 353 (6297): 380–82.

45. Mollayeva, Tatyana, Pravheen Thurairajah, Kirsteen Burton, Shirin Mollayeva, Colin M. Shapiro, and Angela Colantonio. 2016. “The Pittsburgh Sleep Quality Index as a Screening Tool for Sleep Dysfunction in Clinical and Non-Clinical Samples: A Systematic Review and Meta-Analysis.” *Sleep Medicine Reviews* 25 (February): 52–73. https://doi.org/10.1016/j.smrv.2015.01.009.

46. Otaru, Nize, Kun Ye, Denisa Mujezinovic, et al. 2021. “GABA Production by Human Intestinal Bacteroides Spp.: Prevalence, Regulation, and Role in Acid Stress Tolerance.” *Frontiers in Microbiology* (Switzerland) 12: 656895-.

47. Parajuli, Anirudra, Mira Grönroos, Nathan Siter, et al. 2018. “Urbanization Reduces Transfer of Diverse Environmental Microbiota Indoors.” *Frontiers in Microbiology* (Switzerland) 9: 84-.

48. Park, Republic of Korea, J.M., Konkuk University, Seoul, Republic of Korea Shin J.H., Konkuk University, Seoul, Republic of Korea Lee D.W., Konkuk University, Seoul, et al. 2010. “Identification of the Lactic Acid Bacteria in Kimchi According to Initial and Over-Ripened Fermentation Using PCR and 16S rRNA Gene Sequence Analysis.” *Food Science and Biotechnology* (Heidelberg) 19 (2): 541–46.

49. Querdasi, Francesca R., Jessica P. Uy, Jennifer S. Labus, et al. 2025. “Childhood Gut Microbiome Is Linked to Internalizing Symptoms at School Age via the Functional Connectome.” *Nature Communications* 16 (1): 9359. https://doi.org/10.1038/s41467-025-64988-6.

50. Radley, Jason, David Morilak, Victor Viau, and Serge Campeau. 2015. “Chronic Stress and Brain Plasticity: Mechanisms Underlying Adaptive and Maladaptive Changes and Implications for Stress-Related CNS Disorders.” *Neuroscience and Biobehavioral Reviews* (United States) 58: 79–91.

51. Redondo-Useros, Noemí, Esther Nova, Natalia González-Zancada, Ligia E. Díaz, Sonia Gómez-Martínez, and Ascensión Marcos. 2020. “Microbiota and Lifestyle: A Special Focus on Diet.” *Nutrients* (Switzerland) 12 (6): 1776-.

52. Rook, Graham A.W, and Christopher A Lowry. 2008. “The Hygiene Hypothesis and Psychiatric Disorders.” *Trends in Immunology* (England) 29 (4): 150–58.

53. Shu, Wen-Sheng, and Li-Nan Huang. 2022. “Microbial Diversity in Extreme Environments.” *Nature Reviews. Microbiology* (London) 20 (4): 219–35.

54. Stewart, Christopher J., Nadim J. Ajami, Jacqueline L. O’Brien, et al. 2018. “Temporal Development of the Gut Microbiome in Early Childhood from the TEDDY Study.” *Nature (London)* 562 (7728): 583-.

55. Strandwitz, Philip. 2018. “Neurotransmitter Modulation by the Gut Microbiota.” *Brain Research* (Netherlands) 1693 (Pt B): 128–33.

56. Strandwitz, Philip, Ki Hyun Kim, Darya Terekhova, et al. 2019. “GABA-Modulating Bacteria of the Human Gut Microbiota.” *Nature Microbiology* (London) 4 (3): 396–403.

57. Tait, Christopher, and Gregory S. Sayuk. 2021. “The Brain-Gut-Microbiotal Axis: A Framework for Understanding Functional GI Illness and Their Therapeutic Interventions.” *European Journal of Internal Medicine* (Netherlands) 84: 1–9.

58. Zhu, Li-Juan, Huan-Yu Ni, Rong Chen, et al. 2018. “Hippocampal Nuclear Factor Kappa B Accounts for Stress-induced Anxiety Behaviors via Enhancing Neuronal Nitric Oxide Synthase (nNOS)-carboxy-terminal PDZ Ligand of nNOS-Dexras1 Coupling.” *Journal of Neurochemistry* (England) 146 (5): 598–612.

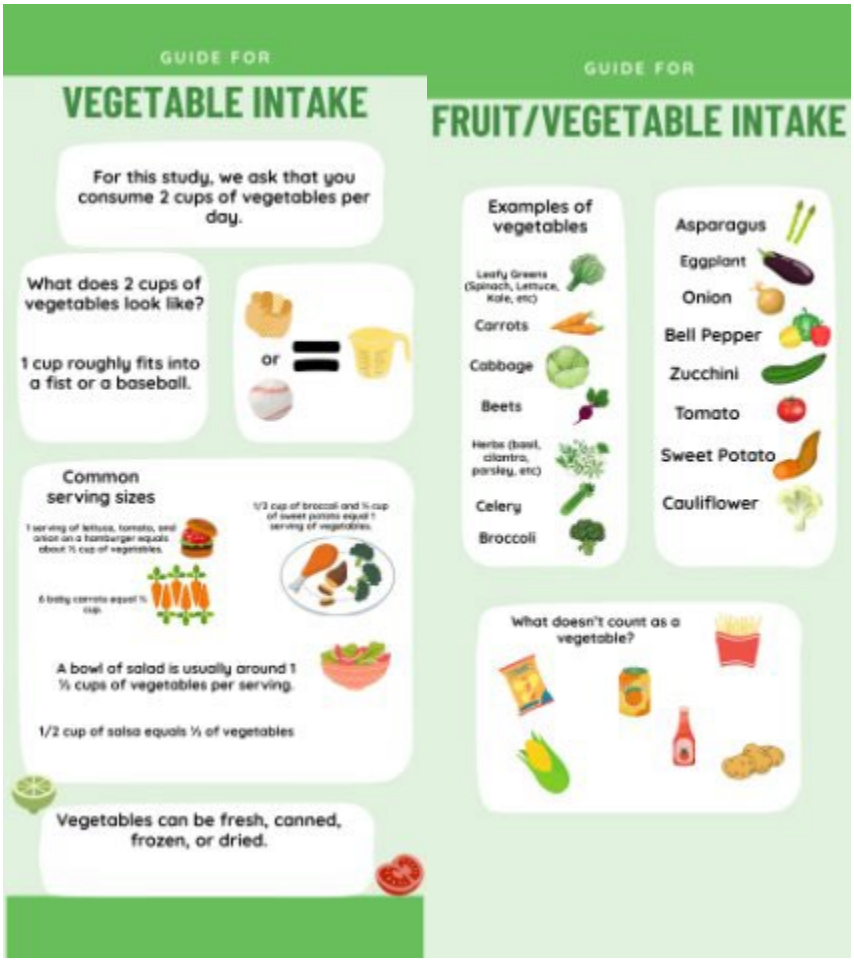
59. Beam, Ashley, Elizabeth Clinger, and Lei Hao. 2021. “Effect of Diet and Dietary Components on the Composition of the Gut Microbiota.” *Nutrients* (Switzerland) 13 (8): 2795-.
60. Bjorksten. 2000. “The Intestinal Microflora in Allergic Estonian and Swedish 2-Year-Old Children (Vol 29, Pg 342, 1999).” *Clinical and Experimental Allergy* (HOBOKEN) 30 (7): 1047–1047.
61. Cao, YuanYuan, YiRan Cheng, WenChao Pan, JianWei Diao, LingZhi Sun, and MiaoMiao Meng. 2025. “Gut Microbiota Variations in Depression and Anxiety: A Systematic Review.” *BMC Psychiatry* (London) 25 (1): 443–14.
62. Dalile, Boushra, Bram Vervliet, Gabriela Bergonzelli, Kristin Verbeke, and Lukas Van Oudenhove. 2020. “Colon-Delivered Short-Chain Fatty Acids Attenuate the Cortisol Response to Psychosocial Stress in Healthy Men: A Randomized, Placebo-Controlled Trial.” *Neuropsychopharmacology (New York, N.Y.)* (England) 45 (13): 2257–66.
63. De Vadder, Filipe, Petia Kovatcheva-Datchary, Daisy Goncalves, et al. 2014. “Microbiota-Generated Metabolites Promote Metabolic Benefits via Gut-Brain Neural Circuits.” *Cell* (CAMBRIDGE) 156 (1–2): 84–96.
64. Donohoe, Dallas R., Nikhil Garge, Xinxin Zhang, et al. 2011. “The Microbiome and Butyrate Regulate Energy Metabolism and Autophagy in the Mammalian Colon.” *Cell Metabolism* (CAMBRIDGE) 13 (5): 517–26.
65. Ezzine, Chaima, Léa Loison, Nadine Montbrion, et al. 2022. “Fatty Acids Produced by the Gut Microbiota Dampen Host Inflammatory Responses by Modulating Intestinal SUMOylation.” *Gut Microbes* 14 (1): 2108280-.
66. Feng, Xiujing, Yuan Zhao, Tianyuan Yang, et al. 2019. “Glucocorticoid-Driven NLRP3 Inflammasome Activation in Hippocampal Microglia Mediates Chronic Stress-Induced Depressive-Like Behaviors.” *Frontiers in Molecular Neuroscience* (Lausanne) 12: 210-.
67. Frost, Gary, Michelle L. Sleeth, Meliz Sahuri-Arisoylu, et al. 2014. “The Short-Chain Fatty Acid Acetate Reduces Appetite via a Central Homeostatic Mechanism.” *Nature Communications* (London) 5 (1): 3611-.
68. Gottschalk, Rachel A., Andrew J. Martins, Bastian R. Angermann, et al. 2016. “Distinct NF-κB and MAPK Activation Thresholds Uncouple Steady-State Microbe Sensing from Anti-Pathogen Inflammatory Responses.” *Cell Systems* (United States) 2 (6): 378–90.
69. He, Haili, Hui He, Li Mo, et al. 2024. “Gut Microbiota Regulate Stress Resistance by Influencing Microglia-Neuron Interactions in the Hippocampus.” *Brain, Behavior, & Immunity. Health* (United States) 36: 100729–100729.

70. Herr, Nadine, Christoph Bode, and Daniel Duerschmied. 2017. “The Effects of Serotonin in Immune Cells.” *Frontiers in Cardiovascular Medicine* (Switzerland) 4: 48-.
71. Hwang, Young Keun, and Jae Sang Oh. 2025. “Interaction of the Vagus Nerve and Serotonin in the Gut–Brain Axis.” *International Journal of Molecular Sciences* (Switzerland) 26 (3): 1160-.
72. Kaisar, Maria M. M., Leonard R. Pelgrom, Alwin J. van der Ham, Maria Yazdanbakhsh, and Bart Everts. 2017. “Butyrate Conditions Human Dendritic Cells to Prime Type 1 Regulatory T Cells via Both Histone Deacetylase Inhibition and G Protein-Coupled Receptor 109A Signaling.” *Frontiers in Immunology* (Switzerland) 8: 1429-.
73. Kalliomäki, M, S Salminen, H Arvilommi, and E Isolauri. 2003. “Probiotics for Prevention of Atopic Disease?” *The Lancet (British Edition)* (London) 362 (9382): 496–496.
74. Ke, Shanlin, Jakob Hartmann, Kerry J. Ressler, Yang-Yu Liu, and Karestan C. Koenen. 2023. “The Emerging Role of the Gut Microbiome in Posttraumatic Stress Disorder.” *Brain, Behavior, and Immunity* (Netherlands) 114: 360–70.
75. Langgartner, Dominik, Christopher A. Lowry, and Stefan O. Reber. 2019. “Old Friends, Immunoregulation, and Stress Resilience.” *Pflügers Archiv* (Berlin/Heidelberg) 471 (2): 237–69.
76. Li, Ming, Wenping Yao, Shenqiang Li, and Juan Xi. 2015. “Norepinephrine Induces the Expression of Interleukin-6 via β-Adrenoreceptor-NAD(P)H Oxidase System -NF-κB Dependent Signal Pathway in U937 Macrophages.” *Biochemical and Biophysical Research Communications* (United States) 460 (4): 1029–34.
77. Liu, Ting, Lingyun Zhang, Donghyun Joo, and Shao-Cong Sun. 2017. “NF-κB Signaling in Inflammation.” *Signal Transduction and Targeted Therapy* (London) 2 (1): 17023-.
78. Loss, Georg J., Martin Depner, Alexander J. Hose, et al. 2016. “The Early Development of Wheeze. Environmental Determinants and Genetic Susceptibility at 17q21.” *American Journal of Respiratory and Critical Care Medicine* (NEW YORK) 193 (8): 889–97.
79. Martinez, Jason E., Doron D. Kahana, Simran Ghuman, et al. 2021. “Unhealthy Lifestyle and Gut Dysbiosis: A Better Understanding of the Effects of Poor Diet and Nicotine on the Intestinal Microbiome.” *Frontiers in Endocrinology (Lausanne)* (Switzerland) 12: 667066-.
80. Mayer, Emeran A. 2011. “Gut Feelings: The Emerging Biology of Gut–Brain Communication.” *Nature Reviews. Neuroscience* (London) 12 (8): 453–66.
81. Michopoulos, Vasiliki, Alex O. Rothbaum, Tanja Jovanovic, et al. 2015. “Association of CRP Genetic Variation and CRP Level With Elevated PTSD Symptoms and Physiological Responses in a Civilian Population With High Levels of Trauma.” *The American Journal of Psychiatry* (United States) 172 (4): 353–62.
82. Mueller, Noel T, Elizabeth Bakacs, Joan Combellick, Zoya Grigoryan, and Maria G Dominguez-Bello. 2015. “The Infant Microbiome Development: Mom Matters.” *Trends in Molecular Medicine* (CAMBRIDGE) 21 (2): 109–17.

83. Oldendorf, WH. 1973. “Carrier-Mediated Blood-Brain Barrier Transport of Short-Chain Monocarboxylic Organic Acids.” *The American Journal of Physiology* (United States) 224 (6): 1450–53.
84. Prandovszky, Emese, Hua Liu, Emily G. Severance, Victor W. Splan, Faith B. Dickerson, and Robert H. Yolken. 2025. “Altered Gut Microbial Diversity, Composition, and Metabolomic Potential in Patients with Major Depressive Disorder and Recent Suicide Attempt.” *Brain, Behavior, & Immunity. Health* (United States) 48: 101081–101081.
85. Reading, Richard. 2001. “Probiotics in Primary Prevention of Atopic Disease: A Randomised Placebo-controlled Trial: Kalliomäki M, Salminen S, Arvilommi H, Kero P, Koskinen P, Isolauri E. (2001) Lancet , 357 : 1076–1079.” *Ambulatory Child Health* 7 (3–4): 334–35.
86. Rook, Graham A.W, and Christopher A Lowry. 2008. “The Hygiene Hypothesis and Psychiatric Disorders.” *Trends in Immunology* (England) 29 (4): 150–58.
87. Santhiravel, Sarusha, Alaa El-Din A. Bekhit, Eresha Mendis, et al. 2022. “The Impact of Plant Phytochemicals on the Gut Microbiota of Humans for a Balanced Life.” *International Journal of Molecular Sciences* (Basel) 23 (15): 8124-.
88. Schmid, Marc W., Sofia J. van Moorsel, Terhi Hahl, et al. 2021. “Effects of Plant Community History, Soil Legacy and Plant Diversity on Soil Microbial Communities.” *The Journal of Ecology* (Oxford) 109 (8): 3007–23.
89. Silva, Ygor Parladore, Andressa Bernardi, and Rudimar Luiz Frozza. 2020. “The Role of Short-Chain Fatty Acids From Gut Microbiota in Gut-Brain Communication.” *Frontiers in Endocrinology (Lausanne)* (Switzerland) 11: 25-.
90. Singh, Rasnik K., Hsin-Wen Chang, Di Yan, et al. 2017. “Influence of Diet on the Gut Microbiome and Implications for Human Health.” *Journal of Translational Medicine* (London) 15 (1): 73–17.
91. Smits, Samuel A., Jeff Leach, Erica D. Sonnenburg, et al. 2017. “Seasonal Cycling in the Gut Microbiome of the Hadza Hunter-Gatherers of Tanzania.” *Science (American Association for the Advancement of Science)* (WASHINGTON) 357 (6353): 802–6.
92. Stanford, Jordan, Anita Stefoska-Needham, Xiaotao Jiang, et al. 2025. “High-Diversity Plant-Based Diet and Gut Microbiome, Plasma Metabolome, and Symptoms in Adults with CKD.” *Clinical Journal of the American Society of Nephrology* (United States) 20 (5): 619–31.
93. Turnbaugh, Peter J., and Jeffrey I. Gordon. 2009. “The Core Gut Microbiome, Energy Balance and Obesity.” *The Journal of Physiology* (Oxford, UK) 587 (17): 4153–58. https://doi.org/10.1113/jphysiol.2009.174136.
94. Turroni, Silvia, Jessica Fiori, Simone Rampelli, et al. 2016. “Fecal Metabolome of the Hadza Hunter-Gatherers: A Host-Microbiome Integrative View.” *Scientific Reports* (London) 6 (1): 32826–32826.

95. Vicentini, Fernando A., Catherine M. Keenan, Laurie E. Wallace, et al. 2021. “Intestinal Microbiota Shapes Gut Physiology and Regulates Enteric Neurons and Glia.” *Microbiome* (London) 9 (1): 210–24.
96. Wood, Jack D. 2011. *Enteric Nervous System : The Brain-in-the-Gut*. In *Enteric Nervous System : The Brain-in-the-Gut*. Integrated Systems Physiology, from Molecule to Function to Disease, # 26. Morgan & Claypool.
97. Ye, Lihua, Munhyung Bae, Chelsi D Cassilly, et al. 2020. “Enteroendocrine Cells Sense Bacterial Tryptophan Catabolites to Activate Enteric and Vagal Neuronal Pathways.” *bioRxiv* (Cold Spring Harbor).
98. Yoshida, Hiroki, Megumi Ishii, and Mitsugu Akagawa. 2019. “Propionate Suppresses Hepatic Gluconeogenesis via GPR43/AMPK Signaling Pathway.” *Archives of Biochemistry and Biophysics* (United States) 672: 108057-.
99. Zhao, Jiayi, Wei Bi, Shu Xiao, et al. 2019. “Neuroinflammation Induced by Lipopolysaccharide Causes Cognitive Impairment in Mice.” *Scientific Reports* (London) 9 (1): 5790-.
100 Zhu, Li-Bin, Yu-Chen Zhang, Han-Hui Huang, and Jing Lin. 2021. “Prospects for Clinical Applications of Butyrate-Producing Bacteria.” *World Journal of Clinical Pediatrics* (United States) 10 (5): 84–92.

1)


GUIDE FOR
VEGETABLE INTAKE
For this study, we ask that you consume 2 cups of vegetables per day.
What does 2 cups of vegetables look like?
1 cup roughly fits into a fist or a baseball.
or
Common serving sizes
A bowl of salad is usually around 1 ½ cups of vegetables per serving.
1/2 cup of salsa equals ½ of vegetables
Vegetables can be fresh, canned, frozen, or dried.
GUIDE FOR
FRUIT/VEGETABLE INTAKE
Examples of vegetables
Leafy Greens (Spinach, Lettuce, Kale, etc)
Carrots
Cabbage
Beets
Herbs (basil, cilantro, parsley, etc)
Celery
Broccoli
Asparagus
Eggplant
Onion
Bell Pepper
Zucchini
Tomato
Sweet Potato
Cauliflower
What doesn't count as a vegetable?