The Effects of Daily Vinegar Intake on Depression Scores and Blood Metabolome

by

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ABSTRACT

Depression has been linked to a significant burden of disease, loss of life, and decreased efficacy of treatment for its comorbidities. Empirical evidence has mounted over the past several decades supporting health benefits from vinegar ingestion, including improvements in blood glucose management, blood cholesterol levels, and inflammation indicators. To date, there have not been any studies in human populations that explore the potential relationship between daily vinegar ingestion and changes in depression indicators and blood metabolomics. This blinded, randomized controlled trial examined the impact of twice-daily vinegar ingestion on mental health measures in healthy young adults recruited from a metropolitan setting. Participants (n=28; aged 25.8±7.0 y; body mass index [BMI] >23 kg/m2) were stratified by age, gender, and BMI and randomly assigned to the liquid (VIN) or pill (CON) groups. VIN participants ingested 2 tablespoons of red wine vinegar (~1550 mg acetic acid; Pompeian Inc., Appendix J) diluted in 8-12 ounces of water, consumed with food at mealtimes twice daily (total ~3100mg acetic acid daily). CON participants consumed 1 vinegar pill daily (22.5 mg acetic acid; Spring Valley, Appendix J). The study lasted four weeks, and anthropometric measurements were conducted in a fasted state at weeks 0 and 4. Study adherence varied slightly (90±17% and 100±14% for VIN and CON respectively, p=0.084); hence, adherence was controlled for in all subsequent analyses. Changes in L-tryptophan (p=0.777, η^2=0.003), peripheral serotonin levels (p=0.348, η^2=0.035), GABA (p=0.403, η^2=0.028), and gut-mediated short-chain fatty acids acetic acid (p=0.355, η^2=0.034), and propionic acid (p=0.383, η^2=0.031), did not differ significantly between VIN and CON groups respectively with the exception of isobutyric acid (p=0.0374, q=0.0473).

However, Patient Health Questionnaire (PHQ-9) depression scores improved significantly for VIN participants in comparison to CON participants (-0.5±1.3 vs. +0.7±2.4 cm [p=0.026] and -0.4±0.7 vs. +0.3±1.0% [p=0.045]. Although these differences between groups are modest, which would be expected given the short study duration, changes were not driven by pharmacological or lifestyle interventions, suggesting the benefits of vinegar ingestion on mental health symptomology and the blood metabolome.

DEDICATION

This thesis is dedicated in memory of my mother, Lisa Barrong (1966-2022).

I would also like to thank my family and community of dear friends, your unwavering encouragement, camaraderie, understanding, and shared curiosity into the world of vinegar have been sources of strength throughout this journey. Your presence, whether in moments of celebration or challenge, has made all the difference. I would not have been able to do this without you.

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CHAPTER 1

Introduction

Depressive disorders are the most prevalent and burdensome mental health disorders in the world. Across all ages, the global burden of disease due to depressive disorders increased by 61% from the years 2009 to 2019 (Vos et al., 2020). Studies also show that individuals with depression are more pessimistic about treatment efficacy for other, potentially more burdensome comorbidities they may live with and have worse treatment outcomes because of that (Bartoli et al., 2018; Hemingway & Marmot, 1999; Lebowitz et al., 2013; Macrodimitris & Endler, 2001; Schroder et al., 2020). The efficacy of antidepressant medications in treating the wide and variable range of experiences in those with depression has long been unclear and lacking in generalizability. In the most recent meta-analyses of antidepressant efficacy, these medications were effective to widely varying degrees based on baseline depression, comorbidities, severity, and duration of symptoms ((Arroll et al., 2005; Gold et al., 2020; Kirsch et al., 2008; Köhler-Forsberg et al., 2023; Remes et al., 2021; Vöhringer & Ghaemi, 2011)). With this lack of clarity surrounding the effectiveness and applicability of treatment options for varying presentations of depression, research is warranted to further understand the etiology and potential treatment methods. Furthermore, this research could improve the treatments of even more prevalent and burdensome chronic health concerns all over the world.

Vinegar is a fermented, water-based acetic acid solution that has become a staple in cuisines around the world throughout history (Zuerich, 2004). In more recent years, research investigating vinegar's role as a functional food has increased in the fields of

nutrition and chronic disease. Researchers have already explored and confirmed its roles in blood sugar management, antioxidant activity, heart disease prevention, brain health, and tumor prevention (Budak et al., 2014; Johnston et al., 2021). These benefits appear to be associated primarily with its acetic acid content (Johnston & Gaas, 2006; Johnston et al., 2021).

In tandem with the increase in vinegar research, interest in the gut-brain axis's relationship with depression has also risen. Looking at both disease states and microbiome health, the field of literature has slowly shifted away from the idea that the cause of depression can be narrowed to a single biomarker (like serotonin, dopamine, or cortisol levels), but rather that it is a mix of genetic, biochemical, and environmental factors (Cowen & Browning, 2015).

One of the dominating factors of interest is tryptophan, an essential amino acid consumed primarily in poultry, milk, tuna, bread, and oats in the American diet (Richard et al., 2009). It is a precursor to serotonin, melatonin, and several other chemicals associated with mental health. Tryptophan is metabolized into these chemicals via three main pathways, which all start in the gut by either the cells of the intestinal walls or by the microorganisms present around them (Gheorghe et al., 2019). Specifically, the Lactobacillus species of bacteria plays a critical role in tryptophan metabolism, and, in animal research, acetic acid has been shown to improve the population and activity of this bacteria (Gheorghe et al., 2019; Li et al., 2022). This tree of relationships is a potential mechanism for the gut-brain axis as it relates to depression.

However, there is a need for more investigation into the potential role of vinegar in mitigating depressive symptoms, as there is only one study to date directly

investigating this relationship. In this study conducted in 2021, daily vinegar ingestion for 4 weeks showed significant decreases in depression indicators, improved mood states, and alterations to urinary metabolites in line with mental health improvement (Johnston et al., 2021). Research into this connection is necessary and scientifically warranted to continue to affirm these relationships between vinegar and depression and shed light on vinegar's specific mechanism of effect.

Purpose of Study

The goal of this study is to examine the effect of vinegar supplementation on depression scores and blood metabolomics related to depression and gut metabolism over 4 weeks in generally healthy overweight or obese adults in the Phoenix metropolitan area. Specifically, the primary objective is to study how two tablespoons of red wine vinegar taken with meals twice per day affect CES-D and/or PHQ-9 questionnaire scores as compared to a placebo vinegar pill. The secondary objective was to identify any changes to tryptophan, serotonin, neurotransmitters, and short-chain fatty acid levels in the blood.

Research Aim and Hypotheses

H_1 Daily red wine vinegar supplementation will be associated with lowered depression questionnaire scores after 4 weeks compared to the placebo treatment (vinegar pill) in a group of generally healthy adults with BMIs from 25-40

H_2 Daily red wine vinegar supplementation will be associated with changes in the blood metabolome consistent with improved mood states and altered tryptophan metabolism – tryptophan, serotonin, gamma-aminobutyric acid (GABA), and three gut-mediated short-chain fatty acids (acetic acid, propionic acid, and isobutyric acid).

Definition of Terms

- Acetic acid: A short-chain fatty acid produced in the gut and the active ingredient in vinegar which gives it its characteristic odor and flavor. Its chemical formula is CH3COOH, and it is generally found in concentrations of 5% or more in commercially available vinegar in the United States.
- Amino acids: Organic compounds that are required for the synthesis of proteins and other important compounds in the human body. The nine amino acids that cannot be produced in the human body and must be consumed in the diet are called essential amino acids.
- Body Mass Index (BMI): A calculation comparing weight to height often used as a predictor of body composition:

  Underweight: <18.5 kg/m2

  Normal weight: 18.5-24.9 kg/m2

  Overweight: 25-29.9 kg/m2

  Obese: ≥30 kg/m2
- Dietary Supplement: A manufactured product designed for ingestion, intended to supplement a person's diet. In the case of this study, dietary supplements specifically refer to non-study-protocol supplements that the participants may be taking.
- Gamma-aminobutyric acid (GABA): An inhibitory neurotransmitter, or chemical messenger, which slows down communication of some nerve cells in the brain. It

produces a calming effect thought to influence hyperactivity associated with seizures, anxiety, stress, and fear.

- Gut-brain axis: The theory of bidirectional communication between the central and the enteric nervous systems, linking emotional and cognitive centers of the brain with peripheral intestinal function (Carabotti et al., 2015)
- Metabolites: A substance made or used when the body breaks down a substance, like food, drugs, or its tissues
- Metabolomics: the scientific study of metabolites that can be found in an organism, organ, tissue, or cell
- Polyphenols: A category of organic compounds found in plants that offer a wide array of health benefits, primarily antioxidative and anti-inflammatory.
- Serotonin: A neurotransmitter, or chemical messenger, that transports communications between neurons in the brain. It plays a role in bodily functions such as mood, sleep, digestion, sexual desire, and blood health. It is a metabolite of tryptophan metabolism.
- Short-chain fatty acid (SCFA): The primary metabolites produced by the microbiota in the human gut. Acetic acid is one of the top three most common SCFAs.
- Tryptophan: an essential amino acid that is used in the biosynthesis of proteins

Delimitations and Limitations

Delimitations

Generally healthy non-smoking adults ages 18-40 were recruited for this study during the spring months in the Phoenix Metropolitan area. Participant BMI values

ranged from 25-40 and participants must be free of chronic disease, including GERD and chronic heartburn. Women may not be pregnant or lactating. The results of this study may not apply to other groups, disease states, or seasons of the year.

Limitations

- Participants cannot be fully blinded to their treatment protocol; they may assume why they are consuming vinegar, the potential benefits, and the differences between liquid vinegar and vinegar pill effects.
- Adherence to the assigned vinegar protocol and unchanged diet, medication/supplement use, and physical activity cannot be guaranteed.
- Subjectivity of 24-hour dietary recall and depression questionnaires means results may carry participant-dependent bias.

CHAPTER 2

Review of Literature

Depression

Depression is a mood disorder with many forms and variations whose underlying causes and pathophysiology are still not as thoroughly understood as other diseases of similar prevalence. According to the American Psychiatric Association, an estimated 1 in 6 (16.7%) people will experience depression in their lifetime, with an estimated 1 in 15 (6.7%) experiencing a depressive episode in any given year (American Psychiatric Association, 2020). This annual prevalence is similar to that of asthma (7.7%) and influenza during flu season (7.1%) (Centers for Disease Control and Prevention, 2023; Tokars et al., 2018). Depression often presents itself as comorbid with other chronic diseases. Globally, comorbid depression appears to worsen overall health outcomes more significantly than other common comorbid diagnoses, like pain, asthma, arthritis, or diabetes (Gold et al., 2020; Moussavi et al., 2007). With these trends in mind, not only are the pursuits of accessible and effective assessments and treatments for depression worthwhile in and of themselves, but they could also have a positive impact on the mitigation of more prevalent and burdensome chronic health concerns all over the world.

Etiology

In some of the oldest texts available for medical study around the 18th century BC in Babylon, their language may have used terms related to fear, suicidality, and distress to describe states that would be interpreted today as depression. Later, Greek influences like Hippocrates and Aristotle (around the 4th century BC) described these states as *melancholia*, caused by imbalances in the body's four "humors" -- blood, black bile,

yellow bile, and phlegm (Telles-Correia & Marques, 2015; Tipton, 2014). Common treatments for melancholia at that time included bloodletting, physical activity, and changes to diet.

Today, there are several different perspectives on the etiology of depression, which cross multiple different disciplines of study representing countless different areas of a depressed person's lived experience. Broadly, these different lenses can be broken down into biological, psychological, and environmental/social categories (National Research Council (US) and Institute of Medicine (US) Committee on Depression et al., 2009; Remes et al., 2021). Influences within the biological category may include physical health conditions, genetic factors including family history of depression, inflammation, sex (females tend to represent a larger proportion of depressed patients), age (studies are conflicted in whether depression is more or less common in geriatric populations) and the body's microbiome (Gold et al., 2020; Remes et al., 2021). Psychological influences may include cognition, emotional regulation, other mental health disorders like substance abuse or anxiety, self-confidence and self-efficacy, personal biases, communication and attachment styles, and other attitudes about themselves (Beck & Alford, 2009; Remes et al., 2021). Environmental and social factors can include physical environments where the patient lives and spends their time, the health of social networks, trauma, stress history, and childhood adversity faced (Beck & Alford, 2009; Remes et al., 2021).

The interplay between these broader etiological categories is also noteworthy. For example, serotonin is well-understood to be involved in the human stress response (aan het Rot et al., 2009). A previous study showed serotonin's moderating role in how

humans respond to stressful situations and, in individuals with a genetic anomaly in the primary neuroactive serotonin receptor, exposure to stressful events correlated with an increase in depressive symptoms (Caspi et al., 2003). This is just one example of how susceptibility to depression can cross multiple etiological influences. Not only can a single type of factor, in and of themselves, cause depressive symptoms, but this interplay with other factors also makes isolating etiologies a virtually impossible task for researchers.

Screening Methods

In the United States, it is generally recommended that all adults are screened for depression, regardless of the presence or absence of risk factors (US Preventive Services Task Force, 2023). Depression screening tools often come in the form of Likert scale surveys and can be broken down into two main categories: those that screen for the presence of depression and those that screen for the severity of depression or degree of suicide risk (US Preventive Services Task Force, 2023). When analyzing the accuracy of these screening tools in identifying those with and without depression, they are usually compared with the widely accepted gold standard of a semi-structured interview with a psychiatric provider. When considering which screening tool is the best fit for practice, it is key to not only evaluate its sensitivity (identifying those with depression) but also its specificity (identifying those without depression). The most popularly used and recommended depression screening tools in the United States include the Patient Health Questionnaire (PHQ) and Center for Epidemiologic Studies Depression Scale (CES-D) tools for adults 19 years of age or older, the Edinburgh Postnatal Depression Scale

(EPDS) in pregnant and post-partum patients, and the Geriatric Depression Scale (GDS) in adults 65 years of age or older (US Preventive Services Task Force, 2023).

Practitioners may choose to start with these screening tools instead of the gold standard interview for a variety of reasons including ease of access, ease of distribution, honesty of self-report with the comparative privacy of a screening survey, and shorter administration time. Studies show that PHQ-9 may be the most accurate self-administered depression screening tool when compared to the widely considered gold standard of Schedules for Clinical Assessment in Neuropsychiatry (SCAN) (Gelaye et al., 2014). After completion of a screening tool, a patient may be referred to a psychiatric or other mental health practice for further evaluation and treatment or moved into a specific workflow if screened at a mental health practice.

Treatment

Treatments for depression come in many forms, including pharmacological interventions, cognitive behavioral therapy, lifestyle changes, and others. Psychotherapy options may come with access concerns, limiting their usefulness or applicability long-term for a number of individuals. Alternatively, the research into the efficacy and side effects of antidepressant medications in the treatment of depression has long been imperfect, debated, and therefore unclear, which highlights the opportunity to search for alternative treatment options.

There have been several meta-analyses over the years examining the effectiveness of these drugs, but they have often returned contradictory or ambiguous results. As of 2005, a meta-analysis of placebo-controlled trials estimated 56-60% efficacy with low to medium effect size compared with placebo when depression was treated with selective

serotonin reuptake inhibitors or tricyclic antidepressants, two of the most common categories of antidepressants (Arroll et al., 2005; Remes et al., 2021). Effect sizes may increase when depression is a comorbid condition (Gold et al., 2020) When pharmacological treatment is focused on depression, effect sizes appear to increase in cases of comorbid major depressive disorder (MDD) compared to cases of an isolated diagnosis (Gold et al., 2020). However, when treatment is focused on the underlying medical condition, outcomes in depressive symptoms are mixed, but depression may be exacerbated in treatments where pain is a resulting side-effect (Gold et al., 2020).

Pharmacotherapies also come with their own inherent risks like negative side effects, limited access, potentially weeks-long delay in symptom relief after medication initiation, and limited efficacy in some forms of depression. Another systematic review looking at randomized control trials (RCTs) where comorbid diseases were also present showed that while antidepressants were safer and more effective at treating and preventing depression than placebo, they increased risk for any adverse events, and their efficacy varied widely depending on the comorbid condition(s) present (Köhler-Forsberg et al., 2023). In another meta-analysis of antidepressant efficacy, it showed that while these medications were effective for acute depressive episodes when they were moderate or severe, they were not effective in mild acute cases or long-term in chronic cases of any severity (Vöhringer & Ghaemi, 2011). Another indicated that, when the baseline severity of depression was controlled, the efficacy of treatment was not significantly higher with medication interventions than with placebo (Kirsch et al., 2008). More than one metanalysis noted that there was a notable lacking in large, high-quality RCTs in the field

that may add more ambiguity to the generalizability and reliability of their results (Arroll et al., 2005; Gold et al., 2020; Köhler-Forsberg et al., 2023)

Public Perception

In 2010, 67% of the public appeared to attribute depression to these neurobiological factors as opposed to childhood upbringing or the depressed individual's character (Pescosolido et al., 2010). Importantly, that same 2010 study showed these attributions to genetics and chemical imbalances correlated with an increased acceptance of treatment but did not appear to decrease stigma around the diagnosis itself. Other studies show that patients who believe their depression is genetic or due to biochemical abnormalities are also more pessimistic about treatment potential, and this prognostic pessimism results in worsened treatment outcomes (Lebowitz et al., 2013, Schroder et al., 2020. Despite these widely popular beliefs, the exact mechanism of depression pathology is, in fact, not well understood, and new theories have been explored in recent years.

This literature around depression indicates that some people not only carry potentially incorrect perceptions about what is causing depression, but also that those incorrect assumptions could be perpetuating stigma around the disease and its treatment. Then, if they are diagnosed, the stigma and misunderstanding can go on to negatively impact their own ability to heal. This academic vacuum without confirmed answers on depression's pathogenesis, combined with the lack of consistently effective treatment options that give patients confidence in their own recovery, presents opportunities for more research in the field that stand to be impactful.

Tryptophan

Tryptophan is one of the 20 amino acids and one of the 9 that are considered essential, being that the human body cannot endogenously produce it (Palego et al., 2016). In the American diet, it is consumed primarily via poultry, milk, tuna, bread, and oats (Richard et al., 2009; Palego et al., 2016). Tryptophan is unique in its chemical structure, as it contains the only bicyclic indole ring of any of the amino acids, giving it a unique metabolic capacity in the body (Palego et al., 2016). This large molecule is a precursor to serotonin, melatonin, and several other chemicals and neurotransmitters associated with health.

Metabolism

After ingestion, tryptophan is absorbed in the gut either by the intestinal wall directly or by the microorganisms present on and around them. There are two stages of absorption by the enterocytes, first via the B^0AT1 epithelial amino acid transport system on the lumen (apical) side and then through the basal epithelium and into the blood stream via the TAT1 (Slc16a10) protein (Keszthelyi et al., 2009) In its initial absorption step into the brush border of the intestinal wall, tryptophan competes with other large neutral amino acids (valine, leucine, isoleucine, histidine, lysine, methionine, threonine, and tyrosine) for use of the B^0AT1 transport system by way of a Na^+ co-transport mechanism (Keszthelyi et al., 2009) Tryptophan has the second lowest affinity for the transport system, except for lysine, and its absorption into the epithelium at this precisely regulated step is significantly impacted by dietary intakes (Keszthelyi et al., 2009; Palego et al., 2016).

Once tryptophan enters the bloodstream, its usable isomer L-tryptophan is distributed to all tissues of the body depending on their need for protein synthesis and energy and is finally taken in by those target tissue cells, including those beyond the blood-brain barrier (BBB) via amino acid transporters (**Gheorghe** et al., 2019; Palego et al., 2016). At the BBB, tryptophan, again, meets the same competition for the B^0AT1 transport system as found in the enterocytes (Palego et al., 2016)

Tryptophan is metabolized primarily via two different pathways within the host organism, marked by either the degradation or retention of the unique indole ring – the kynurenine and serotonin pathways, respectively (Palego et al., 2016). Eventually, tryptophan can be completely oxidized and broken down into terminal products like carbon dioxide and water for excretion, but along the way, it can produce many bioactive compounds via these two primary pathways (Kohlmeier, 2003; Palego et al., 2016; Roth et al., 2021).

The kynurenine pathway is how most tryptophan is metabolized and is primarily carried out within the liver (90-95%), but it can also be found in the kidney, brain, and other tissues (Kohlmeier, 2003; Marsh, 2007; Palego et al., 2016; Roth et al., 2021; Li et al., 2022).

The serotonin pathway, by contrast, is responsible for only a small portion (1-2%) of tryptophan metabolism (Bender, 1983; Li et al., 2022). Up to 90% of the body's serotonin is produced and housed in the GI tract (peripheral serotonin), with the remaining roughly 10% (central serotonin) being produced by the neurons within the Raphe Nuclei of the central nervous system after tryptophan is transported across the BBB (Yabut et al., 2019; Martin et al., 2017; Bakshi & Tadi, 2023; Li et al., 2022). This

conversion from tryptophan to 5-hydroxytryptophan (5-HTP) and then eventually to serotonin (5-HT) is initiated at its first and rate-limiting step by the tryptophan-hydroxylase (TPH1) enzyme which is expressed in the gut and by the TPH2 enzyme expressed in the CNS (Martin et al., 2017).

The third possible metabolic pathway for tryptophan (accounting for ~4-6%) is carried out by microbiota that populate the GI tract rather than the host itself – the indole (indole pyruvate) pathway (Roth et al., 2021). In one branch of this pathway, indole, which is produced by bacteria like *Escherichia coli*, *Clostridium spp*., and *Bacteroides spp*, plays a regulating role in the inflammation processes and health and homeostasis of the gut (Li et al., 2022). By the action of other bacteria like *Xenorhabdus nematophilus*, *Bacillus atrophaeus*, and *Lactobacillus bulgaricus*, another branch produces tryptamine, which can trigger gastric release of serotonin (Li et al., 2022). The indole pathway also appears to play a role in gut immunity, as its key enzyme, IL4I1, is found in high concentrations in the dendric cells of the gut (Li et al., 2022). IL4I1 catalyzes tryptophan conversion to indole-3-aceetic acid (IAA) and indole-3-aldehyde (IAld), two neuroprotective compounds (Li et al., 2022).

Metabolites within all three of these tryptophan metabolic pathways are found across almost every physiological system in the human body, with many potentially playing a role specifically in disease mitigation (Agus et al., 2018).

Tryptophan Metabolites and Mental Health

Given the number of psychoactive metabolites involved in tryptophan metabolism, it's no wonder that it has recently become a focus area for research regarding the gut-brain axis and, more generally, the etiology of neurophysiological

processes. Alterations in tryptophan metabolism may be a shared abnormality in those with many mental health disorders (Gevi et al., 2016; Rothhammer et al., 2018).

Tryptophan (and its interactions with the mechanisms of anti-depressants) may have a role to play in the treatment of patients with previous MDD in remission or healthy individuals with a family history of MDD. In a meta-analysis of different studies that manipulated tryptophan depletion to analyze its effects on depressive symptoms, they showed depression relapse in 47% of patients on anti-depressants and in MDD recovery who were put into tryptophan depletion, and an even higher rate of relapse for patients without antidepressant use (Ruhé et al., 2007). This same meta-analysis did not see a decrease in mood in those who were depressed at the time of tryptophan depletion. This research shows extrapolation potential to patients with either previous depressive episodes or with a genetic predisposition. This link between tryptophan depletion and depressive symptoms is potentially important because if tryptophan depletion shows correlation to depression relapse, and when serotonin does not always show correlation in those with depression, then another portion of tryptophan's metabolism may be an area of additional research opportunity.

There are several bioactive metabolites along the dominant kynurenine pathway, but there are three noteworthy neurotransmitters produced including kynurenine (KYN) itself and its further downstream metabolites kynurenic acid (KA) and quinolinic acid (QA) which are produced after crossing the BBB (Roth et al., 2021). These three substances have been of particular interest in pharmacological research into this pathway for their neuroprotective and neurotoxic properties (Roth et al., 2021). Dysregulation of KA and QA levels has been implicated in various neuropsychiatric disorders, including

autism, anxiety, schizophrenia, depression, and other neurodegenerative diseases (Roth et al., 2021). Whether increases or decreases in these substances is harmful or helpful is often dependent on the specific mental health concern examined, with increases in KA seen in schizophrenic and multiple sclerosis patients but decreases seen in depressed patients and highly stressed stroke patients, for example (Roth et al., 2021).

The bioactive metabolites of the indole pathway mostly play roles in regulating host-microbiota interactions, modulating immune responses, and influencing intestinal motility and barrier function (Roth et al., 2021). However, the antioxidative effects of metabolites like indole-3-propionic acid may protect against weight gain and have a neuroprotective role to play in diseases like Huntington's, Parkinson's, and Alzheimer's diseases (Roth et al., 2021). Additionally, some bacterial strains may be able to produce additional serotonin, adding to peripheral concentrations and contributing to its benefits outlined at length below (Roth et al., 2021).

Serotonin

Peripheral Serotonin

Serotonin is a monoamine neurotransmitter with the chemical name of 5-hydroxytryptamine, often abbreviated as 5-HT. In the gut, this vast majority of serotonin can be found primarily in the enterochromaffin cells (subtype of endocrine found in the stomach) where it influences GI motility and appetite (Yano et al., 2015; Bakshi & Tadi, 2023). Outside of the gut, peripheral serotonin can also be released into the bloodstream and interact with blood platelets, playing critical roles in clot formation and vasoconstriction for wound healing (Bakshi & Tadi, 2023).

There is conflicting evidence about any changes in serotonin production by the EC cells depending on the environmental short-chain fatty acid (SCFA) concentrations (Reigstad et al., 2015; Martin et al., 2017). However, this inconsistency is likely related to the sources of the SCFAs being explored. In those showing a negative correlation, they were often looking at SCFAs generated exclusively via the microbiota alongside a standard diet, rather than direct consumption of or exposure to the acids at higher concentrations (Martin et al., 2017). On the other hand, in the case of direct, concentrated acetic acid exposure, there was a positive correlation with TPH1 expression in the EC cells (Reigstad et al., 2015).

Central Serotonin

Despite its small share of total systemic levels, central serotonin is the version for which the neurotransmitter is often better known. At their most foundational levels, serotonin's roles can be summarized as responses to changes in the environment, playing roles in mood, sleep, libido, aggression, cognition, appetite, and body temperature (Bakshi & Tadi, 2023; Palego et al., 2016).

Serotonin Theory of Depression

The serotonin theory of depression remains well-known and impactful in how our society perceives, studies, and treats depression. The public story and understanding of serotonin and its relationship with depression have gone through many iterations, with varying intensities, since the mid-1900s (Healy, 2015). However, the research to support its link with depression is inconsistent in its ability to quantify how or even if they are directly linked. Looking directly at serotonin levels or its binding ability in the clinically depressed, there do not appear to be any studies that can confirm a causal relationship and

very few can claim a correlation (Moncrieff et al., 2022). Other studies indicate that it may be wiser to attribute an indirect link to monoamines more broadly (serotonin, dopamine, norepinephrine, and epinephrine) (Ruhé et al., 2007). Tryptophan depletion methods have often been used to attempt to show serotonin's role in depression, but this method may inaccurately diminish the role of other tryptophan metabolites and should be interpreted with caution with regard to serotonergic etiology (aan het Rot et al., 2009; L et al., 2011; Ruhé et al., 2007).

The Gut-Brain Axis

Components

The complex, multidirectional communication system between the gut microbiota, gut cells, peripheral nervous system (PNS), and the central nervous system (CNS) of the host is commonly housed under the umbrella term of gut-brain axis (GBA). In the human body, the CNS is comprised of the brain and spinal cord, while the PNS (and all its subsections) includes the other nerves throughout the rest of the body, both working together to ensure adequate signaling from the rest of the body to the brain, and vice versa. The enteric nervous system (ENS) is one of the subsections of the PNS and is the connection point between the digestive system and the central nervous system (Mayer, 2011). It is the largest portion of the PNS, sometimes being called the second brain, and its mass, count of categories of neurotransmitters, and count of neuron types are comparable to those of the spinal cord and the rest of the CNS (Sasselli et al., 2012). The ENS can also function as its own independent system, operating apart from the influence of the CNS, and is comprised of several plexuses responsible for gut hormone secretion, intestinal motility, and fluid balance and blood flow in the lower GI tract

(Sasselli et al., 2012). These local functions are possible, in part, due to the complex and bidirectional neural, immune, and endocrine communication networks that the ENS shares with the microbiota present in the gut (Carabotti et al., 2015).

The Human Microbiota and Microbiome

The human microbiota is the collection of all microbial cells on every surface of the human body, both internal and external, primarily located in the gut lumen (Turnbaugh et al., 2007; Ursell et al., 2012). The human microbiome is the respective collection of genes that these microbial cells carry and express, contributing to the health and function of the human body's systems (Turnbaugh et al., 2007; Ursell et al., 2012). From just minutes after a human's birth, the gut microbiota is developing, and its composition appears to depend on the mode of birth (whether vaginal or via cesarian) and how the baby is fed (Palmer et al., 2007). Then, via mechanisms and patterns that are not well understood, the gut microbiota enters a period of instability over the 6-12 months after birth and closely resembles that of an adult by the end of a baby's first year of life (Palmer et al., 2007). Studies have shown that the development and changes of the gut microbiome through life are mostly determined by environmental, geographic, medical, biometric, and dietary influences, less variation accounted for by genetics (Rothschild et al., 2018; Suzuki et al., 2022). While there have been shared strains identified in the gut microbiomes of parents and their children, similar instances of shared strains have also been identified in mothers and unrelated children, indicating that environmental factors may contribute to these similarities (Suzuki et al., 2022).

The population density of microbes through the typical adult human's gastrointestinal (GI) tract exists in two reversing gradients, with the largest and most

dense populations existing at the distal ends of the system, in the mouth and in the colon, and the lowest microbiota concentrations in and around the stomach due to the impeding effects of digestive acids and enzymes (de Vos et al., 2022). Other than its gradient trends, the gut microbiota can also be described by its alpha-diversity (diversity of microorganisms within one sample), beta-diversity (diversity between different samples, but within the same environment, like the human gut), and whether or not it is in dysbiosis (an altered, maladaptive state of the microbiota, often changes in either alpha- or beta-diversity) (Harding & Bishop, 2022)

Studies of the human microbiome started as early as the 17th century, identifying the differences in microorganism profiles in different environments of the body, and continue today with the exploration of the microbiome's effects, how we can influence them, and why they exist (de Vos et al., 2022; Leeuwenhoek, 1684).

Pre-, Pro-, and Postbiotics

Prebiotics can be broadly understood as the undigestible portions of foods that feed the bacteria of the gut because the host cannot directly digest them. Most commonly, they are found in complex fibers and starches of plant foods (Harding & Bishop, 2022). These often include fructo-oligosaccharides and galacto-oligosaccharides, inulin, and lactulose (Davani-Davari et al., 2019; Harding & Bishop, 2022).

Probiotics are live microbes packaged for ingestion, often in capsule form, beneficially contributing to the human gut microbiota population when taken in sufficient doses (Harding & Bishop, 2022). Common strains found in these probiotic supplements include those from the *Lactobacillus*, *Bifidobacterium*, *Saccharomyces*, *Streptococcus*, *Enterococcus*, *Escherichia*, and *Bacillus* genera (Harding & Bishop, 2022; National

Institutes of Health, 2022). Probiotics administration in animals (mice) have shown to increase plasma concentrations of tryptophan and kynurenic acid compared to controls (Desbonnet et al., 2008).

Finally, postbiotics are the bioactive end-products after the gut's microorganisms complete their digestion of the prebiotics and other nutrients available to them. These include carbohydrates, proteins (enzymes and amino acids), lipids, vitamins (vitamin K and B vitamins), short-chain fatty acids (mainly acetic acid, propionic acid, and isobutyric acid) and other molecules (Harding & Bishop, 2022; Thorakkattu et al., 2022).

Health Effects

It is widely understood and accepted that the gut microbiome, ENS, CNS, and their symbiotic interactions with each other play meaningful roles in host health (Carabotti et al., 2015; Mayer, 2011; Sharon et al., 2016). Abnormalities specifically in bacterial populations have been associated with a wide range of diseases including diabetes, other pancreatic diseases, obesity, cancer, GI disorders, and central nervous system diseases (de Vos et al., 2022; Sharon et al., 2016).

The role of the gut-brain axis specifically in the development of depressive symptoms has become a recent focus of research, looking at the roles that both gastrointestinal disease and gut microbiota appear to play. This field of research has shifted away from the idea that depression's etiology can be narrowed to changes in a single biomarker but, rather, that it is a mix of genetic, biochemical, and environmental factors (Cowen & Browning, 2015). While exact mechanisms, causality, and directionality remain unknown, more recent studies tapping into large microbiome cohort studies and those examining specific bacterial populations continue to affirm that a

relationship between the human gut microbiome and depression status exists (Mireia et al., 2019).

In animals, the absence of gut microbiota in mice seems to be protective against depression, but those same mice were more susceptible to changes in tryptophan ingestion, showing a greater risk for depressive symptoms in acute tryptophan depletion (ATD) (Lukić et al., 2019). While the presence of microbiota showed more susceptibility to depressive symptoms, it also showed protective characteristics when exposed to changes in tryptophan ingestion (Lukić et al., 2019). According to other research in mice, the microbiota may influence the mental health of those with other bowel diseases, independent from changes in inflammatory and ANS indicators (Bercik et al., 2011).

Only study more definitively isolates causation with respect to the human gut microbiota and its relationship to the development of depression indicators, and it was still conducted in animals via fecal transplant (Kelly et al., 2016).

Vinegar

History

Vinegar, whose name gets its roots from the French word for “sour wine” is a water-based acetic acid solution that has become a staple in cuisines around the world, with its first documented uses dating back to the days of Babylon around 3000 BC (Zuerich, 2004). Other ancient citations of its use can be found all over the world in China, Egypt, Greece, and others, but it was not commercially cultivated until roughly the 14th century (Zuerich, 2004). Even in these earliest years of production, we see the invention of methods still used for vinegar production today.

Production

The general process of making vinegar is two steps: fermenting a fermentable carbohydrate source into alcohol by way of yeasts and then converting that alcohol into acetic acid with bacteria (Johnston & Gaas, 2006). Once this second step is done, the final acetic acid concentrations of the vinegar may vary depending on the type of vinegar. To be classified as vinegar and approved in the United States, vinegar must be at least 4% acetic acid, with some common vinegars ranging up to higher percentages (Affairs, 2020b). Vinegar is quite different from diluted acetic acid, as they are produced, categorized, and labeled differently according to FDA guidelines, and should not be used interchangeably in food preparation (Affairs, 2020a).

Today, vinegar production methods are categorized most easily by their speed—fast or slow (Johnston & Gaas, 2006). The slow fermentation process is generally seen in the cases of more traditional vinegars made from fruit juices and have been used since the earliest days of vinegar's history (Johnston & Gaas, 2006; Zuerich, 2004). Slow fermentation is traditionally called a "surface method" because it utilizes barrels or vats of the alcohol source and the acetic acid bacteria (AAB) sit on the surface of the material, usually growing on wood shavings (Budak et al., 2014). This is a slower fermentation process because the surface area is limited and stagnant, so the acetification can take weeks or months to complete (Johnston & Gaas, 2006). In contrast, fast fermentation (also called industrial production or submerged fermentation) includes the introduction of both AAB and oxygen directly into the body of alcohol by spray, air pumps, agitation, or a mixture of these methods (Budak et al., 2014; Johnston & Gaas, 2006). These faster methods, more common in mass production, can usually be completed within 20-24

hours (Budak et al., 2014). The AAB used in both fermentation processes work best in temperatures from 25-30 degrees Celsius and in a pH of 5.0-6.5 (Budak et al., 2014). Acetobacter and Gluconobacter are the two primary genera of AAB used for vinegar fermentation today, and within those categories, over 10 different species can be found at differing concentrations in different vinegars (Budak et al., 2014).

Bioactive Components in Vinegar

In addition to the leftover active bacteria themselves, packaged vinegar may also contain varying amounts of vitamins, minerals, amino acids, sugars, polyphenols, and other organic acids (Johnston & Gaas, 2006; Xia et al., 2020). The concentrations and proportions of each component differ across each type of vinegar, as they retain some of the bioactive components from their original source materials (Xia et al., 2020).

Vitamins and Minerals

The vitamin content of vinegar appears to be dominated by B vitamins and vitamin C, with the leading vitamin varying depending on the source material of the vinegar (Xia et al., 2020). A classic serving size of vinegar is most often considered to be one tablespoon. In its most vitamin-concentrated forms (niacin in grain vinegars and vitamin C in palm vinegars), this serving size provides an estimated 25% and 3.5-4.5% of the RDAs for niacin and vitamin C, respectively (*Online Food Calculator. Food Volume to Weight Conversions*, 2024; Xia et al., 2020). More conservatively, for example, one tablespoon of red wine vinegars provides 0.5mg of vitamin C, 0.5 to 0.6 of the RDA for men and women, respectively (*Online Food Calculator. Food Volume to Weight Conversions*, 2024). Vinegars also contain a wide range of minerals, with 20-21 of the 21 essential and non-essential minerals being provided across the range of vinegars tested

(Xia et al., 2020). One study looking at the mineral content of red wine vinegars found 20 different minerals present in just one type out of Spain and, generally, that concentrations of minerals were higher in aged vinegars when compared with young vinegars (Paneque et al., 2017).

Amino Acids

The literature on the amino acid contents of vinegar centers on both free and nonprotein amino acids, of which there are hundreds of variations. The proportion of each amino acid in the profile of each type of vinegar is directly related to the raw materials used in the production of the vinegar and its fermentation style, with grain vinegars generally being higher in glutamine and alanine, and fruit vinegars leading with proline and leucine (Xia, 2020; Valero et al., 2005). AAB tend to show preferences for consumption of the different amino acids during the fermentation processes that are proportionate to their availability in the source material, with leucine appearing to be the most preferred across all sources (Valero et al., 2005). Amino acids play important roles in metabolism, immunity, and neurological health in the human body (Xia, 2020; Valero et al., 2005).

Sugar

The sugar content of any given vinegar dictates its sweetness classification, with one study distinguishing them as dry (less than 5 g/L), semi-sweet (between 5-69 g/L), sweet (between 70-149g/L), and balsamic (over 150 g/L) (Sánchez et al., 2020). The nature of vinegar production eliminates most food sugars in the vinegar that would have otherwise been found in the original source material, as both the yeast for ethanol fermentation and the bacteria for acetic acid formation use these sugars as their fuel, so

very little is left by the end of the process (Ho et al., 2017). Further, in this chain of reactions, the sugars in vinegars (usually glucose and fructose) will often function as and be documented as reducing sugars (Ho et al., 2017; Xia et al., 2020).

Polyphenols

One study conducted in China in 2019 analyzed the polyphenol contents and antioxidant effects of 23 different fruit vinegar brands from around the world (6 types of vinegar), finding that balsamic and red wine vinegars had the highest average polyphenol contents and antioxidant effect potentials (Liu et al., 2019). This confirmed the results of earlier studies in 2003 and 2017, showing that balsamic vinegar and wine vinegars (aged in wooden barrels, especially) had the highest polyphenol concentrations (Bakir et al., 2017; Natera et al., 2003). Balsamic Vinegar led in gallic acid, hydroxymethyl- furfural, 2-furaldehyde, tyrosol, p-hydroxybenz- aldehyde, caftaric acid, protocatechu-aldehyde, trans-p-coutaric acid, caffeic acid, trans-p-coumaric acid, and ferulic acid. White wine vinegar aged in wood barrels led in p-hydroxybenzoic acid, syringic acid, cis-p-coutaric acid, fertaric acid, vanillin, and syringaldehyde. Red wine vinegar aged in wood barrels had the highest concentrations of protocatechuic acid, p-hydroxybenz- aldehyde, and 2-S-glutathionyl caftaric acid. Apple cider vinegar led in catechin and epicatechin, and honey vinegar led in chlorogenic acid (Natera et al., 2003). Polyphenol contents of different vinegars are of particular interest for this field of research, as literature has shown that they have differing neuroprotective and neurogenerative properties (Dias et al., 2012).

The current body of research points to a complex and multidimensional relationship between polyphenols and depression, indicating that each of the individual polyphenol metabolism pathways may have roles to play to varying degrees (Dias et al.,

2012). One of the proposed pathways may be related to the role of polyphenols in hippocampal neurogenesis, as patients suffering from depression and other mood disorders have been shown to have smaller hippocampi (Dias et al., 2012).

Organic Acids

The organic acid profiles of different vinegars can be categorized as volatile and non-volatile acids and vary widely depending on the original material for the vinegar, with all profiles, considering their nature as vinegar, being predominantly acetic acid (Xia et al., 2020; Natera et al., 2003). Other organic acids found in common vinegar types include volatile types (formic, propionic, butyric, and quinic acids) and organic types like succinic, malic, tartaric, lactic, and citric acids (Natera et al., 2003; Xia et al., 2020). Wine vinegars, apart from balsamic vinegar (51.1 g/L), had the highest acetic acid levels among fruit vinegars (79.6-86.8 g/L), with red wine not fermented in wood carrying the highest among these (Natera et al., 2003). This same red wine vinegar had very low tartaric acid, very high lactic acid, and no malic acid present (Natera et al., 2003). The non-wine vinegars tested (honey, alcohol, apple, and malt) carried much lower levels of acetic acid (50.9 – 67.6 g/L) (Natera et al., 2003). Volatile acids other than acetic acid were not found as consistently, with propionic and butyric only being found in grain vinegars and formic and quinic acids only found in balsamic and some non-wine fruits, respectively (Xia et al., 2020). These organic acids can play many roles in the body. Some have been shown to influence macronutrient metabolism while others may influence insulin balance, affect blood lipid profiles, play a role in neurological, and carry antimicrobial traits (Xia et al., 2020).

Acetic Acid

Biochemistry

Many of the health benefits touted by vinegar ingestion can be attributed to its acetic acid content and this acid's role in metabolic, neurological, and microbial processes in the body. Structurally, acetic acid is a simple monocarboxylic acid with only two carbon atoms and a molecular structure of CH3COOH (National Center for Biotechnology Information, 2004). In the human body, after being absorbed through either the lungs or the GI tract, acetic acid (found in circulation as its anion form of acetate) and its important acetyl group are rapidly utilized and found in virtually every physiological system of the human body (National Center for Biotechnology Information, 2004). It is a non-essential nutrient, as very little of our body's acetic acid is consumed directly through foods (mainly through vinegar, pickled or fermented foods, and some fruits and vegetables) (Kohlmeier, 2003). If not consumed in the body, acetate can also be endogenously produced from the digestion and metabolism of alcohol and macronutrients, including non-digestible fiber, and converted to acetyl-CoA in the liver (Chapp et al., 2024; Kohlmeier, 2003; Moffett et al., 2020). In the GI tract, acetic acid is absorbed in high amounts in the jejunum, and then again in the proximal colon with additional bacterial production of the acid from fiber fermentation (Kohlmeier, 2003; González Hernández et al., 2019). From the gut, acetate travels directly to the brain for parasympathetic activity and into blood circulation as free acetate to fat tissues, muscle tissue, and the pancreas for conversion to acetyl-CoA and then, most often, metabolism within the citric acid cycle (González Hernández et al., 2019).

Health Effects

Once in circulation, acetate appears to have antimicrobial and antifungal properties, may contribute to improved weight control, and studies show it also influences insulin sensitivity and carbohydrate and fat metabolism (National Center for Biotechnology Information, 2004; Xia et al., 2020; González Hernández et al., 2019). The means by which acetic acid positively impacts weight control appear to center on appetite regulation, satiety, and energy expenditure (González Hernández et al., 2019). Its potential benefits for insulin sensitivity and glucose homeostasis have been reviewed at length and appear to be most beneficial when coupled with carbohydrate intake (González Hernández et al., 2019). Specifically, acetic acid has been shown to improve the population and activity of the Lactobacillus species of bacteria, which plays an especially critical role in tryptophan metabolism in animal research, which may highlight potential mechanisms worth exploring more (Gheorghe et al., 2019; Li et al., 2022).

Dosing and Administration

Previous research on acetic acid and its effects on various aspects of health has collectively build preliminary evidence indicating the different doses, modalities, and timing of ingestion required for maximized health impact. Studies have shown improvement in post-prandial glucose levels in a wide range of acetic acid doses (0.5 g, 1.1 g, 1.4 g, and 1.7 g) (Jasbi et al., 2019; Johnston et al., 2010; Ostman et al., 2005). These and others have identified that improved health indicators by vinegar ingestion appear to be dose dependent (Ostman et al., 2005; Johnston & Gaas, 2006; Mimura et al., 2004).

When comparing ingestion of liquid vinegar with commercially available vinegar tablets, it appears that tablets taken whole (1.5 g acetic acid) were not as effective as liquid vinegar (1.25 g acetic acid) at improving blood glucose management (Feise & Johnston, 2020). When dosing of acetic acid is controlled, this higher effectiveness of liquid vinegar may be due to its expedited gastric absorption compared to the post-pyloric absorption of capsulated supplements (Sugiyama et al., 2010). This could partly explain why the effects of vinegar capsules dissolved prior to ingestion are higher than when the capsule is taken whole (Feise & Johnston, 2020).

Vinegar and Depression

In the first of its kind, a 2021 study examined the relationship between vinegar intake and depression scores (Johnston et al., 2021). Participants in the treatment group who supplemented their diets with liquid vinegar showed improvement in depression and mood survey scores and alterations to their urine metabolomics that are in line with improved mood (Johnston et al., 2021). In fact, there was a 20-34% improvement in the self-reported depression scores after the four-week intervention and 17 urine metabolites showed significant change. Specifically, these metabolites appeared to center on the hexosamine pathway and glycine, serine, and threonine metabolism. They concluded that with these improvements in healthy college-age adults, continued research in the area was warranted.

CHAPTER 3

Methods

Recruitment

Investigators recruited generally healthy, non-smoking adults (male and female) ages 18-40 years who are free of chronic disease by self-report (e.g., diabetes, heart disease, cancer, gastrointestinal, liver or kidney disease) with a BMI from 25 to 40 calculated from self-reported height and weight. To adequately power the study, recruiting an estimated 40 participants was desired. Participant recruitment began in January 2022 via flyers (Appendix B), emails, and Canvas platforms for some classes. Individuals with an interest in participating were directed to a SurveyMonkey survey (Appendix C) for further eligibility assessment by investigators. Those who were eligible based on survey results were emailed for any necessary clarification and visit scheduling.

Institutional Review Board (IRB) approval was granted by Arizona State University prior to any recruitment or randomization (STUDY00017204, Appendix A).

Study Design and Participant Procedures

The protocol utilized a placebo-controlled, parallel arm study design for a 4-week trial. At the participants' visits, investigators obtained written informed consent (Appendix D), and participants were stratified by gender, age, and BMI and randomized to the active treatment group of liquid vinegar supplementation (VIN) or the control group of vinegar pill intake (CON). Due to the nature of these two protocols, complete blinding to treatment was not possible, but participants were not made aware of whether

their protocol was considered the active or control treatment and there was no discussion of active dosage discussed with participants.

Participants visited the study site at two points in time: at baseline before protocols began and again at week 4 after protocol completion. At each visit, health questionnaires, fasted blood draws, depression questionnaires, and 24-hour dietary recalls were taken.

Participants in the VIN group were instructed to ingest red wine vinegar at the start of lunch and dinner meals twice per day for the full 4-week duration of the trial starting on the day of the baseline visit. Participants were instructed to dilute the vinegar in at least 8oz of water or another beverage for aspiration mitigation and palatability. Participants in the CON group were instructed to follow the dosing on the bottle of the vinegar capsules—one pill once per day, and to do this in the morning before their first meal. The vinegar capsules had an odor of vinegar and were described to participants during the study visits as 'vinegar pills', ensuring they remained blinded to treatment assignment. Upon successful completion of the study and attendance at their second visit, participants received $50 compensation.

Study Variables

The independent variable in this study is acetic acid ingestion, either at an effective dosage or not. Specifically, those in the VIN group were instructed to ingest 2 tablespoons of red wine vinegar twice daily with meals, which provided an estimated 3,000mg of acetic acid total. Each participant received 4, 16oz bottles of Pompeian Red Wine Vinegar to meet the intake requirements for their protocol, ideally returning 8 oz of remaining vinegar at the end of the study for 100% adherence. Participants in the CON

group were instructed to take 1 apple cider vinegar capsules once per day in the morning (providing 450mg apple cider vinegar, estimated 22.5 mg acetic acid). Each CON participant received one 100-capsule bottle of Spring Valley Apple Cider Vinegar Capsules to meet these requirements for the 28 days of the study. By the end of the protocol, participants should have returned 72 vinegar pills to meet 100% adherence requirements.

The primary dependent variable in this study is any change in depression scores. As secondary dependent variables, any changes to 6 blood metabolites associated with mood states and tryptophan metabolism were analyzed— tryptophan, serotonin, gamma-aminobutyric acid (GABA), and three gut-mediated short-chain fatty acids. It was anticipated that participants in the VIN group would experience a greater improvement (lowering) of depression scores and changes in the blood metabolome consistent with potentially improved mood states and altered tryptophan metabolism**.**

Visit Protocol

At the baseline visit, participants completed a health history questionnaire (Appendix E) the Patient Health Questionnaire (PHQ-9, Appendix H) and the Center for Epidemiologic Studies Depression Scale (CES-D, Appendix G), a stress evaluation survey, (Appendix I), a 24-hour dietary recall (Appendix F), a Tanita body composition analysis, and a fasted (no food or beverage with the exception of water for 10 hours) blood draw, after which they received their assigned protocol materials. At least one investigator was present during each portion of the participants' visits for any questions or concerns the participants had.

The goal of the health history questionnaire was primarily for the evaluation of participant baseline characteristics and determination of eligibility based on inclusion and exclusion criteria, namely physical activity habits, chronic health conditions, adherence to any specific diet plans, pregnancy/lactation status, and any changes to medication or supplementation patterns in the preceding three months. Participants actively training for organized moderate or strenuous sports, with chronic health condition diagnoses, adhering to any specific diet plan including vegetarianism and veganism, who were pregnant or lactating, or who had had any changes to medication or supplementation use were excluded.

The PHQ-9 questionnaire is a 9-question Likert scale assessment designed for the detection of depression in the primary care setting (Kroenke et al., 2010). It also assesses the severity of depression, is a good indicator of treatment effectiveness, and has shown to be equal to or better than other common primary care measures in several studies (Löwe et al., 2004; Kroenke et al., 2010; Williams et al., 2002). Many consider this to be the gold standard for depression detection and treatment monitoring. Should any participant have answered a score of 1 or higher on question 9 of the PHQ-9 survey (indicating suicidal ideation or other risk for death by suicide), the participant would have been immediately disqualified and provided with suicide prevention center and hotline contact information, local nutrition engagement opportunities, and College of Health Solutions research information.

The CES-D is of similar structure to the PHQ-9 with no significant change in its effectiveness to detect and grade depression, but with more of an emphasis on the

affective components of depression and identifying depression in the general population (Radloff, 1977).

The 24-hour dietary recall was conducted by a trained study investigator to ensure clarity and thoroughness. The dietary recall allowed investigators to estimate the dietary consistency of the participants throughout the study and identify shifts in dietary intakes that may have influenced study results. Investigators used the Food Processor software by ESHA Research to calculate the daily energy intake data from the 24-hour dietary recalls completed with participants.

The stress evaluation survey, previously used in research conducted by Arizona State University (Johnston & Curtin, 2020), sought to evaluate whether participants were experiencing higher than normal amounts of stress within the three days before study visits. The survey required that participants select any number of circumstances or events they experienced from a list of 17 options.

The fasted blood draw was conducted by an on-site phlebotomist and then processed in-house by investigators at the Wexford Innovation Center at ASU.

During the assignment of protocol materials, the importance of adherence to the assigned protocol was emphasized and a paper calendar was provided for participants to record their daily treatment adherence, including dosage times, new medications taken, clinical signs and symptoms, and other general notes on health throughout the 4 weeks. Investigators also emphasized that if a dose were missed, it was best to simply note the missed dose on the calendar and then continue with the protocol as normal. Additional materials provided to the vinegar group included a measuring cup and a 2-ounce screw-top bottle for daily vinegar dose transportation as needed for on-the-go consumption.

During the 4 weeks of the study, investigators emailed the participants weekly to check for any questions or concerns and reiterate contact information should the participants have had any during the week.

At their second visit at week 4, participants completed the physical activity portion of the health history questionnaire and the depression questionnaires, provided a fasted blood sample, returned any remaining protocol materials, returned their protocol adherence calendars, and signed their requests for monetary compensation from College of Health Solutions. Any remaining vinegar was weighed, and control protocol capsules counted to check for adherence.

Within 2 weeks of every participant's second visit, the study investigators then mailed their Sonora Quest Laboratories and Tanita results. Monetary compensation was managed externally by the College of Health Solutions administrative staff.

Laboratory Analyses

The fasting blood sample (20 ml) for blood metabolomics was taken by phlebotomy staff at Wexford Labs at ASU and processed by study investigators and other lab staff. Blood metabolites analyzed included tryptophan; serotonin; gamma-aminobutyric acid (GABA); gut-mediated short-chain fatty acids including acetic acid, propionic acid, and isobutyric acid, and others. Previously, investigators had intended to examine changes to quinolinic acid and indoleacetic acid, but targeted metabolomics did not include data on these, so analyses refocused on niacinamide, glutathione, and their respective metabolic pathways.

The targeted detection of aqueous metabolites was detailed previously (Jasbi et al., 2019). Briefly, 50 µL of each plasma sample was mixed with 500 µL MeOH and 50

μL internal standard solution (composed of 13C3-lactate and 13C5-glutamic acid). Following centrifugation, supernatants (450 μL) were dried and then reconstituted in 150 μL of 40% phosphate-buffered saline/60% acetonitrile (ACN). A pooled sample was used for quality control and injected once every 10 experimental samples. All LC-MS/MS experiments were performed on an Agilent 1290 ultra-performance liquid chromatography-6490 triple quadrupole-MS system (Santa Clara, CA). Each sample was injected twice (for negative ionization mode and positive ionization mode) using hydrophilic interaction chromatography on a Waters XBridge BEH Amide column (150 mm × 2.1 mm, 2.5 μm; Waters Corporation, Milford, MA). The flow rate was 0.3 mL min−1, the auto-sampler temperature was kept at 4 °C, and the column compartment was set to 40 °C. The mobile phase system was composed of Solvents A (10 mM ammonium acetate [NH4OAc], 10 mM ammonium hydroxide [NH4OH] in 95% H2O/5% ACN) and B (10 mM NH4OAc, 10 mM NH4OH in 95% ACN/5% H2O). After an initial 1 min isocratic elution of 90% B, the percentage of Solvent B decreased to 40% at t = 11 min. The composition of Solvent B was maintained at 40% for 4 min (t = 15 min), after which the percentage of B gradually went back to 90%, to prepare for the next injection. The mass spectrometer was equipped with an electrospray ionization source. Targeted data acquisition was performed in multiple-reaction-monitoring (MRM) mode. All aspects of the LC-MS system were controlled by Agilent MassHunter Workstation software (Santa Clara, CA). Extracted MRM peaks were integrated using Agilent MassHunter Quantitative Data Analysis (Santa Clara, CA).

Statistical Analyses

In analyzing the data, both depression survey and laboratory data were assessed. Data was reported as mean ± standard deviation (SD) and a p-value of ≤0.05 is considered significant. For baseline data, Mann-Whitney U and chi-squared tests were run for ratio and nominal data, respectively. For depression survey scores (CES-D and PHQ-9), Mann-Whitney U tests were conducted for analysis. A 95% winsorization was applied to change data to limit the impact of outlier data.

Metabolite data were normalized, a standard practice in metabolomics research, to enhance model performance and improve the accuracy of the model using log-transformation and Pareto scaling. This approach functions to minimize the impact of outliers and scaling issues, making the data more suitable for analyses. Metabolite, pathway, and integrating enzyme enrichment analysis were performed and visualized using MetaboAnalyst 5.0 software package (Figures 2-6); significance and impact were calculated using a global test of relative betweenness centrality. For metabolite changes determined to be not significant by MetaboAnalyst 5.0, univariate analyses were subsequently conducted via SPSS.

IBM SPSS Statistics were used for the non-parametric and univariate analyses (IBM Corp. Released 2020. IBM SPSS Statistics for Windows, Version 27.0. Armonk, NY, USA: IBM Corp). Significance was set at $p < 0.05$.

CHAPTER 4

Results

Recruitment

Recruitment began in January 2023 on and around Arizona State University's Downtown Phoenix campus and with faculty members in popular classes on other campuses. Recruitment ended in May 2023. An online Survey Monkey screening survey (www.surveymonkey.com) was advertised via internal CHS research listservs, flyers around campus and the downtown area, My ASU banner ticker on staff My ASU pages, and via email to students across popular ASU classes. A total of 247 respondents completed the screening survey. Of these, 162 were excluded based on their initial responses; 81 were emailed to confirm and validate their responses, obtain written consent, and set up their appointment; with 36 lost due to unresponsiveness at this stage and 4 others lost for other reasons.

The remaining 45 participants were stratified by age, gender, and actual BMI, and then randomized to a protocol group based on a coin flip: the experimental (VIN) group (n=24) or control (CON) group (n=21). These 45 were also scheduled for their first visit, with 9 withdrawing prior to their visit and 7 being excluded during the visit because they did not meet the criteria. Five of these 7 were excluded because they had changed medication or supplement use in the preceding 3 months; one due to previously undisclosed thyroid disease, and one due to mental health restrictions.

Of the remaining 29 subjects who started the protocol, 28 finished the trial. One VIN participant withdrew due to adverse reactions to the vinegar ingestion. Sixteen participants finished in the vinegar group with an average of 90.3% protocol adherence,

and 12 in the control group with an average of 101.1% protocol adherence. Protocol adherence was tracked by weighing the remaining vinegar volume and counting the remaining capsules for VIN and CON, respectively.

Participant and Group Characteristics

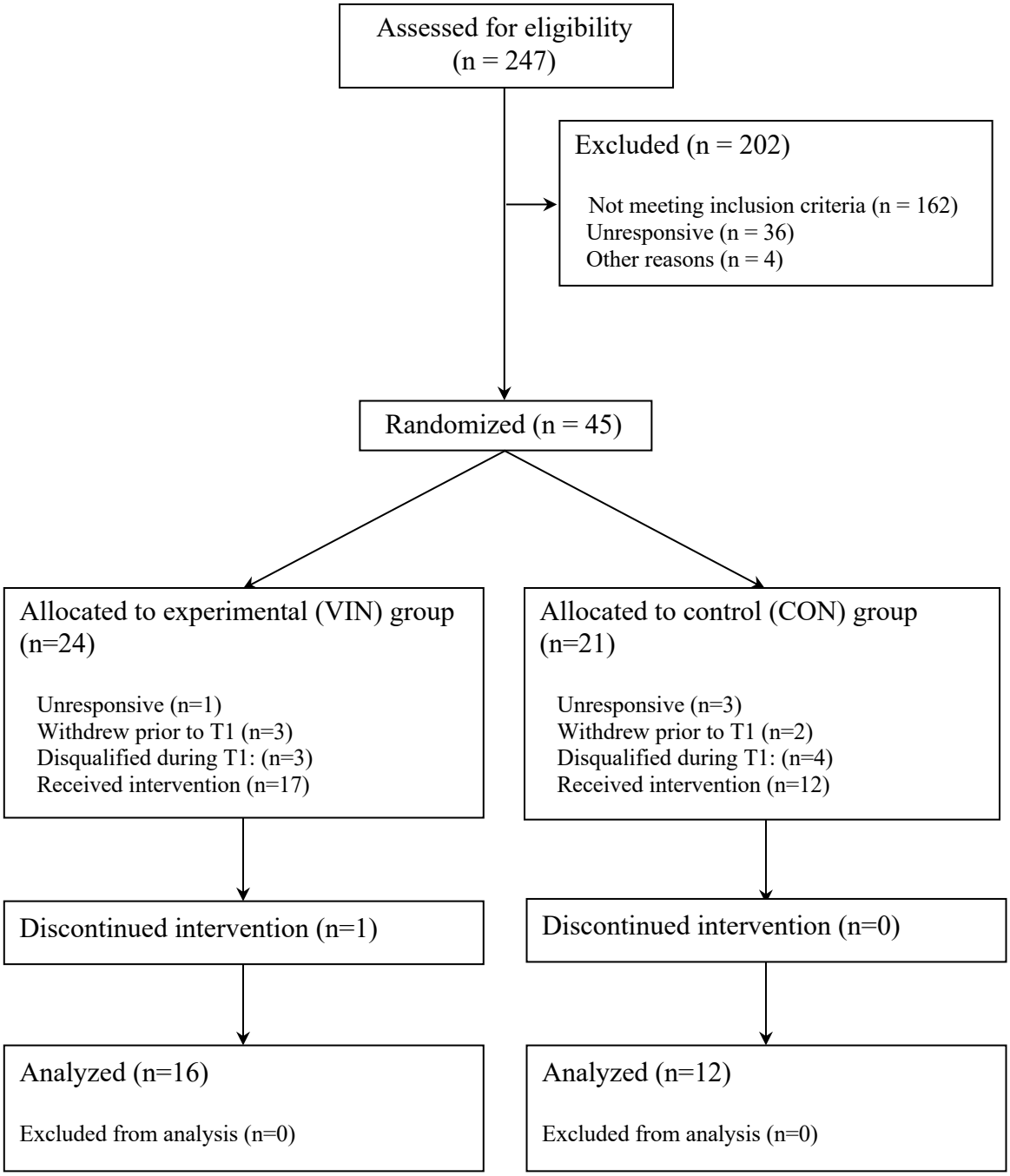
The baseline information collected that was used to determine participant eligibility were age, self-reported BMI at screening, diet history, physical activity, alcohol use, and medication and supplement history. These criteria were evaluated using standard protocol during the screening process and then confirmed or further evaluated at the first visit of the trial. Analysis via Mann-Whitney U test revealed no significant differences between VIN and CON groups at baseline (Table 1).

Ages for participants ranged from 18-41. For the VIN group, participants had a mean age of 25.3±7.3 years, and the CON group had a mean age of 26.3±6.8 years (p= 0.423). BMI by self-reported heights and weights from the screening survey were used for determining eligibility for the study, and then measured BMI for data analysis was determined via Tanita scale at first visit. Measured BMI for participants ranged from 22.6-36.9 kg/m^2. For the experimental group, participants had a mean BMI of 27.3±3.7 kg/m^2, and the control group had a mean BMI of 27.9±3.4 kg/m^2 (p=0.450, formatting).

Across the trial, participants averaged 94.9% compliance with their respective protocols. Compliance was tracked by the weight of remaining vinegar for VIN participants and the count of remaining apple cider vinegar pills for CON participants. The VIN group averaged 90.3±17.1% compliance while the CON group averaged 101.1±13.8% compliance (p=0.029).

Figure 1

Consort Flowchart



Physical activity habits were estimated according to self-reported average weekly activity using Godin Leisure-Time Exercise Questionnaire and subsequent metabolic equivalent tasks (METs) calculations by investigators. For low, moderate, and strenuous activities, values of 3, 5, and 9 METS were assigned, respectively. All participants were below 90 METs of estimated moderate to strenuous physical activity, with the average METs per week coming to 35.6±17.0 for the experimental group and 38.3±20.4 for the control group. The change in average METs per week over the course of the study did not differ between groups (p=0.304).

The average alcohol intake at baseline was 0.56±0.75 drinks per week for the experimental group, and 1.3±2.9 for the control group. Alcohol intake did not change across the course of the study for either group.

Medication and supplement history were reported as number of medications participants took on a consistent basis and whether (yes or no) they took any sort of other dietary supplement. Patients were disqualified if they had any changes to either in the preceding 3 months, anticipated any changes through the course of the study, or were taking vinegar as a dietary supplement already. At baseline, 6 VIN participants (37.5%) and 5 CON participants (41.7%) disclosed medication use, and 6 VIN participants (37.5%) and 5 CON participants (41.7%) disclosed supplement use. Those who disclosed medication use were not always the same participants as those who also disclosed supplement use. The average number of medications taken by those in the experimental group was 0.69±1.08, and 0.50±0.67 for the control group. Total medication use did not change significantly between groups over the course of the study (p=0.365). One participant in the experimental group reported one unexpected new medication added

over the course of the trial. Six (37.5%) and five (41.7%) participants confirmed taking supplements in the experimental and control groups, respectively. There were no reported changes to supplement usage over the course of the trial.

Other participant information gathered that did not dictate eligibility included sex, weight, height, ethnicity, education level, estimated energy intake via 24-hour dietary recall, and stress score (Table 1). The average participant weight was 79.0±15.2 kg for the experimental group, and 79.9±13.1 kg for the control group. The average participant height was 169.9±11.6 cm for the experimental group, and 169.1±10.0 cm for the control group.

There were six options available to participants when selecting their ethnicity: Native American, African American, Caucasian, Hispanic, Asian, and Other. Four of these categories, listed in order of representation, were selected by participants: Caucasian, African American, Native American, and Hispanic. In the experimental group, nine participants (56.3%) identified as Caucasian, five (31.3%) as Hispanic, two (12.5%) as African American, and zero (0%) as Native American. In the control group, six participants (50.0%) identified as Caucasian, three (25.0%) as Hispanic, two (16.7%) as African American, and one (8.3%) as Native American. Due to sample size, non-Caucasian groups were collapsed into a single category for analysis. No participants identified as either Asian or Other.

There were five options available to participants when selecting their level of completed education: high school diploma, AA/vocational degree, college (undergraduate) degree, MS degree, and PhD degree. Four of these categories, listed in order of representation, were selected by participants: high school diploma,

AA/vocational degree, college degree, and MS degree. In the experimental group, six participants (37.5%) completed high school, six (37.5%) completed a college degree, three (18.8%) completed an MS degree, and one (6.3%) completed an AA/vocational degree. In the control group, four participants (33.3%) completed high school, four (33.3%) completed a college degree, two (16.7%) completed an AA/vocational degree, and two (16.7%) completed an MS degree. Due to sample size, high school diploma and AA/vocational degree were collapsed into one category, and college degree and MS degree were collapsed into another for this analysis. No participants reported completed PhD education.

The baseline daily energy intake was 2189.2±565.8 for the experimental group and 2166.5±837.8 for the control group. Over the course of the trial, the experimental group had an average 134.9±708.1 kcal increase in reported energy intake and the control group had an average 2.1±760.2 kcal increase. These increases were not significantly different between the groups ($p=0.479$).

The average stress score at baseline was 3.9±2.5 events for the experimental group, and 2.4±2.3 events for the control group. Changes in stress levels between the groups over the course of the study were not significant ($p=0.907$).

Table 1

Demographics and Baseline Health History Data by Group

Characteristic	VIN	CON	*P* [a]
Sex, n (%)			
Male	4 (25)	3 (25)	0.666
Female	12 (75)	9 (75)	
Age, y	25.3±7.3	26.3±6.8	0.423
BMI, kg/m2	27.3±3.7	27.9±3.4	0.450
Weight, kg	79.0±15.2	79.9±13.1	0.732
Height, cm	169.9±11.6	169.1±10.1	0.982
Ethnicity, n (%)			
Caucasian	9 (56.3)	6 (50.0)	
African American	2 (12.5)	2 (16.7)	0.521
Native American	0 (0.0)	1 (8.3)	
Hispanic	5 (31.3)	3 (25.0)	
Education, n (%)			
High school diploma	6 (37.5)	4 (33.3)	
AA/vocational degree	1 (6.3)	2 (16.7)	0.521
College degree	6 (37.5)	4 (33.3)	
MS degree	3 (18.8)	2 (16.7)	
Alcohol Intake [a]	0.44±0.60	1.21±2.87	0.945
Supplement Use at T1, n (%)			
Yes	6 (37.5)	5 (41.7)	0.823
No	10 (62.5)	7 (58.3)	
Medications [b]	0.69±1.08	0.50±0.67	0.945
Stress Score [c]	3.88±2.45	2.42±2.31	0.159
Adherence, %	90.3±17.1	101.1±13.8	0.029

Note. Ratio data are reported as mean ± standard deviation (SD) and nominal data as n (%). *P* values are significant at $p<0.05$ and represent the Mann-Whitney U test and chi sq. for ratio and nominal data, respectively.

[a] Reflects alcohol intake as reported average number of drinks per week

[b] Reflects the number of medications being consistently taken, by self report

[c] Reflects the number of stressful events in the preceding 3 days, according to Stress Evaluation (Appendix I)

Table 2

Change in Health History Data Over Four Weeks

	VIN	CON	*P* [a]
Energy Intake, kcal per day			
T1	2189.2±565.8	2166.5±837.8	
T2	2324.1±697.5	2178.6±796.8	
Change	134.9±708.1	12.1±760.2	0.479
Physical Activity, METs per week			
T1	35.56±16.99	38.33±20.43	
T2	37.75±19.77	34.96±18.09	
Change	2.19±11.58	-3.38±18.08	0.304
Medication [b]			
T1	0.69±1.08	0.50±0.67	
T2	0.75±1.07	0.50±0.67	
Change	0.06 ± 0.25	0.00 ± 0.00	0.365
Confirmed Other Dietary Supplement Use, n (%)			
T1	6 (37.5)	5 (41.7)	
T2	6 (37.5)	5 (41.7)	-
Change	0.00±0.00	0.00±0.00	
Stress [c]			
T1	3.88 ± 2.45	2.44 ± 2.31	
T2	3.50 ± 2.31	1.67 ± 1.50	
Change	-0.38 ± 2.22	-0.75 ± 2.93	0.907

Note. Ratio data are reported as mean±SD and nominal data as n. Change data calculated as the post-score minus pre-score. *P* values represent univariate and chi squared analyses for ratio and nominal data, respectively, percent adherence as a covariate.

[a] Data are significant at $p<0.05$

[b] Reflects the number of medications being consistently taken, by self-report

[c] Reflects the number of stressful events in the preceding 3 days, according to Stress Evaluation (Appendix I)

Depression Surveys (CES-D and PHQ-9)

At baseline, CES-D results were correlated to participant weight (p=0.047) and PHQ-9 score (p=0.011). However, CES-D (p=0.336) and PHQ-9 scores (p=0.26) at baseline did not significantly differ between groups.

Based on the CES-D scoring at baseline, one experimental participant (6.3%) and zero control participants were at an elevated risk for depression, with scores of 16 or higher. Baseline scores averaged 6.31±5.44 and 4.92±4.96 for the groups respectively (p=0.347). At the second visit, CES-D scores averaged 4.69±3.28 for the experimental group and 4.67±3.87 for the control group, and the changes from baseline were not significant between groups (-1.63±5.07 and -0.25±5.14, p=0.581, ES=0.013). Change data was controlled for adherence and participant weight. At week four, no participants from either group scored 16 or higher, indicating an elevated risk for depression.

Based on the PHQ-9 scoring at baseline, three experimental participants (18.8%) and one control participant (8.3%) scored in the range of 5 to 9, indicating mild depression, and one experimental participant (6.3%) scored in the range of 10 to 14, indicating moderate depression. Baseline scores averaged 3.06±3.065 and 1.75±2.006 for experimental and control groups, respectively (p=0.280). At the second visit, PHQ-9 scores averaged 1.75±1.880 for the experimental group and 1.43±1.929 for the control group. The change data was significant when controlling for adherence (-1.31±2.182 and -0.33±0.985, p=0.049, ES=0.146). At the second visit, one participant from the experimental group (6.3%) and 1 participant from the control group (8.3%) scored in the range of 5-9, indicating mild depression, and no other participants scored higher.

Table 3

CES-D and PHQ-9 Scores at Baseline and at Week 4

	VIN	CON	*P* [a]	η^2
CES-D				
T1	6.31±5.44	4.92±4.96		
T2	4.69±3.28	4.67±3.87		
Change	-1.63±5.07	-0.25±5.14	0.581	0.013
PHQ-9				
T1	3.06±3.065	1.75±2.006		
T2	1.75±1.880	1.43±1.929		
Change	-1.31±2.182	-0.33±0.985	0.049	0.146

Note. Data reported as mean±SD, higher CES-D and PHQ-9 scores are unfavorable. Change data calculated as the post-score minus pre-score. A 95% winsorization was applied to change data prior to analyses (e.g., all data are capped within two standard deviations of the mean). *P* and η^2 values represent univariate analysis results for significance and effect size, respectively, controlling for adherence. Neither CES-D (p=0.347) or PHQ-9 (p=0.280) differed between groups at baseline.
[a] Data are significant at $p<0.05$

Blood Metabolomics

Blood samples were obtained from each participant before and after the intervention, with samples subsequently analyzed using targeted analysis to assess levels of free tryptophan, peripheral serotonin, GABA, and gut-mediated short-chain fatty acids acetic acid, propionic acid, and isobutyric acid. Univariate analyses, controlling for adherence, determined that changes in L-tryptophan (p=0.777, η^2=0.003), peripheral serotonin levels (p=0.348, η^2=0.035), GABA (p=0.403, η^2=0.028), acetic acid (p=0.355, η^2=0.034), and propionic acid (p=0.383, η^2=0.031), did not differ significantly between time between groups. Changes in isobutyric acid were also not identified as significant per univariate analysis (p=0.068, η^2=0.127), but were identified by normalized

metabolomic analyses by MetaboAnalyst 5.0 as significant ($p=0.0374$, $\eta^2=0.127$) (Figure 2).

Aside from those originally hypothesized, there were other exploratory metabolomic data collected related to isolated metabolites and enriched metabolic pathways. These analyses found 4 metabolites with significant change between groups across time points including isobutyric acid ($p=0.0374$, $q=0.0473$), niacinamide ($p=0.0168$, $q=0.0370$), and glutathione ($p=0.0157$, $q=0.0370$). They also identified 9 metabolic pathways with significant changes in activity with vinegar supplementation,

Table 4

Tryptophan, Neurotransmitters and Gut-Derived Short-Chain Fatty Acid Serum Concentrations [a] at Baseline and at Week 4

	VIN	CON	*P* [b]	η^2
L-Tryptophan				
T1	16649.50±4597.99	16923.42±4858.94		
T2	13417.63±5715.14	15125.92±6166.39		
Change	-3231.88±6391.15	-1797.50±8891.17	0.777	.003
Serotonin				
T1	19029.75±4323.19	17411.58±4459.02		
T2	12283.81±4533.08	13830.17±4611.36		
Change	-6745.94±7345.99	-3581.42±6662.42	0.348	0.035
GABA				
T1	7287.38±1592.93	7636.83±1032.96		
T2	7431.81±1210.43	7395.42±1129.20		
Change	144.44±2377.95	-241.42±1650.84	0.403	0.028
Acetic Acid				
T1	9742991.53±3343593.41	9965041.37±3499753.06		
T2	8801391.68±3012460.87	10337203.10±3785243.22		
Change	-941599.84±4271618.82	372161.73±5103060.41	0.355	0.340
Propionic Acid				
T1	387589.93±74584.81	366449.99±74461.75		
T2	377993.33±113186.73	317813.49±59796.42		
Change	-9596.60±111977.30	-48636.50±107506.78	0.383	0.031
Isobutyric Acid				
T1	72019.34±17600.50	56725.29±14981.51		
T2	69576.79±19915.69	74303.75±19978.47		
Change	-2442.55±26547.26	17578.46±18033.17	0.068	0.127

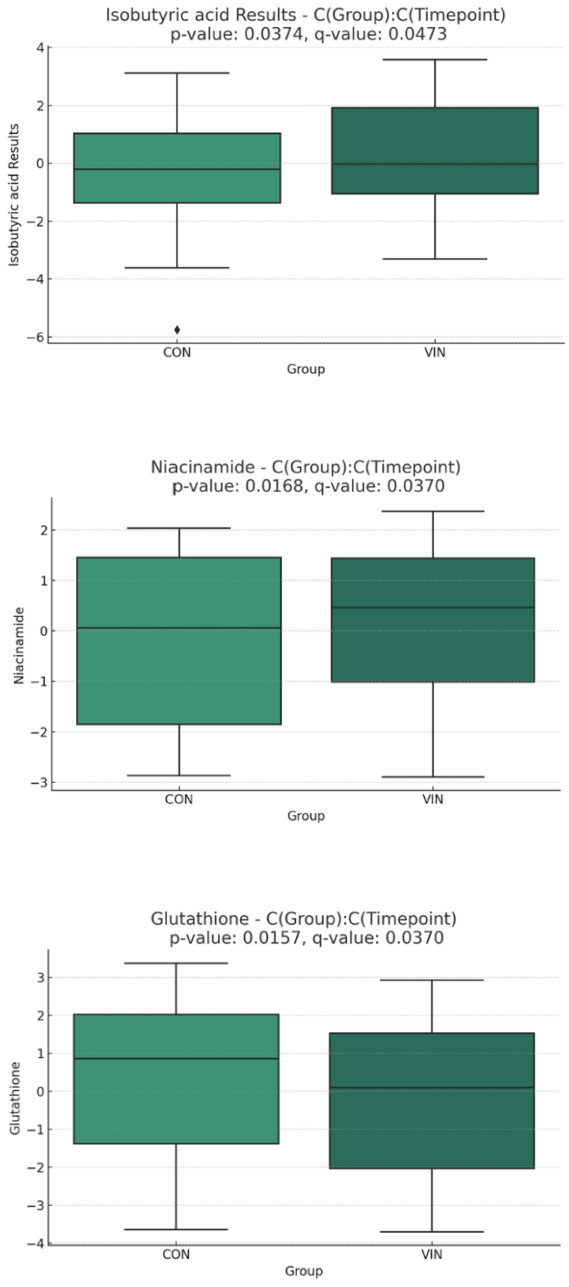
Note. Data reported as mean ± SD. Change data calculated as the post-score minus pre-score. *P* and η^2 values represent univariate analysis results for significance and effect size, respectively, controlling for adherence.

[a] Metabolite concentrations reported as normalized values of abundance

[b] Data are significant at $p<0.05$

Figure 2

Boxplots of Significant Changes in Metabolites Isobutyric Acid, Niacinamide, and Glutathione



Note. P and *q* values represent metabolomics analysis results for significance and significance with an optimized False Discovery Rate (FDR) approach. Metabolic analyses conducted with normalized values of abundance. Data are significant at $p<0.05$ and $q<0.05$.

Table 5

Niacinamide and Glutathione Concentrations [a] at Baseline and at Week 4

	VIN	CON	*P* [b]	η^2
Niacinamide				
T1	2799.13±1329.74	3345.33±1090.56		
T2	5214.44±2844.38	3503.00±1918.24		
Change	2415.31±3591.19	157.67± 2072.21	0.093	0.109
Glutathione				
T1	16728.63±3574.72	20633.25±4140.76		
T2	13788.31±6742.026	13956.08±4587 97		
Change	-2940.31±8224.47	-6677.17± 5501.06	0.238	0.055

Note. Data reported as mean ± SD. Change data calculated as the post-score minus pre-score. *P* and η^2 values represent univariate analysis results for significance and effect size, respectively, controlling for adherence.
[a] Metabolite concentrations reported as normalized values of abundance
[b] Data are significant at $p<0.05$

Figure 3

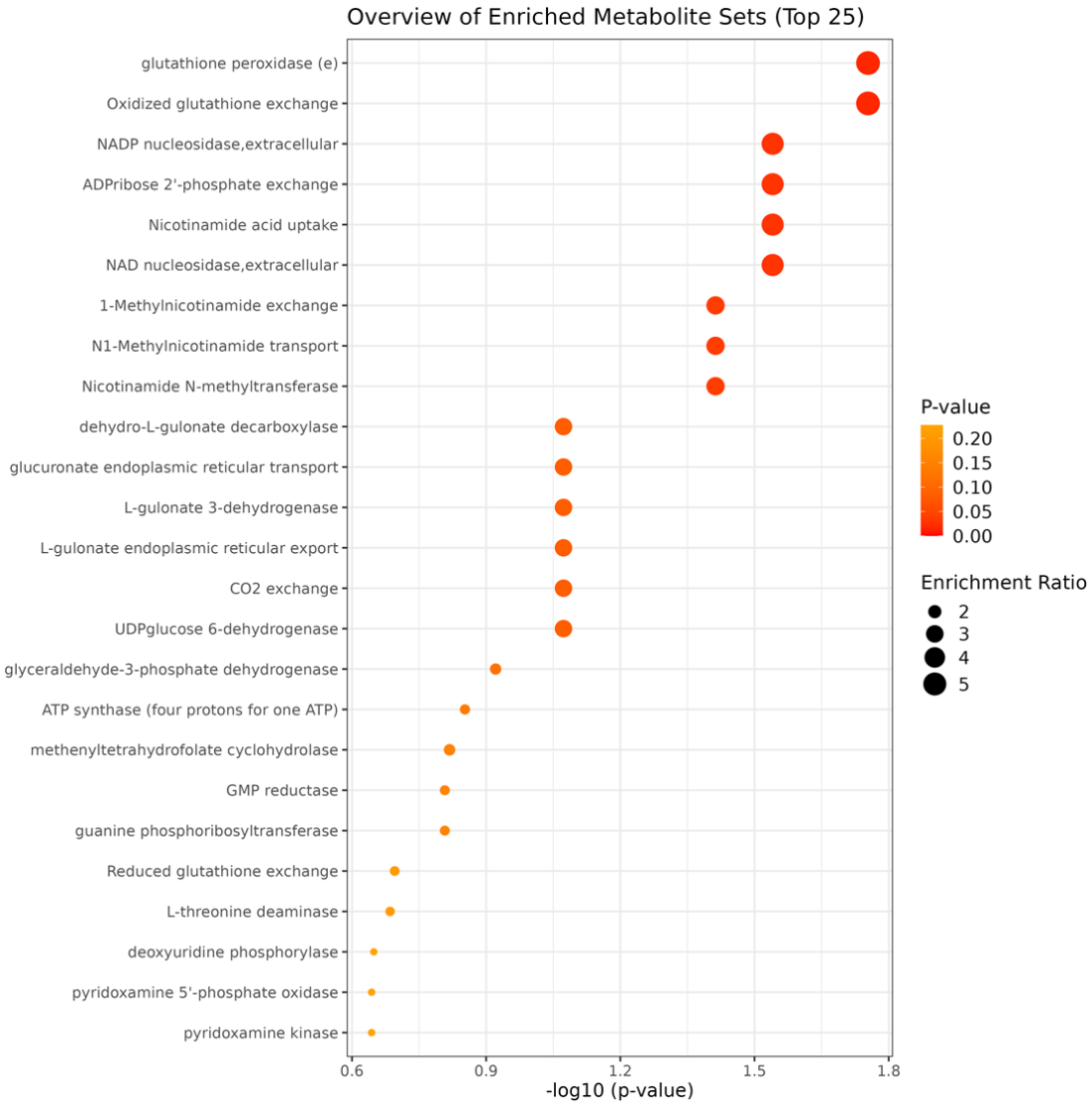
Significant Changes in Metabolic Pathways

Metabolite Set	Total	Hits	Statistic	Expected	P value	Holm P	FDR
glutathione peroxidase (e)	2	1	19.81	3.7037	0.017627	1.0	0.85087
Oxidized glutathione exchange	2	1	19.81	3.7037	0.017627	1.0	0.85087
NADP nucleosidase,extracellular	3	1	17.084	3.7037	0.028801	1.0	0.85087
ADPribose 2'-phosphate exchange	4	1	17.084	3.7037	0.028801	1.0	0.85087
Nicotinamide acid uptake	2	1	17.084	3.7037	0.028801	1.0	0.85087
NAD nucleosidase,extracellular	4	1	17.084	3.7037	0.028801	1.0	0.85087
1-Methylnicotinamide exchange	3	2	11.996	3.7037	0.038652	1.0	0.85087
N1-Methylnicotinamide transport	3	2	11.996	3.7037	0.038652	1.0	0.85087
Nicotinamide N-methyltransferase	3	2	11.996	3.7037	0.038652	1.0	0.85087

Note. P values represent change in metabolic activity between groups across time points, significant at $p<0.05$, reported according to False Discovery Rate (FDR)<0.1

Figure 4

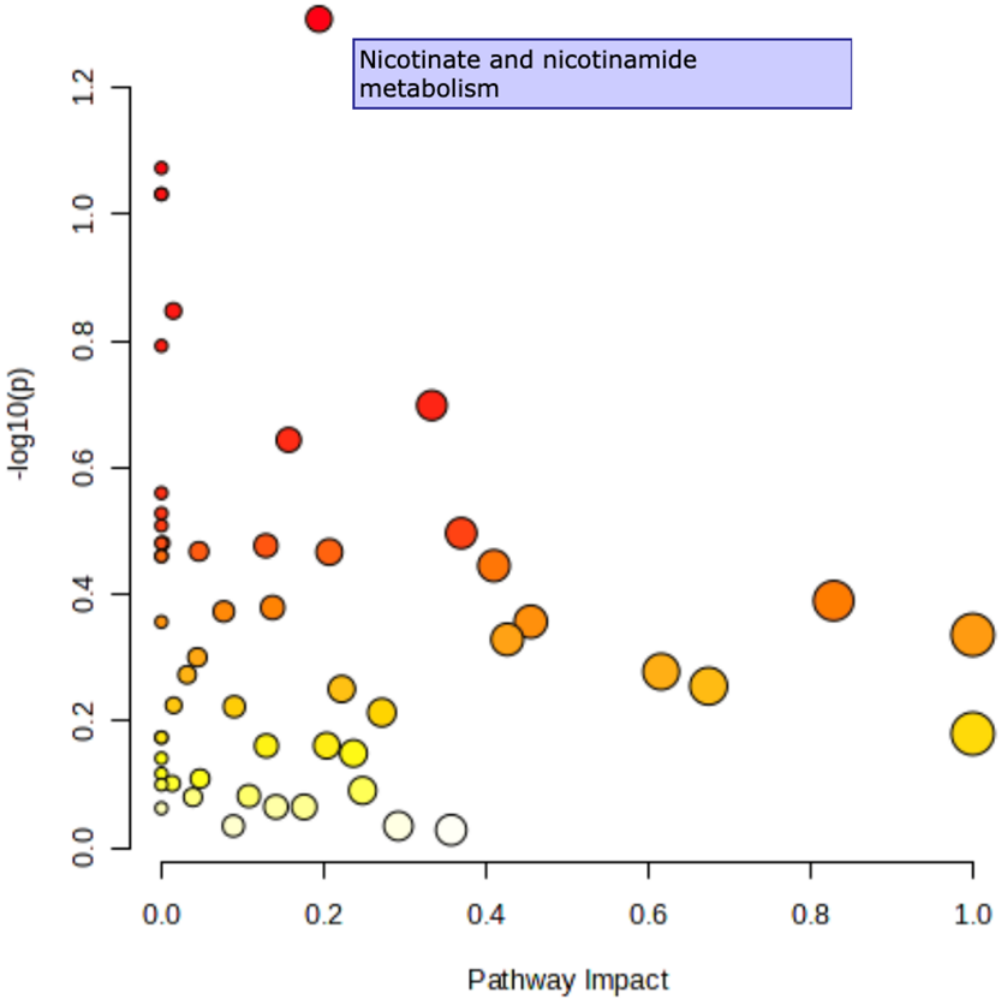
Variance Importance in Projection (VIP) of Pathway Enrichment Analysis Performed Using All Surveyed Metabolites



Note. Displaying the 25 most important enzymatic pathways differentiating between groups between time points, with colored side bar displaying the relative metabolite concentration in each group. Data analyzed between groups after calculating T2/T1 (post/pre). The first nine pathways listed had significant predicted changes ($p<0.05$).

Figure 5

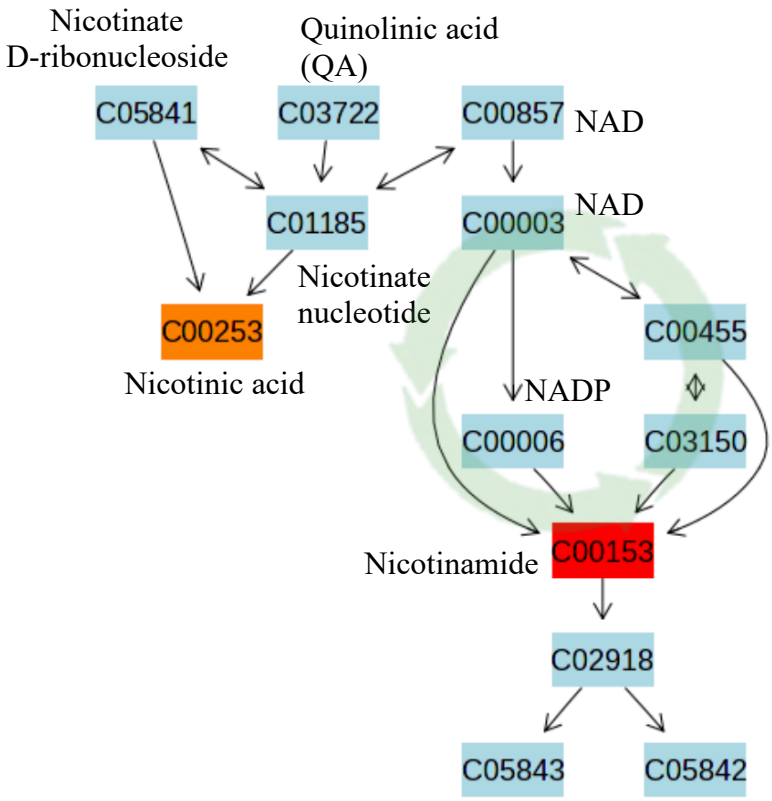
PLS-DA Score Plot of Pathway Enrichment Analysis Performed Using All Surveyed Metabolites



Note. Pathway enrichment analysis performed using all reliably detected metabolites mapped to canonical KEGG pathways. Dots represent change in metabolic activity between groups across time points; size of dot represents the size of the pathway; darker colors (ranging from white to dark red) represent higher hits.

Figure 6

KEGG IDs Referenced in Pathway Diagram



Note. Key metabolite names noted. Colors indicate significance of mapped metabolites. NAD Cycle noted with green arrows

CHAPTER 5

Discussion

This study was conducted to identify the impact that red wine vinegar supplementation has on depression screening survey scores and the blood metabolome in adults free of chronic disease. To date, little research has been conducted to examine the relationship between vinegar intake and its impacts on mental health indicators.

The primary outcome examined in this study was depression symptomology by self-report. Subjective symptoms of depression were quantified in this study with both the CES-D and PHQ-9 surveys, which are validated measures used as screening tools for clinical depression. For the PHQ-9 assessment, when statistical analyses were controlled for protocol adherence, a significant improvement in mean score was observed after liquid vinegar supplementation over the 4-week trial period ($p= 0.049$, $\eta^2=0.146$). When univariate analyses were controlled for both adherence and baseline scores, changes to PHQ-9 were no longer significant but indicated a trend ($p=0.095$, $\eta^2=0.112$). This improvement suggests that daily supplementation of 2 tablespoons of vinegar with meals twice daily (~3100 mg acetic acid) may have a causal relationship with improvement in depressive symptoms in healthy adults.

Depression symptoms according to the CES-D survey were not statistically significantly improved after liquid vinegar supplementation compared with control, even when controlled for adherence and baseline participant weight ($p=0.581$, $\eta^2=0.013$). One explanation for not observing statistically significant changes in survey scores according to the CES-D survey within the same participant pool could be related to the sensitivity with which each survey detected symptomology. At baseline, 25% of VIN participants

tested at mild or moderate depression according to the PHQ-9. Comparatively, only 6.3% of VIN participants scored as depressed according to the CES-D at baseline. This could indicate that the PHQ-9 may have increased sensitivity to symptomology, and therefore a similar potential sensitivity to intervention effects.

It is worth noting that these significant or trending results between intervention groups in the PHQ-9 could be attributed to the VIN group's pre-treatment mean starting higher than that of CON for both the PHQ-9 and CES-D surveys. Although the baseline difference between groups was not statistically significant (CES-D $p=0.347$ and PHQ-9 $p=0.280$), the patterns of means and changes to significance when controlling for baseline scores suggest higher depression scores at baseline for the VIN group may have contributed to the greater changes in survey scores when compared with the control group. This suggests that the efficacy of interventions could improve in groups with worse baseline mental health scores.

Additionally, according to the most commonly accepted definitions for clinical symptom response in the psychology field, there is a treatment response at 50% change on a valid symptom rating scale and a partial response at 25% change (Lecrubier, 2002; Wise, 2004). According to this scale, score changes seen in our study show a partial response in the VIN group in both surveys (25.83% change for CES-D and 42.8% for PHQ-9) compared to no response CON (5.08% for CES-D and 18.86% for PHQ-9).

Throughout the course of the trial, participants gave feedback that they experienced improvements to their mental health from a wide variety of experiences during the 4 weeks of intervention. More than one participant reported that adherence to a routine, self-monitoring, contribution to science, and knowledge of adherence grading

improved their mental health. They also reported improved self-efficacy, confidence, or generally improved mood from these behaviors. These reports were seen across participants from both protocols. This anecdotal evidence supports previous research showing that behavioral strategies for depression treatment may be sufficient to illicit symptom change, irrespective of other interventions (Dimidjian et al., 2006). This idea could be extrapolated to hypothesize that the more behavioral changes implemented, or more of the same positive behavioral change, could further improve depression symptoms. With VIN participants being required to exercise habitual behaviors twice as often as CON participants (vinegar twice per day with meals compared to one pill per day, respectively), the role of behavioral change is another factor to be considered when interpreting these results.

Red wine vinegar is known to have some of the highest polyphenol contents among fruit vinegars, and the antioxidant effects of these compounds should be considered when discussing the noted changes to depression scores in this study (Liu et al., 2019). Red wine vinegar retains comparable antioxidant properties compared to whole black grapes but has a much lower antioxidant capacity than red wines (Pellegrini et al., 2003). Recent research has shown inverse associations between the consumption of high-polyphenol beverages (such as red wine, tea, and coffee) and stress and depression symptomology (Micek et al., 2023).

Based on these results, there is also preliminary evidence that there is a significant association between the Stress Evaluation Survey and PHQ-9 scores ($p=0.05$), with an associative trend emerging with CES-D scores ($p=0.10$). There was no change in average

stress scores in participants before and after interventions, and controlling for stress between groups did not alter the p-values for the depression scores outlined in Table 3.

Moving into the discussion of metabolomics, the study failed to show significant changes in most of the originally hypothesized metabolites, except for isobutyric acid, which showed a significant decrease with vinegar supplementation compared to the increase shown in the control group (Table 4). This short-chain fatty acid decrease could indicate changes to the functionality of the gut microbiota with vinegar supplementation. The results (combined with depression scores) suggest that while the pathophysiology of depression may be influenced by increased ingestion of acetic acid, its exact mechanism may lie at least partially outside of the serotonin pathway of tryptophan metabolism or changes to GABA.

As outlined in the literature review, acetic acid, once absorbed in the body via the GI tract or the lungs, is converted to its anion form as acetate (this conversion is automatic at a pH above 5.5), transported to the liver via the portal vein, and then sent into peripheral tissues (Chapp et al., 2024). In its anion form, to be utilized by the body, it must be converted to acetyl-CoA in tissues all over the body (Chapp et al., 2024; Moffett et al., 2020). Most of the body's acetyl-CoA is quickly utilized within the cytosol, nuclei, and mitochondria in tissues around the body in the citric acid (also TCA or Krebs) cycle as the final step in the breakdown of food to energy within the body (Chapp et al., 2024; Moffett et al., 2020). With this physiological pH regulation of the blood, we would not have expected blood levels of neutral acetic acid to increase if the dosing was not in excess. Further, free acetate is so quickly utilized within the body for cellular respiration (the conversion of food to energy), that an increase in acetate was also not necessarily

expected. While statistically insignificant, our results did show a high effect size in the acetic acid changes in the blood.

Of the additional metabolites analyzed outside of those originally hypothesized, nicotinamide (NAM) and glutathione are of specific interest, as they center on two different pathways within the body that have previously been connected with depression in the literature. One of these pathways is that of the Kynurenine Pathway, the primary tryptophan degradation pathway initiated by stress hormones, which has an end-product of quinolinic acid (QA). QA is a molecule with neurotoxic properties that can be further metabolized into the important coenzymes nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide-P (NADP). This process by which tryptophan eventually converts to NAD and NADPH is known as the de novo NAD Synthesis Pathway, and it has been shown to play a beneficial role in aging, the stress response, longevity, and depression (Zuo et al., 2016; Dugué et al., 2022; Goetzl et al., 2021). Increases in NAM has been associated with improved depression symptomology in humans and has shown to ameliorate depressive symptoms in animals (Prousky, 2010; Liu et al., 2020). This current experiment showed significant changes to the metabolites and enzymatic activity associated with the upregulation of NAM and NAD cycling, with a significant increase in serum levels of NAM ($p=0.0168$) and a significantly increased expression of the extracellular enzyme NADP/NADP nucleosidase (Figures 3 and 4). Previous studies have seen that, independent of other neuroactive metabolic pathways, upregulation of this cycle may attenuate depressive systems (Liu et al., 2020; Wang et al., 2022; Xie et al., 2020).

Previous research has seen a relationship between exogenous acetic acid and alterations in tryptophan metabolism specifically when red wine vinegar is used, as opposed to apple cider vinegar, which has been attributed to their differing polyphenol contents (Jasbi et al., 2019; Johnston et al., 2021). With other research pointing to changes in tryptophan metabolism because of polyphenols in red wine vinegar, it is possible that changes noted herein could be at least partially due to the polyphenol content of red wine vinegar, as opposed to its acetic acid content (Jacobs et al., 2012).

Another pathway identified by which depressive symptoms may be impacted was that of glutamate, another free amino acid that functions as the most abundant neurotransmitter in the human brain (Y. Zhou & N.C. Danbolt, 2014). Glutamate is produced directly from the amino acid glutamine, or it can be endogenously produced from α-ketoglutaric acid, an intermediary of the citric (also TCA or Krebs) cycle, several steps down from the introduction of acetyl-CoA previously discussed (Ubuka, 2021). Glutamate then catabolizes to GABA, and the balance between these two factors is considered by some to be the most important and delicate balance between any two neurotransmitters (Rowley et al., 2012). Excitatory and inhibitory in nature, respectively, keeping glutamate and GABA in balance is the goal of many pharmacological interventions for a wide range of CNS and mood disorders (Cutler et al., 2023; Rowley et al., 2012). Down an alternative metabolic pathway, glutamate converts to glutathione, which (in its reduced form, GSH) is a potent free radical detoxifier catalyzed by the glutathione peroxidase enzyme. This alternative pathway to GSH is upregulated in states of oxidative stress, a potential contributing factor to development of MDD, especially in older adults (Duffy et al., 2015). How exactly GSH and depression are related is still

unclear, according to the literature (Duffy et al., 2015; Godlewska et al., 2015). Duffy et al. (2015) showed that in cases where patients are at risk for depression but not diagnosed, increases in GSH were associated with worsening depressive symptoms in the setting of oxidative stress. However, most other studies conclude that GSH levels are lower in those with depression (Godlewska et al., 2015; Samuelsson et al., 2012; Zalachoras et al., 2020). This current experiment showed significant changes to the metabolite and enzymatic activities associated with this glutathione pathway, with a significant difference in changes to serum levels of glutathione between groups ($p=0.0157$) and significantly increased expressions of the glutathione peroxidase enzymes and oxidized glutathione exchange (Figures 3 and 4). The etiologies for these metabolic shifts are unclear at this stage but could support previous research by showing lower glutathione in a group with worse depression symptomology at baseline. Alternatively, our research could be an early indicator that, in populations at-risk but undiagnosed for depression, vinegar ingestion could influence oxidative stress, thereby altering the GSH response.

Both themes of results, depression symptomology and metabolomics, are crucial findings, as they indicate that vinegar supplementation may improve symptoms of a major contributor to worldwide disability even though the exact mechanism to explain why is still to be clarified. The results of this study provide valuable progress for future researchers to build upon when examining the relationships between acetic acid, depressive symptoms, and the blood metabolome in various human populations.

Limitations

There were several limitations within this study related to our primary and secondary objectives that should be considered when evaluating the results.

Potentially the most significant limitation of this study was its small sample size. According to a reverse power calculation, this study had a 12% and 33% probability of detecting a treatment difference at a two-sided 0.05 significance level for CES-D and PHQ-9 respectively. The exclusion criteria for this study significantly inhibited recruitment efforts, with only 28 of 247 applicants accepted. This small sample size significantly limits its generalizability to other populations.

Other limitations include short intervention duration and lack of control over participant adherence and lifestyle factors like dietary intake and physical activity. While dietary intake was monitored via 24-hour recall, and physical activity assessed via at each visit.

With this protocol structure, there is also always the potential for lack of complete blinding, as participants were aware of which protocol they were receiving. A true blinded placebo (no acetic acid intake) is not possible with this study protocol, although effective placebo has been established according to the predetermined effective dose of acetic acid. Assuming that participants are not aware of this previously determined effective dose, blinding can also be assumed.

CHAPTER 6

Conclusion and Applications

In conclusion, the current study supports both our primary hypothesis that vinegar intake over four weeks can improve depression screening survey scores, and our secondary hypothesis that it would significantly alter the blood metabolome associated with tryptophan metabolism, GABA, and gut-mediated short-chain fatty acids. These results do reflect others identified in the only other study looking at vinegar intake and its potential impact on mental health indicators (Johnston et al., 2021).

Future research is warranted to continue to explore the possible mechanisms by which depression comes to be, and therefore how the addition of acetic acid into its physiology is influencing the lived experiences of participants. Specific attention to metabolic mechanisms is warranted, especially related to SCFAs in the gut, the Kynurenine to NAD pathway, and Glutamate metabolites GABA and glutathione.

When developing future research in this field, studies must be adequately powered with larger participant counts and that the metabolomic factors studied represent alternative physiological processes. Future research could also be enhanced by focusing on clinically depressed or at-risk populations, all BMI ranges, and those on antidepressant medications.

These findings support that recommendations of no more than 2 tablespoons of vinegar twice daily with meals may improve self-reported depression symptomology in generally healthy adults. This is valuable guidance, as vinegar ingestion in healthy adults is generally understood to be well-tolerated, non-invasive, and widely accessible.

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APPENDIX A

IRB APPROVAL

ASU Knowledge Enterprise Development

APPROVAL: MODIFICATION

Carol Johnston
CHS: Health Solutions, College of
602/496-2539
CAROL.JOHNSTON@asu.edu

Dear Carol Johnston:

On 1/17/2023 the ASU IRB reviewed the following protocol:

Type of Review:	Modification / Update
Title:	Effect of daily vinegar ingestion for four weeks on mood state, inflammatory state, and risk for metabolic syndrome in healthy adults
Investigator:	Carol Johnston
IRB ID:	STUDY00017204
Funding:	Name: Graduate College (GRAD)
Grant Title:	None
Grant ID:	None
Documents Reviewed:	• GCP certificate Barrong, Category: Vitaes/resumes of study team; • GCP certificate Coven, Category: Vitaes/resumes of study team; • GCP certificate Fessler, Category: Vitaes/resumes of study team; • pre-screening for stressful events, Category: Measures (Survey questions/Interview questions /interview guides/focus group questions); • protocol, Category: IRB Protocol; • resource guide, Category: Technical materials/diagrams; • Roberts CITI training, Category: Vitaes/resumes of study team; • Screener, Category: Screening forms; • verbal script and ad, Category: Recruitment Materials;

The IRB approved the modification.

When consent is appropriate, you must use final, watermarked versions available under the "Documents" tab in ERA-IRB.

In conducting this protocol you are required to follow the requirements listed in the INVESTIGATOR MANUAL (HRP-103).

Sincerely,

IRB Administrator

APPENDIX B

RECRUITMENT FLYER AND MY ASU BANNER

ASU Nutrition Study: *Vinegar and Health*

THE ASU NUTRITION PROGRAM IS RECRUITING ***HEALTHY ADULTS*** TO EXAMINE HOW VINEGAR IMPACTS HEALTH

Participation is voluntary

If you are a healthy adult 18 to 40 years of age, a non-athlete, and not adhering to a specific diet plan - and if you are willing to incorporate vinegar into your diet daily for 4 weeks – please complete our survey.

Participation includes:

- Two site visits (ASU downtown campus) to meet with study investigators and complete health questionnaires and assessments including height, weight, and blood pressure (<45 minutes)
- At each visit, a fasting blood sample (<1 tbsp) will be collected to measure metabolites linked to health
- Consuming a vinegar supplement daily for 4 weeks (all vinegar supplements provided free of charge)
- You will receive $50 at the completion of the study.
- INTERESTED??? Please visit our recruitment site: https://www.surveymonkey.com/r/ASUVinegarStudy

APPENDIX C

RECRUITMENT SCREENING SURVEY

Background

Thank you for your interest in this research study conducted by ASU Nutrition Professor Carol Johnston and MS students Haley Barrong, Lexie Lish, and Hannah Coven. This research study will examine health effects of vinegar supplementation. We are inviting your participation in the screening process, which will consist of answering questions regarding health history, demographics, and scheduling availability. You have the right to not answer any question, and to stop participation at any time.
We are recruiting upto 40 healthy adults between the ages of 18 and 40 years. Your participation in this survey is voluntary. If you choose not to participate or to withdraw from the survey at any time, there will be no penalty. Your responses to this survey will be confidential. If you meet the criteria for this study, you will be contacted to schedule an in-person appointment at the downtown campus of Arizona State University. If you have any questions concerning the research study, please contact Dr. Carol Johnston: carol.johnston@asu.edu. If you have any questions about your rights as a subject/participant in this research, or if you feel you have been placed at risk, you can contact the Chair of the Human Subjects Institutional Review Board, through the ASU Office of Research Integrity and Assurance, at (480) 965-6788.

By completing this survey, you are agreeing to be contacted by investigators (via email) to schedule an appointment, should you qualify.

* 1. Please provide your email address.

* 2. Are you able and willing to consume vinegar daily for four weeks (liquid or pill form)? (note: if you suffer from heart burn or GERD, you should answer 'no')

- ◯ Yes
- ◯ No

3. How old are you? (report in years)

4. How tall are you? (report in inches) (5 feet=60 inches)

5. How much do you weigh? (report in pounds)

6. Do you compete in a sport or exercise strenuously more than 75 minutes weekly?

- ◯ Yes
- ◯ No
- ◯ Not sure

7. Has your physician diagnosed you with diabetes, heart disease, cancer or other chronic disease condition?

- ◯ Yes
- ◯ No
- ◯ Not sure

8. Do you adhere to a special diet, such as vegan, Paleo, or intermittent fasting?

- ◯ Yes
- ◯ No

If yes, please enter:

9. Do you take any of the following medications: e.g. beta-blockers, ACE inhibitors, diphenhydramine or cyproheptadine (allergy medications), lithium carbonate, corticosteroids, thiazolidinediones (Actos, Avandia or Avandamet), sulfonylureas, biguanides, meglitinides, incretins, sodium valproate, or thyroid replacement therapy?

- ◯ Yes
- ◯ No
- ◯ Not sure

10. Do you take supplements daily (including vinegar)?

- ◯ Yes
- ◯ No

If yes, please list:

11. Are you OK with having two fasting blood samples taken (<1 tablespoon) four weeks apart?

- ◯ Yes
- ◯ No

12. Will you be able to maintain your current diet and physical activity patterns during the 4-week study?

- ◯ Yes
- ◯ No

13. Women: what is your dress size? e.g., 8, 10, 12, etc.

14. Men: what is your pant size? e.g., 42, 44, 46, etc.

15. Are you willing and able to travel to the ASU Downtown Campus to meet with the research investigators on two separate mornings? (parking costs will be covered) (location near Roosevelt and 5th Streets)

- ◯ Yes
- ◯ No

APPENDIX D

INFORMED CONSENT FORM

College of Health Solutions
Arizona State University

Informed Consent
Vinegar and Health in Young Adults

INTRODUCTION
The purposes of this form are (1) to provide you with information that may affect your decision as to whether to participate in this research study, and (2) to record your consent if you choose to be involved in this study.

RESEARCHERS
Dr. Carol Johnston (ASU Nutrition professor) and MS students Lexie Lish, Hannah Coven, and Haley Barrong have requested your participation in a research study.

STUDY PURPOSE
The purpose of this study is to investigate the effects of vinegar on health parameters in young adults.

DESCRIPTION OF RESEARCH STUDY
You have indicated to us that you are a non-smoker 18 to 45 years of age and healthy. You are not a competitive athlete, and you do not follow specific diets for weight loss, and if female, you are not currently pregnant or planning a pregnancy. Also, you are willing to ingest vinegar, either in liquid or pill form, daily for four weeks. You will be randomly assigned to the liquid vinegar group (2 tablespoons diluted in water and drunk with meals twice daily) or the vinegar pill group (2 pills daily). Otherwise, your diet and daily activities will remain constant. We ask that you maintain your normal physical activities and not initiate a new exercise protocol. You will come to the test site in a fasted state (no food or drink aside from water for 10 hours) on two occasions (study weeks 1 and 4; each visit ~45 minutes) to provide a blood sample (<1 tablespoon per draw), complete several surveys on health, and undergo measurements (e.g., height, weight, blood pressure, and waist circumference). Your blood will be analyzed for markers related to health and immunity (including these common markers: glucose, cholesterol, and the inflammatory marker, CRP) You will be provided with vinegar (liquid or pills) at the first visit, and you will receive emails weekly during the study to answer any questions you may have. At study completion, you will receive $50.

Your blood results (glucose, cholesterol, CRP) can be made available to you once the study is complete. To receive results, you will need to sign the Research Results Acknowledgement Statement at the start of the study.

A description of this clinical trial will be available on http://www.ClinicalTrials.gov. This Web site will not include information that can identify you. At most, the Web site will include a summary of the results. You can search this website at any time.

RISKS
There are no known risks to this study other than a change to your daily routine by incorporating vinegar ingestion along with meals twice daily. Vinegar has a distinctive taste, which many find unappealing and may lead to nausea and an upset stomach. *You should not participate in this study if you cannot tolerate the taste of vinegar.* The blood draw may result in faintness, nausea, dizziness, and bruising at the site of the needle insertion. A certified phlebotomist, trained to handle these transient effects will be performing the blood draw using standard, sterile procedures.

BENEFITS
This trial is examining possible health benefits of daily vinegar ingestion. Although you may not benefit directly from this research, the data derived from this research will expand our understanding of how vinegar influences metabolism.

NEW INFORMATION
If the researchers find new information during the study that would reasonably change your decision about participating, then they will provide this information to you.

CONFIDENTIALITY
All information obtained in this study is strictly confidential unless disclosure is required by law. The results of this research study may be used in reports, presentations, and publications, but your name or identity will not be revealed. To maintain confidentiality of your records, Dr. Johnston will use subject codes on all data collected, maintain a master list separate and secure from all data collected, and limit access to all confidential information to the study investigators.

WITHDRAWAL PRIVILEGE
You may withdraw from the study at any time for any reason without penalty or prejudice toward you. Your decision will not affect you any manner. We ask that you notify us in a timely manner if you decide to withdraw from the study, and we will ask you the reason for withdrawing for our records.

COSTS AND PAYMENTS
You will receive $50 after completing the study at visit 2. There are no payments required for this study. You will receive the vinegar (liquid or pills) free of charge, and parking at the test site will be covered.

COMPENSATION FOR ILLNESS AND INJURY
If you agree to participate in the study, then your consent does not waive any of your legal rights. However, in the event of harm, injury, or illness arising from this study, neither Arizona State University nor the researchers are able to give you any money, insurance coverage, free medical care, or any compensation for such injury. Major injury is not likely but, if necessary, a call to 911 will be placed.

VOLUNTARY CONSENT
Any questions you have concerning the research study or your participation in the study, before or after your consent, will be answered by Dr. Carol Johnston, 550 N. 3rd St., Phoenix, AZ 85004. [carol.johnston@asu.edu]

If you have questions about your rights as a subject/participant in this research, or if you feel you have been placed at risk, you can contact the Chair of the Human Subjects Institutional Review Board, through the ASU Research Compliance Office, at 480-965 6788.

This form explains the nature, demands, benefits and any risk of the project. By signing this form, you agree knowingly to assume any risks involved. Remember, your participation is voluntary. You may choose not to participate or to withdraw your consent and discontinue participation at any time without penalty or loss of benefit. In signing this consent form, you are not waiving any legal claims, rights, or remedies. A copy of this consent form will be given to you.

Your signature below indicates that you consent to participate in the above study.

_____	_____	_____
Subject's Signature	Printed Name	Date

_____

Contact phone number Email

INVESTIGATOR'S STATEMENT

"I certify that I have explained to the above individual the nature and purpose, the potential benefits, and possible risks associated with participation in this research study, have answered any questions that have been raised, and have witnessed the above signature. These elements of Informed Consent conform to the Assurance given by Arizona State University to the Office for Human Research Protections to protect the rights of human subjects. I have provided the subject/participant a copy of this signed consent document."

Signature of Investigator_____
Date_____

APPENDIX E

HEALTH HISTORY QUESTIONNAIRE

HEALTH HISTORY QUESTIONNAIRE ID#_____

1. Gender: M F

2. Age: _____

3. Have you lost or gained more than 10 lbs. in the last 12 months? Yes No
   If yes, how much lost or gained? _____ How long ago? _____

4. Ethnicity: (please circle one) Native American African-American Caucasian Hispanic Asian Other

5. Education (please circle) High school diploma AA/vocational degree College degree MS degree PhD degree

6. Do you smoke? No, never _____
   Yes _____ # Cigarettes per day = _____
   I used to, but I quit _____ months/years (circle) ago

7. Women only: Are you pregnant now or plan a pregnancy in the next 3 months?
Yes No

8. Do you take any medications regularly? Yes No *If yes, list type and frequency:*

Medication Dosage Frequency

_____
_____
_____
_____
_____

9. Do you currently take supplements (vitamins, minerals, herbs, etc.) ? Yes No
If yes, list type and frequency:

Supplement Dosage Frequency

_____
_____
_____

10. Please check (YES/NO) if you currently have or if you have ever been clinically diagnosed with any of the following
diseases or symptoms:

	YES	NO		YES	NO
Coronary Heart Disease			Chest Pain		
High Blood Pressure			Shortness of Breath		
Heart Murmur			Heart Palpitations		
Rheumatic Fever			Any Heart Problems		
Irregular Heart Beat			Coughing of Blood		
Varicose Veins			Feeling Faint or Dizzy		
Stroke			Lung Disease		
Diabetes			Liver Disease		
Low Blood Sugar			Kidney Disease		
Bronchial Asthma			Thyroid Disease		
Hay Fever			Anemia		
Leg or Ankle Swelling			Hormone Imbalances		
Eating Disorder			Depression		

Please turn over →→

11. Have you ever had abdominal surgery? Yes No

12. Do you have any of the conditions listed below? Yes No

acid reflux, ascites, pancreatitis, diverticulitis/diverticulosis, Crohn's disease, and/or irritable bowel syndrome

13. Please circle the **number of times** you did the following kinds of exercises **for more than 15 minutes** last week.

Mild exercise (minimal effort):
Easy walking, golf, gardening, bowling, yoga, fishing, horseshoes, archery, etc.

Times per week: 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14+

Moderate exercise (not exhausting):
Fast walking, easy bicycling, tennis, easy swimming, badminton, dancing, volleyball, baseball, etc.

Times per week: 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14+

Strenuous exercise activities (heart beats rapidly):
Running, jogging, hockey, football, soccer, squash, basketball, cross country skiing, judo, roller skating, vigorous swimming, vigorous long distance bicycling, etc.

Times per week: 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14+

14. Are you healthy and fit? Yes No

Comments:
_____
_____

15. How much alcohol do you drink? (average #drinks per week) _____

16. Are you a current smoker (cigarettes or cannabis)? Yes No
Are you a former smoker? Yes No *if yes,* when was the last time you smoked?
_____

17. Do you have any food allergies? Yes No
If yes, explain:
_____
_____

18. Do you follow a special diet? Yes No
If yes, explain: _____
_____

19. Do you plan to change your diet in the next 8 weeks? Yes No
If yes, explain: _____
_____

20. Do you plan to change your exercise level in the next 8 weeks? Yes No

If yes, explain:

_____

_____

21. Are you willing to consume vinegar supplements daily for 4 weeks? Yes No

APPENDIX F

24-HOUR DIETARY RECALL

Subject #_____ Date _____

Upon waking, what food and beverages did you consume?

Food/Beverage	Quantity	Portion Size

What was the next thing you ate or drank?

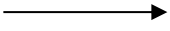
Food/Beverage	Quantity	Portion Size

What did you have to eat and drink for lunch?

Food/Beverage	Quantity	Portion Size

Did you have any snacks or beverages next?

Food/Beverage	Quantity	Portion Size

What did you have to eat and drink for dinner? 

Food/Beverage	Quantity	Portion Size

Did you eat or drink anything else throughout the day or night?

Food/Beverage	Quantity	Portion Size

Is there any condiment, topping, seasoning or food you may have missed, such as: sugar, butter, ketchup, salt, cream cheese, etc.?

Think for a minute. Was there any food, beverage or anything else you may have missed that you consumed yesterday?

Was this a typical day in terms of dietary choices and eating patterns? What differs?

APPENDIX G

CENTER FOR EPIDEMIOLOGIC STUDIES DEPRESSION SCALE (CES-D)

Center for Epidemiologic Studies Depression Scale (CES-D), NIMH

Below is a list of the ways you might have felt or behaved. Please tell me how often you have felt this way during the past week.

	During the Past Week			
	Rarely or none of the time (less than 1 day)	Some or a little of the time (1-2 days)	Occasionally or a moderate amount of time (3-4 days)	Most or all of the time (5-7 days)
1. I was bothered by things that usually don't bother me.	☐	☐	☐	☐
2. I did not feel like eating; my appetite was poor.	☐	☐	☐	☐
3. I felt that I could not shake off the blues even with help from my family or friends.	☐	☐	☐	☐
4. I felt I was just as good as other people.	☐	☐	☐	☐
5. I had trouble keeping my mind on what I was doing.	☐	☐	☐	☐
6. I felt depressed.	☐	☐	☐	☐
7. I felt that everything I did was an effort.	☐	☐	☐	☐
8. I felt hopeful about the future.	☐	☐	☐	☐
9. I thought my life had been a failure.	☐	☐	☐	☐
10. I felt fearful.	☐	☐	☐	☐
11. My sleep was restless.	☐	☐	☐	☐
12. I was happy.	☐	☐	☐	☐
13. I talked less than usual.	☐	☐	☐	☐
14. I felt lonely.	☐	☐	☐	☐
15. People were unfriendly.	☐	☐	☐	☐
16. I enjoyed life.	☐	☐	☐	☐
17. I had crying spells.	☐	☐	☐	☐
18. I felt sad.	☐	☐	☐	☐
19. I felt that people dislike me.	☐	☐	☐	☐
20. I could not get "going."	☐	☐	☐	☐

SCORING: zero for answers in the first column, 1 for answers in the second column, 2 for answers in the third column, 3 for answers in the fourth column. The scoring of positive items is reversed. Possible range of scores is zero to 60, with the higher scores indicating the presence of more symptomatology.

APPENDIX H

PATIENT HEALTH QUESTIONNAIRE (PHQ-9)

PATIENT HEALTH QUESTIONNAIRE (PHQ-9)

ID #: _____ **DATE:** _____

Over the last *2 weeks*, how often have you been bothered by any of the following problems? *(use "✓" to indicate your answer)*	Not at all	Several days	More than half the days	Nearly every day
1. Little interest or pleasure in doing things	0	1	2	3
2. Feeling down, depressed, or hopeless	0	1	2	3
3. Trouble falling or staying asleep, or sleeping too much	0	1	2	3
4. Feeling tired or having little energy	0	1	2	3
5. Poor appetite or overeating	0	1	2	3
6. Feeling bad about yourself—or that you are a failure or have let yourself or your family down	0	1	2	3
7. Trouble concentrating on things, such as reading the newspaper or watching television	0	1	2	3
8. Moving or speaking so slowly that other people could have noticed. Or the opposite — being so figety or restless that you have been moving around a lot more than usual	0	1	2	3
9. Thoughts that you would be better off dead, or of hurting yourself	0	1	2	3
		add columns	+	+
(Healthcare professional: For interpretation of TOTAL, please refer to accompanying scoring card).		TOTAL:		

10. If you checked off *any problems*, how *difficult* have these problems made it for you to do your work, take care of things at home, or get along with other people?

- Not difficult at all _____
- Somewhat difficult _____
- Very difficult _____
- Extremely difficult _____

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PHQ-9 Patient Depression Questionnaire

For initial diagnosis:

1. Patient completes PHQ-9 Quick Depression Assessment.
2. If there are at least 4 ✓s in the shaded section (including Questions #1 and #2), consider a depressive disorder. Add score to determine severity.

Consider Major Depressive Disorder

- if there are at least 5 ✓s in the shaded section (one of which corresponds to Question #1 or #2)

Consider Other Depressive Disorder

- if there are 2-4 ✓s in the shaded section (one of which corresponds to Question #1 or #2)

Note: Since the questionnaire relies on patient self-report, all responses should be verified by the clinician, and a definitive diagnosis is made on clinical grounds taking into account how well the patient understood the questionnaire, as well as other relevant information from the patient.
Diagnoses of Major Depressive Disorder or Other Depressive Disorder also require impairment of social, occupational, or other important areas of functioning (Question #10) and ruling out normal bereavement, a history of a Manic Episode (Bipolar Disorder), and a physical disorder, medication, or other drug as the biological cause of the depressive symptoms.

To monitor severity over time for newly diagnosed patients or patients in current treatment for depression:

1. Patients may complete questionnaires at baseline and at regular intervals (eg, every 2 weeks) at home and bring them in at their next appointment for scoring or they may complete the questionnaire during each scheduled appointment.
2. Add up ✓s by column. For every ✓: Several days = 1 More than half the days = 2 Nearly every day = 3
3. Add together column scores to get a TOTAL score.
4. Refer to the accompanying **PHQ-9 Scoring Box** to interpret the TOTAL score.
5. Results may be included in patient files to assist you in setting up a treatment goal, determining degree of response, as well as guiding treatment intervention.

Scoring: add up all checked boxes on PHQ-9

For every ✓ Not at all = 0; Several days = 1;
More than half the days = 2; Nearly every day = 3

Interpretation of Total Score

Total Score	Depression Severity
1-4	Minimal depression
5-9	Mild depression
10-14	Moderate depression
15-19	Moderately severe depression
20-27	Severe depression

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APPENDIX I

STRESS EVALUATION

Stress Evaluation **ID#_____** **Date: _____**

This evaluation aims to determine if you are currently experiencing higher than normal amounts of stress. Please complete the following questions based on your experience over the last **three days**. Circle yes or no for each question – if the statement is true, circle yes. Take your time and answer all questions honestly.	
I feel more stressed than usual.	Yes / No
My job has been a major source of stress lately.	Yes / No
I have many tests coming up in the next week that I am worried about.	Yes / No
I have many assignments due in the next week.	Yes / No
I feel that I do not have enough time to complete all of my upcoming tasks.	Yes / No
I have been working long hours at my job.	Yes / No
I have been spending many hours studying each day.	Yes / No
I have a speech or presentation coming up that I am nervous about.	Yes / No
I have recently been harassed or bullied.	Yes / No
I have had a conflict with a person close to me.	Yes / No
I have been sleeping less than usual.	Yes / No
My quality of sleep has been very poor.	Yes / No
I feel more tired than usual.	Yes / No
My home life has been exceptionally chaotic.	Yes / No
I have recently experienced a family crisis.	Yes / No
A family member or loved one has recently been injured or critically ill.	Yes / No
I have been exceptionally worried about my current financial situation.	Yes / No

APPENDIX J

INTERVENTION PRODUCTS

Pompeian Red Wine Vinegar (16 oz) Bottle and Nutrition Facts Label

Nutrition Facts

about 32 servings per container

Serving size **1 tbsp (15mL)**

Amount per serving

Calories **0**

	% DV*
Total Fat 0g	0%
Saturated Fat 0g	0%
Trans Fat 0g	
Sodium 0mg	0%
Total Carbohydrate 0g	0%
Protein 0g	

Not a significant source of cholesterol, dietary fiber, total sugars, added sugars, vitamin D, calcium, iron, and potassium.

* %DV = % Daily Value

Ingredients:
Red Wine Vinegar diluted with water to 5% acid strength.
Contains Sulfites.

Spring Valley Apple Cider Vinegar Capsules (100 Count) and Nutrition Facts Label

Supplement Facts

Serving Size 1 Capsule
Servings Per Container 100

Amount Per Serving	% Daily Value
Apple Cider Vinegar 450 mg	†

†Daily Value not established.

OTHER INGREDIENTS: Gelatin Capsule, Rice Powder, Vegetable Magnesium Stearate, Silica.