THESIS

IMPACTS OF MICROALGAE CONSUMPTION ON PSYCHOLOGICAL STRESS AND CARDIOVASCULAR FUNCTION

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ABSTRACT

IMPACTS OF MICROALGAE CONSUMPTION ON PSYCHOLOGICAL STRESS AND CARDIOVASCULAR FUNCTION

Cardiovascular disease (CVD) is the leading cause of death in the United States, killing 702,880 people annually. Psychological (or mental) stress has gained attention as a modifiable risk factor for CVD, showing links to hypertension, dyslipidemia, insulin resistance, atherosclerotic CVD, and stroke. The extent of how stress translates to pathological changes which result in CVD is not fully understood. One potential approach to managing mental stress is through the gut-brain-axis. A limited body of research in humans and rodents suggests that dietary microalgae supplementation may reduce gastrointestinal (GI) inflammation, leading to improvements in stress via the gut-brain axis which may have implications for cardiovascular health. We performed a randomized, double-blind, placebo-controlled, parallel-arm clinical trial evaluating the efficacy of four weeks of daily supplementation with 250 mg microalgae extract from *Tetradesmus obliquus* Mi175.B1.a vs. placebo on stress and anxiety in healthy adults aged 18-55 years with mild to moderate GI distress. Fifty-six adults (age: 31.9±7.7 years; body weight: 71.8±12.6 kg; BMI: 24.6±2.8 kg/m^2) were enrolled. Participants were administered subjective assessments of perceived stress, blood pressure responses to environmental stress (i.e., cold pressor test, CPT) were evaluated, and salivary cortisol concentrations were measured. After four weeks of microalgae supplementation, our results showed improved scores on Positive and Negative Affect Schedule questionnaires which may reflect improvements in mental stress. No major treatment effects were observed for blood pressure responses to CPT or salivary cortisol. Our results suggest daily microalgae supplementation may have implications to improved mental health; however, more evidence is needed to

understand the mechanisms of microalgae on stress reduction and implications for cardiovascular health.

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INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death worldwide and accounts for one third of all deaths in the United States (US)[1]. Atherosclerotic CVD complications comprise of myocardial infarction, strokes, and thrombosis and is considered to be the most common cause of CVD related death in the US, contributing to over $400 billion in healthcare costs annually[2,3]. Hypertension is a significant risk factor for CVD and is characterized by the chronic elevation of blood pressure which has direct adverse effects on the vascular endothelium[4,5]. Endothelial dysfunction can be exacerbated by the presence of hypertension while significantly progressing the onset of atherosclerosis[6,7]. The primary characteristic of endothelial dysfunction is the reduction in endothelial-dependent vasodilation perpetuated by the inhibition of nitric oxide (NO) bioavailability[6]. The presence of hypertension and endothelial dysfunction may perpetuate a feudal cycle which accelerates the onset of CVD as hypertension may accelerate the progression of endothelial dysfunction while endothelial dysfunction derived vasoconstriction may worsen the severity and the progression of hypertension[4,5]. Non-modifiable risk factors such as age, sex, ethnicity, and family history contribute to the development of endothelial dysfunction and hypertension; however, modifiable risk factors like diet and stress (and their management) can significantly impact the progression of hypertension and endothelial dysfunction through the modulation of the gut microbiota and the gut-brain axis (GBA)[5,7-11].

Psychological stress and anxiety may significantly increase the incidence of hypertension and endothelial dysfunction via sympathetic nervous system (SNS) overactivation[12]. SNS activity is regulated by the hypothalamus and involves the release of catecholamines like norepinephrine to target organs such as the heart, blood vessels, and kidneys to regulate blood pressure[12,13]. Chronic SNS activation may result in the chronic

elevation of blood pressure and increase oxidative stress[12,13]. The hypothalamic-pituitary-adrenal (HPA) axis comprises organs which regulate the body's endocrine system which are responsible for regulating the body's reaction to stress through a series of feedback loops[14,15].The presence of chronic stress and anxiety may result in the dysregulation of the HPA activity and hormonal signaling, resulting in the onset or progression of hypertension, endothelial function, as well other chronic inflammation[14,15].

The GBA is a bidirectional communication network between the gut and the brain. It encompasses pathways of the central nervous system (CNS), endocrine system, and the immune system[10,16]. Adverse alterations to the gut microbiota lead to increased permeability at the intestinal epithelium, resulting in proinflammatory gut microbiota-derived metabolites such as lipopolysaccharides (LPS) entering the bloodstream. LPS and other pathogenic metabolites down-regulate tight junction proteins at the blood-brain barrier (BBB) and activate microglia, triggering the synthesis of proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin 6 (IL-6), resulting in neuroinflammation and the upregulation of immune responses[8,14,17]. The increase in inflammation and decrease in BBB integrity may induce HPA dysregulation, allowing for the increase in SNS activity and the progression of hypertension[8]. Additionally, the upregulation of macrophage activity and pro-inflammatory cytokines synthesis, along with increased oxidative stress, causes damage to endothelial cells, allowing the formation of atherosclerotic plaque[14,17].

Microalgae are photosynthetic unicellular microscopic algae found in fresh water, brackish, marine, and hypersaline aquatic environments[18-20]. They contain a unique profile of bioactive compounds such as phenolic compounds, pigment proteins, polysaccharides, polyunsaturated fatty acids (PUFAs), amino acids, vitamins, and minerals[18,20-25]. Microalgae may contribute to reduced CVD risk through their influence on gut microbiota composition and/or modulating the GBA[25-28]. Studies in animals and humans suggest the supplementation

of microalgae and microalgae-derived bioactive compounds such as polysaccharides, fibers, phenolics, and pigments support improvements of intestinal permeability through the production of microbe-derived short-chain fatty acids (SCFAs)[26,29-31]. The improvements in gut health via microalgae supplementation have been shown to reduce psychological stress and stress related mood disorders, suggesting improvements in HPA regulation and decreased sympathetic tone[32,33].

Cold pressure tests (CPT) have been used in limited studies to assess sympathetic tone in humans with varying degrees of stress-related mood and neurological disorders[34]. CPTs are used to induce a stress response using controlled exposures to ice cold water to the hands or feet[34-36]. During the CPT, changes in blood pressure and cardiac wave reflection are measured, which can be used to assess arterial stiffness or impedance. The purpose of this Plan A project is to assess the impact of four weeks of supplementation with a microalgae extract from *Tetradesmus obliquus* Mi175.B1.a (TOME) on measurements of psychological stress, blood pressure, and wave reflection responses to a CPT in free-living participants with mild to moderate gastrointestinal (GI) distress.

Specific Aim 1: Evaluate the extent to which four weeks of microalgae supplementation influence blood pressure and wave reflection responses to a cold presser test (CPT) in healthy adults aged 18-55 years with mild to moderate GI.

Hypothesis 1: Four weeks of microalgae supplementation will significantly reduce blood pressure responses, particularly systolic blood pressure, to a CPT in healthy adults aged 18-55 years with mild to moderate GI.

Specific Aim 2: Determine the degree to which four weeks of microalgae supplementation impact psychological stress/anxiety.

Hypothesis 2: Four weeks of microalgae supplementation will significantly reduce reported stress/anxiety and saliva cortisol concentrations.

LITERATURE REVIEW

Cardiovascular Disease

Incidence and Prevalence

Cardiovascular disease (CVD) remains a significant public health concern worldwide, with various risk factors contributing to its incidence and prevalence. Globally, CVD is the leading cause of mortality and morbidity, with its prevalence growing due to aging populations and lifestyle factors[1]. CVD encompasses a number of disorders which impact the heart and blood vessels. Such disorders include coronary artery disease, hypertension, heart failure, stroke, and atherosclerosis[37,38]. As an individual ages, the likelihood of developing serious cardiovascular conditions associated with lifestyle characteristics and age-related alterations in the heart and vasculature, to include arterial thickness and stiffness, calcification, and increased endothelial dysfunction, dramatically increases[39]. In the United States, alone, the prevalence of hypertension, coronary artery disease, heart failure, and stroke increases from roughly 40% in adults aged 40 to 59 years to over 80% by the age of 80, with over 75% of Americans suffering from CVD by the age of 60 to 70 years[39]. Additionally, cardiovascular outcomes continue to be impacted by racial and ethnic inequities, particularly among Black/African American, Hispanic, American Indian, and Alaska Native populations, as well as subgroups of Asian American populations[38]. Individuals within these racial and ethnic groups, as well as individuals in all racial and ethnic populations who are living in poverty, and those living in rural areas are reported to have higher rates of incident and prevalent CVD, stroke, and cardiovascular mortality, with rates expected to continuously increase by the year 2030[38]. CVD is considered to be the most common causes death in the United States, with CVD accounting for one third of all deaths in 2023, 80% of which were considered premature[2,3]. Furthermore, CVD continues to strain economies as the United States reported an average annual cost of

CVD estimated at $407.3 billion in 2018 to 2019[2]. While CVD rates have fluctuated among different populations over the last decade, rates continue to increase on average, year after year[2,38]. This increase in incidence and prevalence suggests further surveillance and targeted interventions are necessary to address this persistent public health concern.

Risk Factors and Pathophysiology

With CVD leading national public health concerns, research has prompted the identification of the many risk factors associated with development and progression of CVD. Non-modifiable and modifiable risk factors may be taken into consideration as research and medical guidance seek to address CVD mortality and morbidity[37,38,40]. Non-modifiable risk factors have remained relatively unchanged over time and include factors such as age, sex, race and ethnicity, and family history[39,41]. Modifiable risk factors continue to evolve as populations grow, demographics change, and environments shift to the needs of modern civilization. Such factors include, but are not limited to hypertension, hyperlipidemia and dyslipidemia, obesity, diabetes mellitus, sedentary lifestyles, smoking, diet, and psychological stress[1,38,40-42]. Both modifiable and non-modifiable risk factors have the potential to impact cardiovascular outcomes independently; but, often have synergistic effects as some risk factors may exacerbate the physiological implications of another. For example, type 2 diabetes (T2D) and insulin resistance may develop independently and alongside CVD but may accelerate the development and progression of CVD as homeostasis becomes more severely disrupted[37,41,42]. This interplay between risk factors emphasizes the importance of comprehensive management through lifestyle interventions, pharmacological approaches, and preventative treatment[41]. Age is the primary risk factor for CVD as age strongly correlates with increased risk of CVD with advanced aging. As age increases, vascular stiffening and reduced arterial elasticity become more predominant, oxidative stress and inflammation increase, and

the capacity to regenerate cardiovascular tissue declines[39,43]. In these circumstances, systolic hypertension (i.e., high systolic blood pressure with normal or reduced diastolic blood pressure) becomes more prevalent with increasing age, which may be exacerbated by co-morbidities such as obesity or diabetes mellitus[4,37,43-45]. Additionally, endothelial dysfunction become more prevalent as aging accelerates arterial lipid accumulation and bioavailability of nitric oxide (NO) is reduced, impeding vasodilation altogether[46]. This can increase the development and progression of atherosclerosis. The role of risk factors such as high blood pressure or hypertension have been extensively researched for decades; however, the relationship between these CVD risk factors and other factors that are less known should be further evaluated for their contributions to CVD. Psychological stress and anxiety have gained increased attention over recent decades as these risk factors play significant roles in emerging research focused on the gut-brain axis (GBA). This field of scientific research suggests an interconnectedness between the gut environment, including the gut microbiome, psychological stress, and CVD, with each domain having direct and indirect implications towards each other[8,9,47-49].

Hypertension

Elevated blood pressure (BP) and hypertension are significant risk factors for CVD, particularly for their direct implications on the heart. Hypertension is characterized by the chronic elevation of BP, most notable during systole in middle-aged and older individuals[4]. This increase in BP can directly affect the vascular endothelium and promote vascular dysfunction and remodeling[5,39,43]. One of the primary outcomes of hypertension is the thickening of the myocardium in the left ventricle, or left ventricular hypertrophy, which results from elevated systemic vascular resistance and increased arterial afterload[5,45,50]. This increased resistance causes the left ventricle to work harder in order to pump blood throughout the body, also

referred to as left ventricular systolic load[5,43]. If left on managed, risk of heart failure or atrial fibrillation significantly increases[39,43].

Another significant implication of hypertension is arterial stiffness. Arterial stiffness further complicates the risk of CVD as it can be a cause and a consequence of hypertension but is ultimately initiated through vascular dysfunction increases throughout aging and is influenced by the same nonmodifiable and modifiable risk factors associated with CVD[4,5,45,50,51]. Arterial stiffening is identified as the reduction in the ability of the arteries to expand and contract in response to pressure changes; however, this inability to expand and contract simultaneously contributes to the systemic increase blood pressure over time[50,51]. Stiffening of the arteries can exacerbate left ventral hypertrophy; however, as stiffening progresses, the risk of damaging other organs such as the kidneys and brain also increases[45]. If left unmanaged, arterial stiffening may cause damage to the brain as cerebral perfusion decreases, increasing the risk of stroke[2,38,52]. In other cases, chronic kidney disease may develop as renal function is impaired due to damage to glomeruli caused by elevated BP[43,45,50,53].

While hypertension plays a key role in the development and progression of arterial stiffness, which significantly influences pulse pressure and arterial afterload, the role of the sympathetic nervous system (SNS) in regulating arterial pressure is becoming increasingly recognized. Increased sympathetic activation affects target organs which regulate BP, such as the blood vessels, kidneys, and the heart[12,13,44,50]. Evidence in animal models and humans have shown SNS activity contributes to cardiovascular effects through catecholamine release, which contributes to increased oxidative stress and progression of hypertension[12,13]. There are a number of internal and external factors that can cause increased SNS activation, or in some cases chronic activation, referred to as overactivation. One of the major causes of SNS overactivation, and a primary focus of this literature review, is psychological stress and anxiety. Stress and anxiety are increasingly being recognized as contributors to the development of CVD

through hypertension and endothelial damage by way of SNS overactivation[12,13,32,33,50,54]. When events take place which cause psychological stress to occur, or when stress is prolonged, chronic psychological stress activates the hypothalamic-pituitary-adrenal (HPA) axis, resulting in the release of glucocorticoids such as cortisol, catecholamines such as epinephrine and norepinephrine, and proinflammatory cytokines[32,33]. The metabolic function of cortisol is to make energy substrates more available in the event the body needs to fight or flee to safety. This metabolic function results in a multitude of physiological changes; however, sustaining these physiological conditions for prolonged durations can consequently result in insulin resistance and hyperglycemia[32,55,56]. In the context of CVD, cortisol also increases blood pressure and heart rate (HR), increasing the risk of hypertension when these conditions become chronic[32,33].

Endothelial Dysfunction and Atherosclerosis

Much like hypertension and arterial stiffness, endothelial dysfunction adds to the complexity of CVD risk as endothelial dysfunction can be both the cause or the consequence of hypertension or arterial stiffness, while being significantly influenced by chronic psychological stress and anxiety. Endothelial function is a necessary component of cardiovascular health as the endothelial cells which line the blood vessels allow for the vessels to dilate and constrict to optimize blood flow to the heart and other organs[6,33,57]. The primary characteristic of endothelial dysfunction is insufficient nitric oxide (NO) bioavailability; however, it can also result from mitochondrial dysfunction of the endothelial cells, chronic inflammation, immune system activation, and metabolic disorders[6,7,58-60]. When conditions exist that are physiologically harmful to the endothelial lining (e.g., during prolonged psychological stress), chronic elevation of glucocorticoids and catecholamines impedes endothelial NO production by disrupting endothelial nitric oxide synthase (eNOS) activity and expression[32,57]. This reduction in NO

bioavailability promotes vasoconstriction and increased vascular resistance. For this reason, endothelial dysfunction serves as a precursor to atherosclerosis, as these conditions promote the infiltration of lipids into the arterial walls and the formation of plaque[6,7,33,57]. The release of glucocorticoids and catecholamines by SNS overactivation increases progression of endothelial dysfunction and atherosclerosis as these hormones have been shown to increase levels of reactive oxygen species (ROS)[33,61,62]. When excessive ROS release from psychological stress leads to an imbalance of ROS production and antioxidant capabilities (i.e., oxidative stress), NO bioavailability is decreased, endothelial cells are damaged, and chronic low-grade inflammation is increased [59,60]. Chronic inflammation activates the immune system and endothelial cells, which promotes the accumulation of pro-inflammatory cytokines at the damaged site of the blood vessel[57,59,60]. Most notable of these pro-inflammatory cytokines are interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α)[63]. IL-1, IL-6 and TNF-α are primary drivers in the pathogenesis of atherosclerosis as they perpetuate further vascular inflammation, causing the formation of foam cells and adhesions molecules in which the development of atherosclerotic plaque takes place[63].

Role of the Gut-Brain Axis

Another key factor in the connection between stress and CVD is the GBA which is a bidirectional communication network that links the gut-microbiota to the brain, allowing the two to effectively share information with each other[10]. The human gut microbiota refers to the trillions of microorganisms which live insider our GI tract. These organisms are primarily bacteria such as Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes, as well as Fusobacteria and Verrucomicrobia to a lesser extent; however, other microorganisms such as yeasts, archaea, helminths, viruses, and Protozoa also contribute to the composition of induvial gut-microbiota[10]. It is currently understood that the gut-microbiota plays a significant role in human

health and disease status, while significantly impacting regulation of host physiology[10]. Recent evidence suggests the GBA significantly impacts the regulation of both psychological and cardio-respiratory homeostasis[10,14,16]. The modalities in which the gut microbiota and the brain exchange such information via GBA are sophisticated and encompassing of afferent and efferent pathways of the central, enteric, and autonomic nervous systems, as well as the HPA axis[14,15]. Alterations in the bidirectional interactions between the gut microbiota and the brain via the GBA are believed to significantly influence blood pressure regulation[8]. Studies have shown imbalances in sympathetic/parasympathetic activity to be a symptom of neuroinflammation of the central nervous system (CNS). It is believed that alterations in the gut microbiota and their metabolites are associated with regulation of neuroinflammation and SNS mediated BP regulation, linking the gut microbiota and neuroinflammation to hypertension and increased risk for CVD[8,17,64]. There is a limited understanding of the mechanisms to support this hypothesis, but evidence suggests gut dysbiosis, which refers to the imbalance or disruption in the composition and function of the microbiota, in the context of hypertension results in an imbalance of circulatory anti- and proinflammatory mediators and host systemic inflammation. Through signaling via the vagus nerve and blood circulation, gut dysbiosis induced systemic inflammation may directly and indirectly activate the microglia of the CNS and induce neuroinflammation, as well as heightened immune system activation[8,10,14,47]. Activated microglia are believed to accumulate in the hypothalamic paraventricular nucleus (PVN) which are the brain nuclei responsible for regulating the HPA axis, triggering autonomic responses which can result in increased SNS activity and blood pressure[8,17]. Additionally, it has been observed that CNS neuroinflammation triggers the upregulation of pro-inflammatory cytokines such as TNF-α and IL-6, further exacerbating the effects of increased SNS activity and the progression of hypertension and atherosclerosis[8,17,64].

The bidirectionality of the GBA between the gastrointestinal tract and brain creates feedback loops which up-regulate conditions that increase the risk for CVD, as well as mood disorders such as increased psychological stress and anxiety. As described previously, alterations in gut microbiota or dysbiosis may promote neuroinflammation of the CNS, leading to an increase in autonomic imbalance. During such conditions within the intestinal tract, endotoxins like lipopolysaccharides (LPS) and other pathogen-derived compounds increase tight-junction permeability in the epithelium, allowing various pro-inflammatory compounds and endotoxins to translocate, activate microglia, enter the bloodstream, and modulate immune responses, resulting in the production of pro-inflammatory cytokines [11,49]. Consequently, evidence has shown increased permeability of the epithelium to coincide with increased permeability of the blood-brain barrier (BBB) as LPS and inflammatory cytokines can degrade the tight junction proteins of the BBB [11,49,65]. The increase in BBB permeability has been linked to observed increases in the development of mood disorders such as depression, as well as increased stress and anxiety. In this context, IL-1, IL-6, TNF-α, and other immune system derived monocytes permeate across the BBB and upregulate HPA axis activity. Additionally, other parts of the brain related to stress regulation, such as the amygdala have been shown to be impacted by BBB permeability. The amygdala helps regulate emotional learning, social behavior, and processing of stressful stimuli responsible for triggering fear and anxiety [11]. It should be taken into consideration that the mechanisms described above have mostly only been observed in rodent models; however, evidence continues to emerge which highlights the complexity of GBA.

Inverse to the mechanisms and pathways outlined previously, other animal models have brought to light the mechanisms in which psychological stress triggers dysbiosis through afferent signaling. Whether the causation of stress and anxiety is derived from physiological or environmental factors, the increase in HPA axis and SNS activity via stressful stimuli has been

observed to potentate gut-microbiota alterations and neuroinflammation through the release of glucocorticoids and catecholamines[11]. While not fully understood, it is hypothesized that the changes in BBB permeability and enhanced neuroinflammation are causal to the downregulation of tight junctions in the intestine and the upregulation of intestinal inflammation markers followed by altered microbial diversity[11]. Nonetheless, animal models, as well as limited evidence in humans, suggest the role of the GBA in the modulation of CVDs and mood regulation should not be ignored.

Prevention and Treatment of Cardiovascular Disease

The prevention and treatment of CVD encompasses the prevention and treatment of the development and progression of hypertension, endothelial dysfunction, atherosclerosis, and chronic inflammation. Research suggests prevention may require interventions which target modifiable risk factors such as diet, physical activity, stress, and other lifestyle habits such as smoking [66]. Organizations such as the American heart association (AHA) emphasize diet modifications for the prevention of CVD, which include increased plant food intake and diets low in saturated fats and added sugar[67,68]. Diets like the Mediterranean and the DASH diet are some of the most studied and prescribed diets for meeting prevention standards[67,68]. Such diets have shown beneficial outcomes in reducing the risk of hypertension and atherosclerosis, as well as co-morbidities such as obesity and diabetes, mainly for their relatively lower abundance of added sugars and saturated fats, but also for their higher abundance of dietary fibers, phytochemical and antioxidant contents, as well as vitamins and minerals[67-69]. Additionally, the use of pharmacological interventions such as lipid-lowering medications, glucose-lowering medications, antiplatelet agents, and anti-hypertensive treatments may be used in patients at high risk[66,70].

Methods of treating CVD may vary based on the severity of the CVD and the etiology. Surgical interventions such as arterial revascularization and valve repairs have been used in more severe cases requiring immediate care. For chronic treatment of CVD, methods exist for targeting modifiable risk factors such as modified diets and physical activity programs. Pharmacological interventions have also shown to be effective in target pathways of blood pressure regulation by using ACE inhibitors and betablockers, lipid management can be treated using statins, and antiplatelet therapies for the reduction of thrombotic risk[66]. While invasive procedures and pharmaceuticals are essential for managing acute and advanced CVD and diseases which lead to CVD development, non-pharmaceutical interventions such as micronutrient-rich diets like the Mediterranean and DASH diets, as well as vegetarian and plant-based diets are also effective tools for managing CVD[67-69]. Additionally, interventions involving weight management, cessation of smoking, and stress reduction are becoming increasingly recognized for the effectiveness in CVD treatment. In acute cases of CVD, such non-pharmaceutical approaches may not be effective on their own to treat CVD but typically recommended instigated into treatment plans which include pharmaceuticals or other invasive procedures[66-68].

Measurements and Biomarkers

Sympathetic and Parasympathetic Nervous System Activity

The sympathetic and parasympathetic nervous system (PNS) are the subsystems which make up the autonomic nervous system (ANS). This section of the nervous system is regulated by control centers located in the midbrain, hypothalamus, and the medulla [71]. Through cervical, cervicothoracic (stellate), and thoracic ganglia, cardiac sympathetic innervation directly impacts the heart as these nerve fibers terminate on the surface of the hearts' sinoatrial node,

atrioventricular node, and the myocardium[12,13]. The methods used in clinical practice and in research to measure the mechanisms of ANS activity involve heart rate variability (HRV) analysis. This measurement requires the analysis of beat-to-beat changes in the R-wave to R-wave intervals, which can be translated to the overall state of the cardiac autonomic nerves [71]. In this context, a lower HRV indicates reduced variation in the time intervals between heartbeats and is associated with increased or high SNS activity. Low HRV may be indicative of autonomic dysfunction characterized by sympathetic overactivity and is often used as a marker of poor cardiovascular health. Inversely, higher HRV is associated with increased PNS activity as the time intervals between heart beats increase [13,71]. High HRV may indicate healthy autonomic nervous system activity which is associated with relatively increased adaptability to stress and improved cardiovascular outcomes[71].

Another common and traditional approach to analyzing the ANS is the measurement of plasma norepinephrine. Measurement of blood levels of norepinephrine during fluctuations in sympathetic and parasympathetic activity can be performed, but this method has been recognized to be more appropriate for assessing ANS function rather than to measure activity levels. Measuring blood norepinephrine has been shown to lack sensitivity and reproducibility as circulating norepinephrine has been shown to be a fraction of what is actually secreted from sympathetic nerve terminals[12]. Researchers have demonstrated methods of measuring regional and total norepinephrine spillover using norepinephrine isotope dilution, as well as direct intraneural microneurographic recording of postganglionic efferent sympathetic nerve activity to skeletal muscle and cutaneous circulations to be exceptional complimentary methods of neurotransmitter secretion; however, these methods are often costly and require highly specific equipment[12].

Cold Pressor Test

The cold pressor test (CPT) has been a widely used method for evaluating sympathetic neural control and cardiovascular reactivity. By immersing an individual's hand or foot in temperature specific ice water or exposing the forehead to ice, sympathetic responses can be triggered, resulting in increased BP and HR. Different simulation sites (hand, foot, or forehead) and postures such as supine or seated have demonstrated varying cardiovascular response patterns. Cold exposure to the foot has been shown to cause relatively significant increases in systolic BP, while forehead exposure may cause greater increases in HR[72]. This method is predominantly used in research settings; however, it has demonstrated clinically predictive value as it has aided in the identification of impaired sympathetic responses due to concussions or autonomic disorders[34,73]. Additionally, earlier research has demonstrated the potentiality of CPT to identify increased risk for CVD and predict hypertension in younger individuals who exhibit specific alterations in physiological responses[35,36]. The autonomic responses demonstrated in CPT, such as reduced variability in CPT responses and the higher BP values could be a mode for identifying autonomic dysfunction and sympathetic overactivity, implying an increased risk for the development of hypertension or even coronary artery disease and atherosclerotic conditions related to vascular dysfunction[74]. Furthermore, the connection between cardiovascular response and autonomic function has also been demonstrated in concussed athletes who were shown to have blunted cardiovascular responses during a CPT, indicating potential sympathetic dysfunction following their concussion[73]

Measures of Stress

Cortisol is used as a biomarker for psychological stress due to its release from HPA axis activation in response to stress. Salivary cortisol is often used in research related to psychological stress to assess HPA activity; however, salivary cortisol levels do not consistently

correlate with plasma cortisol levels due to variations in other biological factors which impact adrenal sensitivity, capacity, cortisol binding, as well as free cortisol levels in the blood[56]. Some research has shown correlations in salivary and plasma cortisol levels with reported stress. Moreover, researchers have also shown individuals with higher basal cortisol levels demonstrate increased resilience to stress when presented with stressful situations. In this same context, those who had lower basal salivary cortisol levels demonstrated greater increases in cortisol levels and reported higher stress levels when presented with the same stressful situation within a controlled environment. These data suggest decreased HPA sensitivity may correlate strongly with higher basal cortisol levels and greater resilience to stress[55].

Studies have shown saliva and plasma chromogranin A (CgA) levels to be reliable indicators of stress due to the association with sympathetic tone and adrenomedullary activity[75]. Salivary CgA levels have been found to significantly and positively correlate with mood disturbances measured through emotional subscales[75,76]. Other studies have demonstrated plasma CgA levels to significantly and positively correlate with measures of anxiety and depression in multiple populations, as well as those experiencing increased workload stress[75,77]. CgA is known to be co-stored and co-released with catecholamines from the adrenal medulla but is thought to be a more reliable biomarker than catecholamines like norepinephrine as it is more stable, easier to handle during storage, transport, and analysis, and has a longer half-life. Additionally, catestatin, a CgA-derived fragment and endogenous multifunctional neuroendocrine peptide, has been shown in numerous studies to be closely tied to CVD, specifically hypertension, acute coronary syndrome, and heart failure[77-80].

Alongside the many quantifiable biochemical markers available for measuring stress in the context of ANS and HPA axis activity are some qualitative measurement tools. These tools provide insights into subjective experiences of stress and anxiety, which may compliment

objective biochemical markers such as catecholamine levels, HRV, and BP. Tools like the Perceived Stress Scale (PSS) and the Generalized Anxiety Disorder 7 (GAD-7) questionnaire are helpful for identifying perceived stress and anxiety and are widely used across healthcare disciplines and clinical research[81,82]. PSS is considered a gold standard instrument for measuring stress perception and is a self-reported questionnaire which assesses the respondent's subjective appraisal of their life circumstance and experienced events over the previous two weeks[82]. The Perceived Stress Scale consists of 10 items scored on a 5-point Likert scale ranging from 0 (never) to 4 (very often). The questionnaire helps capture the frequency and intensity of stress-related feelings. A higher PSS score indicates greater perceived stress and has been validated across various demographics[82]. The GAD-7 is used to assess frequency of symptoms of generalized anxiety disorder over the previous two weeks. It consists of seven items which are rated on a 4-point Likert scale with 0 equating to "not at all" and 3 equating to "nearly every day." Total scores of the GAD-7 seven range from 0-21 with 5-9 indicating mild anxiety levels, 10-14 equating to moderate levels, and 15-21 indicating severe anxiety levels[81]. When used in conjunction with biochemical markers of stress, these tools may provide a more comprehensive and reliable understanding of perceived stress and the connection to physiological changes associated with stress.

The Gut-Brain Axis

Recent studies have suggested the use of measuring alterations in gut microbial composition and diversity as a method for predicting outcomes of mood disorders associated with chronic psychological stress such as major depressive disorder (MDD) and general anxiety disorder (GAD) scores, as certain gut microbiota may have direct implications on GBA activity[83-85]. While few studies have made distinct claims as to what microflora significantly correlates to the development of stress related mood disorders, researchers have observed

genera such as *Christensenellaceae_R7_group*, *Faecalbacterium*, *Fucatenibacter*, and *Sutterella* to be associated with affecting disease phenotype[83]. Additionally, probiotic administration of *Bifidobacterium longum* and *Lactobacillus helveticus* have demonstrated the potential to decrease symptoms of anxiety in rodents and humans[86,87]. There are a multitude of additional microbiota that have identified as correlating to the signs and symptoms of psychological stress and associated mood disorders. The exact mechanisms for which many of these interacts through the GBA is unclear, but research has suggested alterations in the microbiota, especially during dysbiosis, lead to increased permeability along the intestinal epithelium, allowing for increased translocation of pro-neuroinflammatory pathogens and microbial metabolites across the epithelial barrier[16,47,83,88,89]. Inversely, short-chain fatty acid (SCFA) levels may serve as an indicator of improved composition of, or a relatively healthy status of the gut-microbiome, suggesting positive implications towards mental health via GBA[90]. SCFA's such as acetate, propionate, and butyrate are metabolites produced through microbial fiber fermentation which are associated with maintaining gut barrier integrity, neurotransmitter synthesis, immuno-, and neuroinflammation modulation. While Animal and human studies are currently ongoing, current evidence has shown butyrate levels correlating more strongly with levels of depression and anxiety. These results have primarily been observed in microbial transplants from humans to rodents; however, these studies show significant correlations to fecal SCFA levels and reduced levels of stress that may translate to improved cardiovascular outcomes[90-93]. In a recent human trial, an intervention using colon-delivered SCFAs of varying doses was shown to attenuate cortisol responses following a series of stress tests. Results of this study showed increased levels of serum SCFA following the intervention; however, fecal SCFA levels were unchanged, indicating a high absorption rate of SCFAs. The results also suggest that SCFAs may directly modulate HPA activity by permeating across the BBB[93]. While SCFAs show potential in their ability to attenuate stress, suggesting downstream

benefits towards cardiovascular health, the exact mechanisms which outline the resulting improved biomarkers of stress are not fully understood[90,93].

Bioactive Compounds of Microalgae

Dietary supplements, mainly those which contain phytochemicals or bioactive food components, may be beneficial for promoting health, particularly in mental health and cardiovascular function through modalities of the gut. Microalgae supplements in particular have gained increased attention for their nutrient density and potential health promoting properties[94,95]. Microalgae supplements like *Chlorella, Spirulina*, as well as others available on the market are relatively inexpensive to cultivate and are rich in compounds such as prebiotic polysaccharides, antioxidants, polyphenols, pigment proteins, polyunsaturated fatty acids (PUFAs), health promoting amino acids, essential vitamins, and mineral[21,24,96,97]. Polysaccharides, polyphenols, and even lipids to some extent, have demonstrated prebiotic characteristics which promote resilience and improved health within the gut, resulting in outcomes of improved intestinal permeability, improved markers of stress, and reduced oxidation stress[98-100]. Additionally, compounds such as polyphenols, pigment proteins, and PUFAs exhibit potent antioxidant properties following intestinal absorption, which may result in significant reductions in neuroinflammation and improved immune function, cascading towards improved ANS activity, and reducing vascular damage[101-104]. Microalgae and their secondary metabolites should be further investigated to better understand the extent of their potential health benefits and the mechanisms in which their nutrients act upon. The following sections take a deeper look into the current literature revolving around the nutrients found in microalgae.

Polysaccharides and Fibers

Marine-derived ingredients from microalgae are attracting increased attention as sources of nutrients and phytochemicals with the potential improve gut-health and microbiota composition[21,105]. Polysaccharides from algae are primarily found in the cell walls of the marine organisms, and they have shown prebiotic effects on gut microbiota in several *in vivo* studies[21]. Due to their high molecular weight, polysaccharides can serve as prebiotics and undergo microbial fermentation in the lower intestine, allowing for the beneficial modulation of the microbiota. This alteration in microbiota has been shown to reduce harmful bacteria which produce LPS, namely *E. coli*, while simultaneously promoting the production of SCFAs by beneficial bacteria like *Bacteroides* and *Prevotella*[21,106]. Polysaccharides from marine derived algae, *Sargassum fusiforme* have demonstrated the ability to promote SCFA production and the up-regulation of tight junction proteins such as claudin-1 and ZO-1 in mouse models[107]. Additionally, polysaccharides derived from *Undaria pinnatifida*, another marine derived alga, has shown to regulate dysbiosis induced by high-fat diets (HFD) by improving colonic tissue damage and reducing levels of LPS[108]. The derivatives of polysaccharides, such as dietary fiber, also pose individual beneficial effects on the gut microbiota. Some of the marine derived fibers which have been identified are soluble fibers such as alginates, fucoidans, carrageenan, and exopolysaccharides which may be partially fermented to produce SCFAs[19]. Such fibers are commonly found in algae such as *Chlorella, Spirulina,* and *Undaria*, with other algae such as *Fucus, Laminaria, Chondrus*, and *Porphyra* also being rich in a multitude of other soluble and insoluble fibers. The abundance of polysaccharides and fibers provide benefits which go beyond modulation of the gut microbiome which include lipid regulation, glucose digestion and insulin sensitivity, and other metabolic benefits[19].

Phenolics and Pigments

Microalgae are a rich source of secondary metabolites such as phenolic compounds and pigment proteins. Phenolic compounds are characterized by their benzene ring and attached hydroxyl group, which have been demonstrated to be potent antioxidants which may translate to human health benefits[22,96,103]. However, the biological relevance of the *in vitro* models used to evaluate antioxidant activity has been questioned due to lack of consideration of digestion, absorption, and metabolism[109]. Polyphenols are considered a subclass of phenolic compounds which contain two or more phenol rings. Phenolic compounds, to include polyphenols are divided into several classes which include flavonoids, phenolic acids, lignans, tannins, and anthocyanins, with each comprising of different types within their respective class. Polyphenols are classified on the basis of the number of phenol rings which they contain and the structural elements that bind the rings to each other.[110]. Studies have evaluated total phenolic content (TPC) and total flavonoid content (TFC) across various algae genus to include *Scenedesmus*, *Tetraselmis*, and *Chlorella*, as well as their varying species, and have discovered high variability between the different strains[96]. Some of the most notable phenolic compounds observed in microalgae are phenolic acids, flavonoids (to include flavones flavanols, and anthocyanidins), stillbenoids, and tannins such as ellagitannins and phlorotannins for their capacity to reduce ROS and suppress signaling pathways associated with inflammation[22,29,96,103]. Other phenolic compounds discovered within microalgae are phenolic acids such as gallic acid and caffeic acid, flavonoids known as epicatechin as kaempferol, and resveratrol which is a stillbenoids. These polyphenols exhibit properties that allow them to participate in hydrogen atom transfer or single electron transfer. While the extent of the benefits of these compounds is not fully understood, they have been exhibited with cardioprotective and neuroprotective characteristics due to their ability to attenuate oxidative stress[96]. While polyphenols poses potent antioxidant characteristics when absorbed, the

reality is that many polyphenols have low bioavailability. The phenolic compounds which never cross the intestinal epithelium reach the colon on unaltered forms, allowing for direct interaction with the gut-microbiota[102]. Polyphenols poses prebiotic characteristics similar to polysaccharides as they also resist gastric acidity, hydrolyzation of mammalian enzymes, and can be fermented by intestinal bacteria. Due to these characteristics, the interaction between polyphenols and bacteria in the gut result in the production of secondary metabolites which can cross the intestinal epithelium, contributing to human health[102].

Microalgae-derived pigments such as carotenoids, chlorophylls, and phycobiliproteins are also major phytochemicals found in microalgae and have benefits to health ranging from antioxidant, anti-inflammatory, to neuroimmune-enhancing properties[111]. Of the carotenoids found in microalgae, ß-carotene (a precursor to vitamin A), lutein, fucoxanthin, and zeaxanthin are noted for reducing oxidative stress, anticancer effects, as well as cardioprotective effects[111-114]. Chlorophylls and phycobiliproteins provide the vibrant colors found in microalgae[111-113]. Phycobiliproteins, particularly phycocyanin, have been shown to antioxidant and anti-inflammatory properties which my contribute to the modulation of the immune system as well as cancer prevention[111-113].

Lipids

Another key characteristic of microalgae is their lipid content and fatty acid profiles. Microalgae are considered a sustainable source of essential lipids, with benefits extending across multiple industries due to their diverse fatty acid profile and abundance[115]. Nearly all the microalgae genus and species available for commercial and nutraceutical use have been shown to contain various PUFAs or long-chain fatty acids which are widely known for their health benefits. They are also considered to be primary producers of omega-3-fatty acids, with many algae comprising docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA)[115,116].

Omega-3 polyunsaturated fatty acids are well known for their lipid lowering properties within the vasculature, as well as their implication on inflammation reduction; however, Omega-3 fatty acids have recently shown potential for possessing prebiotic properties similar to fermentable dietary fibers. In a study where Omega-3 fatty acid supplementation was compared to inulin supplementation for 6 weeks, participants taking the Omega-3 intervention displayed significant changes of bacterial fermentation products such as butyrate. These results were also accompanied with increased levels of *Bifidobacterium, Lachnospiraceae, Coprococcus*, and *Bacteroides* which were similar to those supplementing with inulin[100]. Furthermore, PUFAs such as Omega-3 fatty acids have been studied for their ability to improve depression symptoms, anxiety, stress responses, and overall cognitive function[99,101,117]. Omega-3 PUFAs may promote antidepressive-like effects specifically by modulating HPA as function by increasing expression brain-derived neurotropic factor (BDNF) and levels of serotonin, which are necessary for mood regulation[101]. Other studies support associations between Omega-3 PUFA intake and improved cognitive function and reduction in mental disorders related to stress and anxiety[99,117]. Included in the fatty acid profile of microalgae are phytosterols which have potential application in functional foods and pharmaceuticals due to their anti-inflammatory and neuroprotective properties[118]. Additionally, an *in vitro* study has purported glycolipids and phospholipids as having anti-inflammatory and anti-thrombotic characteristics[119].

Proteins

The protein contents of algae differ significantly across different species and strains. *Spirulina* and *Chlorella* contain complete proteins with protein level reaching up to 70% dry weight protein with amino acid (AA) profiles mimicking that of eggs[95,120]. Many analyses of AA composition in marine algae show glutamic acid and aspartic acid being some of the highest concentrated in most algae, with red algae also containing higher ratios of taurine[95]. In

conjunction with the AA contents of microalgae, algae-derived amino acids have shown to provide bioactive peptides which have additional cardiometabolic benefits including blood-pressure lowering effects[121]. Identification of these peptides can be accomplished using bioinformatic and proteomic tools. Using such tools has identified AA derived peptides as significantly ranging in structure, resulting in vastly diverse functionality and added antioxidant and antimicrobial properties[121,122]. Additionally, research has shown the implications of metabolic health from the hydrolysis of AA during digestion and peptide production through properties responsible for inhibiting Angiotensin I-Converting Enzyme (ACE), which has direct implications to improving blood pressure and hypertension[122].

Effects of Microalgae on Cardiovascular Health

Studies have investigated the potential for microalgae to impact CVD through several modalities. First, microalgae have been studied for implications in hypertension. Several *in vitro* and *in vivo* studies suggest compounds found within algae possess antihypertensive properties, as well as properties which reduce inflammation and improve lipid profiles[123-125]. In particular, bioactive peptides have been identified for their ability to regulate hypertension. Studies using strains of Chlorella identified the marine derived peptide's ability to regulate BP by promoting ACE inhibitor activity, suppressing concentrations of angiotensin II, allowing for improved vasodilation[123,124,126]. In addition to the antihypertensive peptides found in microalgae, pigments such as carotenoids and phycobiliproteins synthesized within algae exerts antioxidant and anti-inflammatory effects in vivo, potentially resulting in reduced hypertension and risk for CVD. Studies using *Spirulina*, *Chlorella*, *Dunaliella*, *Gracilariopsis*, and *Undaria* which have high concentrations of antioxidants, may reduce oxidative stress by reducing endothelial cell damaging ROS, preventing further damage to the endothelial lining and reducing risk for hypertension vascular dysfunction[123,126]. The anti-inflammatory and antioxidant effects of

microalgae on hypertension have also been translated to having significant implications towards atherosclerosis. Evidence has shown the protective properties of algae derived pigments and phenolic compounds reducing ROS production and the formation of plaque caused by oxidized LDL cholesterol within the endothelial lining of arteries[53,125]. Additionally, research has shown the previously mentioned compounds found in algae, as well as their high concentrations of PUFAs to be beneficial towards reducing levels of inflammatory cytokines like IL-6 and TNF-α which are known to contribute to atherosclerosis[30,125]. The fatty acid profiles of microalgae which include DHA and EPA also have also been studied with evidence of their lipid-lowering qualities. Evidence in humans and in rodents have exhibited DHA and EPA to support the lowering of circulating triglycerides, while increasing levels of high-density lipoprotein (HDL) cholesterol, which may result in decreased lipid accumulation in the endothelial lining and reduced progression of atherosclerotic plaque formation[124,127-130]. HDL cholesterol (HDL-C) is significant in promoting cardiovascular health as they are multifunctional particles known for their reverse cholesterol transport, which facilitates the removal of cholesterol from tissues, aiding in the prevention and treatment of atherosclerosis[131]. Furthermore, the effects of microalgae on CVD can also be outlined in studies which have explored their implications on other components of metabolic syndrome which significantly increase the risk of CVD. Briefly, metabolic syndrome is characterized by a combination of conditions which include insulin resistance, obesity, dyslipidemia, and hypertension, and is associated increased risk for CVD and type 2 diabetes[132]. Such studies have explored the effects on microalgae interventions on hyperlipidemia, and diabetes mellitus. *Chlorella, Spirulina*, and other strains of algae have been associated with improving lipid profiles while increasing insulin sensitivity and weight loss[30,53,124,125].

Effects of Microalgae on Psychological Stress

The potential for microalgae to promote mental health in individuals with and without psychological stress has gained increased attention in recent years. The bioactive compounds found within the various strains of microalgae have been shown to exhibit neuroprotective and anti-inflammatory properties which may translate to improved stress outcomes due to improvements in sleep quality, mood, and reduced gastrointestinal discomfort[133,134]. One randomized control trial in human subjects demonstrated significant reductions in irritability and tension related to work stress following an intervention using *Euglena*[135]. Euglena, along with other microalgae strains are believed to reduce mental stress by regulating ANS activity through the production of neurotransmitters which could derive either directly from microalgae or the interaction of their bioactive compounds with gut microflora[135]. Neurotransmitters which are commonly associated with microalgae intake are dopamine and serotonin. Intake of *Botryoccus terribilis* has been shown to decrease symptoms of depression due to decreases in neuroinflammation related to increased gene expression in dopamine and serotonin[136,137]. Interventions which include the supplementation of popular microalgae like *Spirulina* and *Chlorella* which are rich in pigments, polyphenols, and phytosterols have also exhibited improvements in mental health outcomes. These phytochemicals can exert antioxidant effects, leading to improvements in neuroinflammation and immune responses, and improvements in sleep and mood regulation[134]. Furthermore, PUFAs such as DHA and EPA are abundant in marine-derived algae. These omega-3 fatty acids may reduce depressive-like symptoms and chronic stress[136]. EPA has been observed to have anti-inflammatory properties by reducing proinflammatory cytokines which may increase sympathetic tone, while also modulating neurotransmitter systems which may improve cell signaling with the hippocampus[136,138]. DHA, synergistically modulates receptor function of neural membranes, leading to improved cognitive function and management of stress related depression[136,139]. In addition to long-

chain PUFAS, the SCFAs produced from microalgae digestion and fermentation have also shown to be a promising modality for improving stress and mood disorders. Studies have observed the association between SCFA levels and mental health, showing increased levels to correlate strongly with reduced symptoms of mood disorders like depression, anxiety, and stress[90]. SCFAs exhibit properties which allow them to interact with immune, neural, and endocrine pathways. Using these pathways, SCFAs have been linked to modulation of the HPA[93]. Finally, a few studies have explored the efficacy of reducing stress by improving symptoms of irritable bowel diseases (IBD) such as Crohn's disease and ulcerative colitis, and irritable bowel syndrome (IBS)[140]. The regulation of these diseases and syndromes has shown to be useful for reducing stress and anxiety by reducing symptoms of pain and discomfort, while simultaneously decreasing stress and mood disrupting neuroinflammation by improving intestinal epithelial integrity[133,140].

Effects of Microalgae on Gut Health

Research on microalgae and microalgae extracts have emerged showing several potential benefits to gut health. The bioactive compounds found within microalgae give these organisms the potential to modulate the intestinal environment (e.g., gut microbiota composition) which may trigger a cascade of downstream effects linked to improved intestinal health and reduced inflammation-related intestinal discomfort. There is increasing evidence that the bioactive compounds, particularly sulfated polysaccharides, found in algae such as *Sargassum fusiforme, Chlorella,* and *Spirulina* possess prebiotic effects which alleviate gut microbiota dysbiosis[21,98,141]. It has been reported that these marine derived polysaccharides are responsible for aiding in the proliferation of beneficial bacteria such as *Lactobacillus*, *Akkermansia*, and *Bifidobacterium*, which are associated with improved gastrointestinal

health[21,141]. Sulfated polysaccharides derived from algae supplementation have also been observed to decrease unfavorable bacteria such as *Actinobacteria*, *Verrucomicrobia*, as well as *Pseudomonas*, and *Yershinia* in high-fat diet fed mice[98,141]. Additionally, the delivery of polysaccharides has been shown to boost the production of SCFAs, which have been shown to decrease inflammation throughout the gastrointestinal tract, while improving the integrity of intestinal lining. The sulfated polysaccharides and the SCFA produced from the fermentation of polysaccharides are both associated with the upregulation of gene expression of tight junction proteins such as claudin-1 and ZO-1, as well as mucusin (Muc-2)[21,31,98,140,142]. The presence of improved intestinal epithelial integrity has been accompanied by improved physical characteristics of intestinal health in several animal models such as reduced inflammation of the intestine, restored villus height in the jejunum, and total energy digestibility[142,143]. Furthermore, much of the research focused on the interaction between microalgae supplementation and gut health have explored the benefits on IBD and IBS. Research in humans and in animals with colitis and IBS have reported significant improvements in symptoms related to gastrointestinal discomfort, bloating, bowel movement frequency, and reduced incidence of diarrhea[142-144].

Microalgae and the Gut-Brain Axis

The research which directly measures the impact of microalgae on GBA activity is limited; however, there is evidence supporting beneficial impacts of microalgae's bioactive compounds on the major components of the GBA such as the gut microbiota, intestinal epithelial activity, nervous system function, and HPA activity. In the context of microalgae acting as a prebiotic, or as a vehicle which provides bioactive compounds with prebiotic characteristics, supplementation has been shown to reduce stress responsiveness, anxiety, and depressive-like behaviors, while enacting changes in hippocampal synaptic efficacy and hypothalamic neurol

activity[10,11]. These findings suggest a direct interplay between microalgae and GBA activity. This change in psychoactive behavior during intervention which includes microalgae, or the compounds extracted from micro algae have been observed to associate strongly with activity in the gastrointestinal tract. Stated previously, microalgae supplementation has been shown to modulate gut microbiota composition by inhibiting harmful bacteria and allowing beneficial bacteria to proliferate[98,141]. It has been observed that such changes in the gut microbiota can alter regulation of microbial gene expression of metabolites and neurotransmitters which serve as neuromodulators that interact more directly in messaging to HPA or other parts of the brain. Gamma-aminobutyric acid (GABA), glutamine, histamine, LPS, SCFAs, bile acids, and catecholamines are some but not all of the gut microbiota-derived metabolites or compounds which significantly modulate GBA activity through interactions with glial cells, myelination, synaptic pruning, and functions of the BBB[9-11,145,146]. Additionally, the compounds within microalgae, whether prebiotic-like phenolic compounds, polysaccharides, and/or fatty acids, or microbe-algae derived SCFAs or other bioactive compounds may significantly improve intestinal permeability through the upregulation of tight junction protein synthesis[29,47]. The improvement of intestinal epithelial integrity allows for improved sampling of immune cells within the gut through microfold cells, which has been shown to improve markers of neuroinflammation in rodent models, further the implications of microalgae supplementation on bidirectional communication between the gut and the brain.[47]

The following methods, results, and discussion sections include content that have been directly taken from the original research article in which this thesis is a part of. These manuscripts are presented in their original or minimally modified form to maintain consistency with the submitted work. Proper citations and acknowledgements are provided to credit co-authors of the original source.

METHODS

Clinical Study

Purpose

This project is part of a larger randomized, double-blind, placebo-controlled, parallel-arm clinical trial evaluating the efficacy of four weeks of microalgae supplementation on stress and anxiety in healthy adults aged 18-55 years with mild to moderate GI distress. Here, we evaluated the extent to which microalgae supplementation for four weeks impacts markers of psychological stress and anxiety and physiological changes in blood pressure measures caused by cold stress[147].

Study Design

This was a randomized, double-blind, placebo-controlled, parallel-arm intervention trial (Figure 1) at Colorado State University's Food and Nutrition Clinical Research Laboratory (FNCRL) within the Department of Food Science and Human Nutrition. Briefly, healthy adults with mild to moderate gastrointestinal distress were recruited from Fort Collins, Colorado, and surrounding areas through referrals from local healthcare providers, flyers, emails, and word of mouth. Initial eligibility was assessed via a phone screening questionnaire and confirmed onsite at the FNCRL. Healthy, free-living adults aged 18–55 years with a BMI between 18.0-29.9 kg/m² who experienced mild to moderate gastrointestinal distress, confirmed by one positive response to the Rome IV criteria were enrolled into the study. Exclusion criteria included: (A) individuals <18 or >55 years of age; (B) BMI outside the 18.0-29.9 kg/m² range; (C) pregnancy or breastfeeding; (D) current smoker or use of tobacco products; (E) diagnosis of intestinal diseases such as Celiac disease, Crohn's disease, Ulcerative Colitis, or gastrointestinal

cancers; (F) regular use of statins, metformin, steroids, NSAIDs, or MAO inhibitors; (G) a clinical diagnosis of mental health disorders (e.g., depression, bipolar disorder, Alzheimer's disease, etc.); (H) a known allergy to any ingredients in the microalgae extract or maltodextrin; and (I) a history of alcohol or substance abuse within the past 12 months. Participants were asked to maintain their regular eating and exercise habits throughout the study duration[147].

Eligible participants were enrolled and given sequential study codes then randomized to consume two powder-encased capsules per day of either a maltodextrin placebo (PL) or 250 mg of a microalgae Tetradesmus obliquus Mi175.B1.a (TOME) via randomization codes generated in Matlab version R2021b (MathWorks, Natick, MA, USA) and printed on capsule packages. Study participants, principal investigators, and clinical personnel (FNCRL, CSU) were blinded to the treatment groups until all primary data analyses were complete[147].

Participants attended clinic visits for eligibility screening and at baseline, 2-week, and 4-week time points. Participants arrived at the clinic after fasting for at least 8 hours, and refraining from strenuous exercise, alcohol consumption, and use of medications or dietary supplements for at least 24 hours. During screening, medical history, health status, and demographic information were collected, followed by anthropometric measurements (height, weight, and waist and hip circumferences). Participants meeting all eligibility criteria were asked to complete a 24-hour dietary recall on three separate days (two weekdays and one weekend day) prior to the baseline and final study visits, using the National Cancer Institute's Automated Self-Administered 24-hour dietary assessment tool (ASA24; https://asa24.nci.nih.gov). Participants were instructed to record their bowel movements using the Bristol Stool Scale (BSS) for at least 7 days prior to their baseline clinic visit to capture their normal stool habits. At the baseline, 2-week (midpoint), and 4-week (final) visits, in addition to anthropometric and blood pressure measurements, participants were asked about their physical activity levels over the previous seven days using a validated questionnaire. They also completed several

questionnaires, including the Gastrointestinal Symptom Rating Scale (GSRS), the Generalized Anxiety Disorder 7-item scale (GAD-7), the Positive and Negative Affect Schedule (PANAS), and the Perceived Stress Scale (PSS). Blood and saliva samples were collected following standard procedures to analyze biomarkers related to gastrointestinal health, stress, inflammation and metabolic/lipid panels. Participants were also instructed to collect a stool sample at home the day before each study visit using a provided collection kit. Stool samples were used to assess fecal markers of intestinal permeability and function. At the end of the baseline visit, participants received their 4-week treatment supply and a daily treatment log. At the final visit, participants returned unused treatment capsules to assess compliance. Primary outcomes included safety and tolerability of the product and indicators of gastrointestinal health, including changes in gastrointestinal symptoms and bowel movements, microbiota composition, gut function and permeability biomarkers. Secondary outcomes included assessing impacts on stress and anxiety through mental health assessments and acute stress biomarkers, blood pressure and pulse wave analysis assessment at baseline and during a CPT, and changes in lipid profiles. Finally, responses to inflammation were determined by measuring inflammatory biomarkers in LPS-stimulated PBMCs. The trial is registered at ClinicalTrials.gov as NCT06425094 and was approved by the Colorado State University Institutional Review Board (CSU #5162), in accordance with the Declaration of Helsinki[147].

Intervention and compliance

Participants were randomly assigned to take supplements containing either a PL or TOME (i.e., microalgae extract from Tetradesmus obliquus Mi175.B1.a) daily for 4 weeks. The experimental supplement consisted of capsules containing 125 mg of microalgae extract, standardized to 0.3% lutein (Zengut™, Microphyt, Baillargues, France). Microalgae composition is described in Table 1. The PL capsules were identical in appearance, containing 125 mg of maltodextrin, and were formulated to taste the same as the experimental supplement. Participants ingested 2 capsules daily, consistent with the dosage approved by the United States Food and Drug Administration (FDA) for microalgae extract from Tetradesmus obliquus Mi175.B1.a. The product manufacturers provided a certificate of analysis to verify the dosage and confirm the absence of contaminants. Supplementation began on the first study day following baseline testing, and participants were instructed to take the capsules daily at lunchtime with 8 ounces of water. Both the PL and TOME supplements were distributed in blister packs and stored at 4°C. To assess treatment compliance, participants completed treatment diaries and returned any unused capsules at their final study visit[147].

	Composition	**Methods**
Tetradesmus obliquus Mi175.B1.a extract	20 – 35% (w:w)	/
MCT oil based on coconut oil (carrier)	65 – 80% (w:w)	/
Mix of non-GMO tocopherols (antioxidant) – E 306	0.45 – 0.55% (w:w)	/
Total lipids	≥ 80% (w:w)	Gravimetry
Proteins	≤ 5% (w:w)	Kjeldahl (Titrimetry)
Humidity	< 5% (w:w)	Thermogravimetry
Total MUFAs	3.5 – 11.5% (w:w)	GC-FID
Carbohydrates	≤ 20% (w:w)	Calculation
Total PUFAs	1 – 5.5% (w:w)	GC-FID
Lutein	0.3 – 0.9%(w:w)	HPLC
Sterols content (g/100g product)	0.3 – 0.9	NF en ISO 12228-1

Table 1. *Tetradesmus obliquus* Mi175.B1.a composition

Anthropometrics

Height (cm) was measured using a scale-mounted stadiometer to the nearest 0.5 cm, and weight (kg) was measured with a digital scale (Health-O-Meter Professional Scale, Sunbeam Products, Ibc.) Waist and hip circumferences were recorded using Gulick measuring tape with a tension handle (Creative Health Products, Inc.) in accordance with standard procedures[147,148].

Blood and saliva collection and ELISA assays

Blood and saliva were collected from participants at each study visit. Venous blood samples were collected in lithium heparin and ethylenediaminetetraacetic acid (EDTA) tubes. Plasma was collected by centrifugation of the EDTA tubes and stored at -80°C. Saliva samples were collected and stored at -80°C until use[46]. Biomarkers of stress, sympathetic activation, and intestinal function were measured in saliva and/or plasma using ELISA assays according to manufacturer's instructions. Stress markers included: plasma and salivary cortisol (MP Biomedicals, Solon, OH, USA), plasma and salivary a-amylase (Novus Biologicals, Centennial, CO, USA), plasma and salivary chromogranin A (Novus Biologicals, Centennial, CO, USA), plasma blood-derived neurotrophic factor (BDNF; BioLegend, San Diego, CA, USA), and plasma adrenocorticotropic hormone (ATCH; Eagle Biosciences, Amherst, NH, USA)[147].

Hemodynamics and pulse wave analysis at rest and during the cold pressor test (CPT)

Brachial systolic blood pressure and diastolic blood pressure were measured in triplicate (and averaged) on the upper left arm in the supine position after 10 minutes of rest, using an automated blood pressure monitor (Omron HEM-907XL Professional Blood Pressure Monitor). Pulse wave analysis was performed in triplicate (and averaged) following brachial blood

pressure measurement using the SphygmoCor XCEL (AtCor Medical Inc., Naperville, IL, USA) as described[46]. Outcomes for pulse wave analysis included aortic systolic and diastolic pressure, mean arterial pressure (MAP), pulse pressure, aortic pressure (AP), heart rate, augmentation index (AIx), and AIx normalized to a heart rate of 75 bpm (AIx@75). For the CPT, participants submerged their right hand just above the wrist into an ice/water slurry bath (1-4°C confirmed with a digital thermometer and water kept circulating with a pump) for 2 minutes. Brachial blood pressure measurements were performed at 1- and 2-minute time points during the CPT, while pulse wave analysis measurements were performed at the 2-minute time point during the CPT. After the 2-minute period, the participant's hand was removed from the bath, wrapped in a towel, and brachial blood pressure was measured every 1 minute for a total of 3 minutes[147].

Statistical Analysis

All statistical analyses related to biomarkers and participant-reported outcomes were performed using GraphPad Prism 10 Version 10.2.2. Data were assessed for normality using QQ plots and Shapiro-Wilks statistical tests. All biomarkers and participant-reported outcomes were analyzed using linear mixed effects models that included treatment, time, and time*treatment as fixed effects and subject as a random effect. Any biomarkers and participant-reported outcomes showing significant ($P<0.05$) relationships with fixed covariates were brought forward into additional analyses (e.g., multiple comparisons test and change/% change from baseline). Post hoc multiple comparisons were conducted using Tukey's or Sidak's tests; accordingly, all reported P-values for the multiple comparisons are adjusted P-values. All mixed effects models were run as per protocol (PP; n=53). Two-tailed T-tests or non-parametric Wilcoxon Rank Sum tests were used to determine if there were any differences in change from

baseline-to-final values between the microalgae extract and comparator treatments. For all statistical tests, p-values < 0.05 were considered statistically significant, with p-values < 0.1 being reported as non-significant trends. Effect sizes for primary outcomes were determined using partial ETA squared (ηp2), which was calculated as the SSeffect/(SSeffect+SSerror). Calculated values range from 0-1 with values between 0.01-0.05 considered a small effect, 0.6-.13 is a medium effect, and >0.14 is a large effect[147].

RESULTS

Participant characteristics

A total of 145 individuals responded to study advertisements and were assessed for eligibility. Of these, 68 individuals passed the online pre-screening and were invited for the clinical screening visit (Figure 1). At this visit, individuals were familiarized with the study and provided written informed consent. A total of 56 individuals met all criteria and were enrolled into the intervention. Twenty-eight participants were randomized into the PL group and 28 into the TOME group. A total of 53 participants completed the study and are included in the statistical analyses (per protocol). Participants that did not complete the study dropped out because of inability to attend scheduled clinic visits (n=2), a change in medication (n=1). Participant demographics are included in Table 1[147].

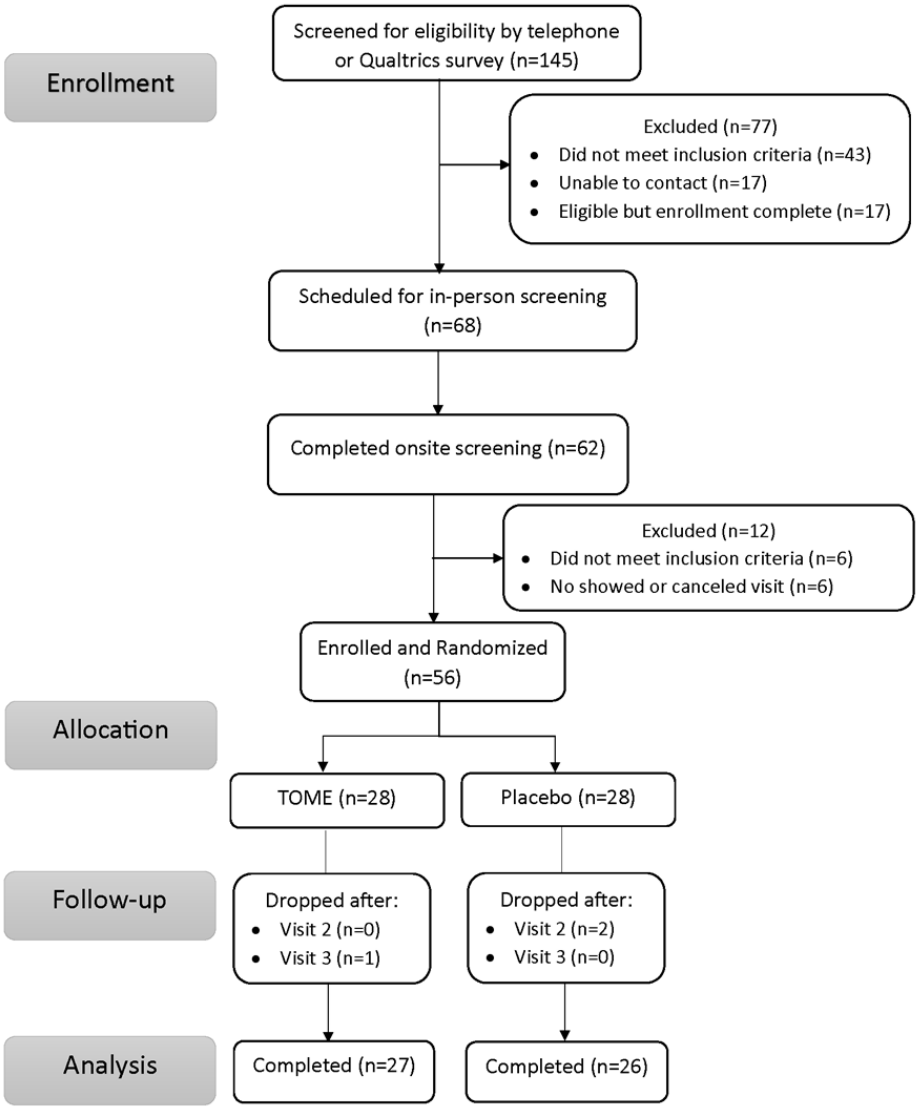

Figure 1: Study consort diagram.

Table 1: Participant Demographics. Data are presented as means ± standard deviations.

Variable		N	Mean	P-Value
Gender F(M)	Placebo	28	16 (12)	0.64
	TOME	28	18 (10)	
Age	Placebo	28	30.1 ± 6.25	0.11
	TOME	28	33.7 ± 8.68	
	Total	56	31.9 ± 7.72	
Height (cm)	Placebo	28	170.7 ± 9.97	0.94
	TOME	28	170.5 ± 10.03	
	Total	56	170.6 ± 10.00	
Body Mass Index (kg/m^2)	Placebo	28	24.9 ± 2.82	0.35
	TOME	28	24.2 ± 2.73	
	Total	56	24.6 ± 2.77	
Weight (kg)	Placebo	28	72.9 ± 12.43	0.53
	TOME	28	70.8 ± 12.92	
	Total	56	71.8 ± 12.61	
Waist Circumference (cm)	Placebo	28	81.5 ± 8.43	0.50
	TOME	28	79.9 ± 9.08	
	Total	56	80.7 ± 8.72	
Hip Circumference (cm)	Placebo	28	101.6 ± 7.31	0.16
	TOME	28	99.1 ± 5.63	
	Total	56	100.3 ± 6.58	
Resting Heart Rate (beats/min)	Placebo	28	61.4 ± 8.75	0.84
	TOME	28	60.9 ± 8.66	
	Total	56	61.2 ± 8.63	
Systolic Blood Pressure (mmHg)	Placebo	28	113.1 ± 10.13	0.34
	TOME	28	110.8 ± 7.10	
	Total	56	112.0 ± 8.72	
Diastolic Blood Pressure (mmHg)	Placebo	28	67.8 ± 8.05	0.85
	TOME	28	67.4 ± 6.27	
	Total	56	67.6 ± 7.13	

Compliance, safety, tolerability, and diet

Compliance with the intervention protocol was determined by calculating the percent of consumed capsules. Average compliance for the PL group was 97% (range=82-100%) and average compliance for the TOME intervention group was 94% (range=75-100%). To accurately assess the impact of the intervention, participants were instructed to adhere to their habitual diet and physical activity regime. No significant differences in total caloric, macronutrient, or micronutrient intake were noted between or within groups at any time point (Table 3).

Study-related adverse events were reported to be mild to moderate and included one report of constipation, one report of diarrhea, one report of bloating and dizziness, and three reports of increased anxiety in participants on the placebo. Treatment related adverse events included six reports of increased flatulence, four reports of increased bloating, two reports of borborygmi, and one report of increased abdominal pain. No participants withdrew from the study due to adverse events. Metabolic panels for liver function and blood chemistry remained within standard reference ranges throughout the study, although there was a significant main effect of time observed for glucose (P=0.03), sodium (P=0.03), AST (P=0.002), and albumin (P=0.002). Post hoc analysis revealed that AST was significantly reduced between baseline and 4 weeks in the TOME group (P=0.03) and albumin was significantly reduced between baseline and week 4 in the placebo group[147].

Self-reported assessments of stress, anxiety, and affect

Several questionnaires were used to assess whether the intervention impacted stress, anxiety, and mood. The Generalized Anxiety Disorder-7 (GAD-7) is a seven-item screening tool used by healthcare providers to assess severity of anxiety. Scores from 0-4 indicate minimal anxiety, 5-9 is mild anxiety, 10-14 is moderate anxiety, and 15-21 is severe anxiety. Both groups reported low levels of anxiety at baseline (TOME mean=5.3; placebo mean=4.7) and there were no significant differences in GAD-7 scores between intervention groups or over the course of the study (Figure 2A). The Perceived Stress Scale (PSS) is a classic instrument that helps to evaluate how different situations affect perceived stress. Scores range from 0-40 with a score of 0-13 indicating low stress, 14-26 indicating moderate stress, and 27-40 indicating high perceived stress. At baseline, participants average scores fell into the mild/moderate perceived stress range (TOME mean=14.46; placebo mean=13.92). The mixed effects models showed a significant effect of time (Figure 2B, P<0.001) and post hoc Sidak's analysis showed that there

was a significant reduction in perceived stress in both the TOME (P=0.028) and Placebo group (P=0.014) at Week 4 compared to baseline. Finally, we used the Positive and Negative Affect Scale (PANAS), which assesses levels of positive and negative emotions. There was a significant effect for time (P=0.010), and post hoc comparisons showed that there was a significant decrease in positive affect in the TOME group between Weeks 2 and 4 (Figure 2C; P=0.003). There were no other statistically significant comparisons; however, at the week 2 timepoint there was a trend towards significance in the difference between the TOME group and PL (P=0.059). There was a significant effect of time (P=0.006) as well as an interaction effect (P=0.021) for the negative aspect scores, which indicate negative emotions and perceptions (Figure 2D). Specifically, there was a significant decrease in negative affect scores individuals in the TOME group between Weeks 2 and 4 (P=0.009) and between baseline and Week 4 (P<0.001). To further explore the differences that we observed in positive and negative affect scores, we calculated changes at week 2 and week 4 from baseline were compared. The change from baseline between week 2 and week 4 in the TOME group for positive affect (Figure 2E; P= 0.001) and negative affect (Figure 2F; P=0.005) were statistically significant. In addition, the change in negative affect at the end of the study was significantly different compared to the Placebo (P=0.033)[147].

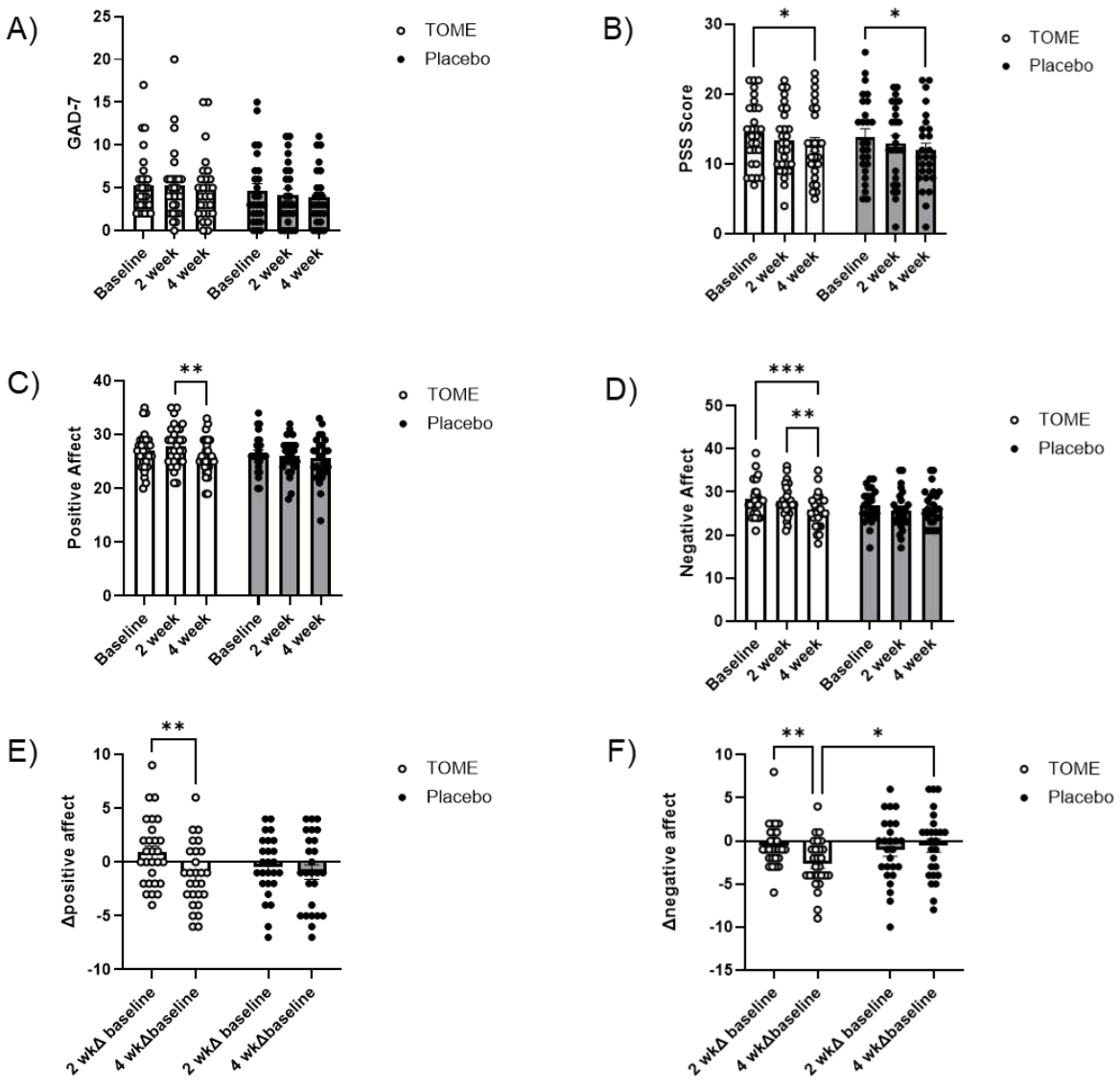

Figure 2: Self-reported assessments pf stress, anxiety, and affect. Results of GAD- 7 (A); PSS (B); Positive Affect (C); Negative Affect (D); Delta change in Positive Affect (E); and delta change and Negative Affect (F). Data are based on mean scores at baseline, weeks 2 and 4 of supplementation and 95% confidence intervals. * indicates statistical significance between group differences (* = p<0.05; ** = p<0.01). 2-way mixed model ANOVA were used for repeated measures and Tukey's post hoc analysis for multiple comparisons on scores changes.

Blood and saliva markers of stress

Several biological markers of stress and anxiety were measured in saliva and/or plasma. Cortisol, a stress hormone produced by the adrenal glands, α-amylase and CgA, markers of the

sympathetic-adrenal-medullary activation of sympathetic nervous system, were measured in both the saliva and plasma. There was a significant effect of time (P=0.04) for plasma alpha-amylase. Pairwise comparisons showed that there was a trending decrease in α-amylase in the placebo group (Figure 3A; P=0.09). In addition, there was a trending effect for time for plasma CgA levels (P=0.06). Post hoc analysis showed that CgA was significantly decreased after TOME intervention at Week 4 compared to baseline (Figure 3B; P=0.03) as well as a trending decrease compared to the placebo group (P=0.08). We also measured BDNF and ACTH in plasma. BDNF serves to protect neurons and tends to decrease with chronic stress, while ACTH is produced by the pituitary glands and signals the body to produce cortisol. Neither of these markers were significantly altered by either the treatment or placebo over the course of the study[147].

Table 2: Saliva and plasma ELISA assays. Data are expressed as means ± standard deviations. Data were analyzed using mixed-effects analysis for multiple comparisons. P-values represent comparison from baseline to final for each treatment group. † = p<0.05

Variable	Group	N	Baseline	Week 4	P-Value
Saliva cortisol	Placebo	26	3.31 ± 1.67	3.33 ± 1.36	0.998
(ug/dl)	TOME	27	3.48 ± 1.50	3.39 ± 1.28	0.926
Serum cortisol	Placebo	25	15.09 ± 6.84	15.74 ± 7.41	0.834
(ug/dl)	TOME	25	18.07 ± 7.82	17.23 ± 9.17	0.742
Salivary α-amylase	Placebo	25	95.81 ± 77.12	104.21 ± 75.82	0.511
(U/L)	TOME	23	126.22 ± 77.00	114.02 ± 88.05	0.361
Serum α-amylase	Placebo	25	50.39 ± 13.55	45.74 ± 16.48	0.090
(U/L)	TOME	25	46.81 ± 11.60	44.88 ± 12.16	0.640
Saliva CgA	Placebo	25	394.9 ± 453.5	467.8 ± 559.2	0.516
(ng/ml)	TOME	22	421.5 ± 730.4	333.3 ± 463.3	0.461
Serum CgA	Placebo	25	1305.9 ± 1063.5	1305.9 ± 1063.5	0.947
(ng/ml)	TOME	25	1053.6 ± 1116.1	1260.5 ± 1054.3†	0.037
Serum BDNF	Placebo	24	15.85 ± 12.80	13.76 ± 8.34	0.689
(ng/ml)	TOME	26	14.83 ± 12.99	11.59 ± 8.53	0.389
Serum ATCH	Placebo	24	14.41 ± 8.82	16.15 ± 11.07	0.269
(pg/ml)	TOME	26	16.14 ± 27.58	17.32 ± 30.22	0.433

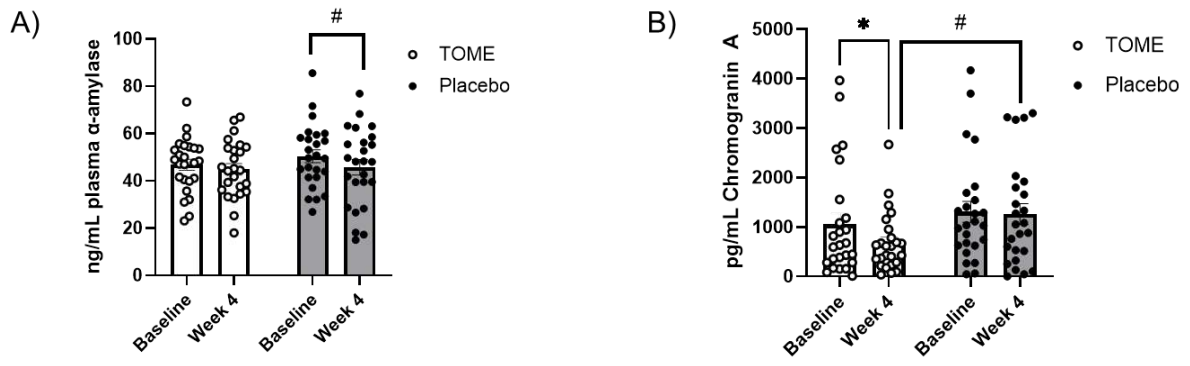

Figure 3: Plasma α-amylase and Chromogranin A. Baseline and final measure of plasma α-amylase (A); and plasma CgA (B). Data are based on mean scores at baseline and week 4 of supplementation and 95% confidence intervals. * Indicates statistical significance between group differences ($p<0.05$). # Indicates statistical trend towards significance ($p<0.07$). 2-way mixed model ANOVA were used for repeated measures and Tukey's post hoc analysis for multiple comparisons on scores changes.

Blood pressure and pulse wave analysis at rest and during CPT

Blood pressure and other parameters assessed through pulse wave analysis are shown in Table 3. At baseline and before the CPT (i.e., resting), there were no statistically significant differences between groups. However, the PL group had a significantly (P=0.021) lower AIx response at the 2-minute time point during the CPT at baseline and the 4-week time point compared to the TOME group suggesting baseline differences in cardiovascular function. Resting aortic systolic blood pressure significantly increased (P=0.028) in the PL group but not in the TOME group from baseline to 4-weeks but there were no differences between groups. Brachial diastolic blood pressure responses after 2 minutes of the CPT were significantly higher at the 4-week timepoint than baseline in the TOME group but not PL (Figure 4), though there were no differences between groups. At the 4-week time point, the TOME group had higher AIx@75 at the 2-min CPT than the PL group. No other significant differences were observed within or between groups[147].

Table 3: Brachial and aortic blood pressure and augmentation index (AIx) at rest and during the CPT. Comparisons were made using a repeated measure two-way analysis of

variance (ANOVA) with Tukey's correction. Post hoc significance P < 0.05 *compared with baseline, † compared with Treatment 1. Abbreviations: a, aortic; b, brachial; Tx, treatment.

Outcome	Condition	TOME		Placebo		*P*-Value		
		Baseline	4-Weeks	Baseline	4-Weeks	Time	Tx	Time x Tx
bSBP	Rest	106.17 ± 1.36	106.33 ± 1.36	108.42 ± 2.14	109.65 ± 2.33	0.420	0.233	0.518
	CPT – 1 min	131.30 ± 3.16	132.93 ± 3.61	130.52 ± 2.44	130.79 ± 2.41	0.667	0.678	0.655
	CPT – 2 min	117.85 ± 5.46	123.26 ± 2.77	121.31 ± 2.50	124.12 ± 2.39	0.140	0.627	0.614
	1 min post CPT	111.44 ± 1.95	111.37 ± 1.81	113.73 ± 2.44	115.12 ± 2.55	0.520	0.295	0.478
	2 min post CPT	109.85 ± 1.60	109.11 ± 1.70	110.08 ± 2.02	112.80 ± 2.70	0.329	0.453	0.099
	3 min post CPT	108.96 ± 1.76	107.44 ± 1.25	110.20 ± 2.18	111.0 ± 2.52	0.818	0.366	0.236
bDBP	Rest	65.98 ± 1.36	66.86 ± 1.32	65.46 ± 1.57	67.01 ± 1.33	0.051	0.944	0.550
	CPT – 1 min	90.59 ± 2.64	89.96 ± 3.10	87.24 ± 1.72	88.38 ± 1.53	0.894	0.414	0.578
	CPT – 2 min	74.8 ± 3.71	81.0 ± 2.08*	77.81 ± 1.67	80.48 ± 1.71	0.016	0.712	0.307
	1 min post CPT	69.67 ± 1.92	70.30 ± 1.88	70.04 ± 1.66	71.32 ± 1.51	0.357	0.792	0.799
	2 min post CPT	67.22 ± 1.70	68.15 ± 1.67	67.23 ± 1.71	68.84 ± 1.65	0.197	0.874	0.728
	3 min post CPT	66.96 ± 1.49	67.04 ± 1.57	65.96 ± 1.73	66.88 ± 1.67	0.549	0.745	0.604
aSBP	Rest	98.59 ± 1.42	99.85 ± 1.30	99.35 ± 1.68	102.2 ± 1.51*	0.028	0.404	0.374
	CPT – 2 min	114.67 ± 2.67	117.78 ± 2.36	113.62 ± 2.13	115.08 ± 2.01	0.076	0.523	0.461
aDPB	Rest	67.89 ± 1.18	69.07 ± 1.10	68.27 ± 1.57	70.24 ± 1.21*	0.045	0.622	0.589
	CPT – 2 min	78.19 ± 2.39	79.04 ± 1.77	77.46 ± 1.71	78.76 ± 1.52	0.269	0.839	0.822
aMAP	Rest	79.37 ± 1.34	80.67 ± 1.17	80.19 ± 1.69	82.28 ± 1.31	0.052	0.471	0.608
	CPT – 2 min	91.74 ± 2.54	93.78 ± 1.93	90.73 ± 1.80	92.08 ± 1.61	0.10	0.614	0.739
HR	Rest	60.93 ± 1.67	63.22 ± 1.75	61.42 ± 1.72	61.32 ± 2.17	0.419	0.780	0.457
	CPT – 1 min	70.33 ± 2.09	70.56 ± 2.27	68.64 ± 2.70	66.67 ± 3.07	0.557	0.462	0.431
	CPT – 2 min	59.93 ± 2.08	61.15 ± 1.57	59.42 ± 1.80	60.56 ± 2.30	0.273	0.869	0.938
	1 min post CPT	61.11 ± 2.03	61.56 ± 1.72	59.42 ± 1.65	59.52 ± 2.13	0.710	0.484	0.968
	2 min post CPT	59.85 ± 1.89	60.67 ± 1.78	59.46 ± 1.94	59.12 ± 1.95	0.559	0.796	0.778
	3 min post CPT	60.41 ± 2.04	61.52 ± 1.81	60.44 ± 1.65	59.36 ± 2.03	0.706	0.654	0.465
AIx (%)	Rest	15.37 ± 2.99	14.78 ± 3.05	10.65 ± 2.81	9.52 ± 2.28	0.565	0.195	0.889
	CPT – 2 min	22.81 ± 3.17	25.0 ± 3.04	14.46 ± 2.22†	16.36 ± 2.09†	0.137	0.021	0.996
AIx@75 (%)	Rest	8.56 ± 3.32	9.07 ± 3.13	4.08 ± 3.12	2.92 ± 2.72	0.928	0.213	0.677
	CPT – 2 min	15.48 ± 3.54	18.30 ± 3.13	7.12 ± 2.52	9.40 ± 2.44†	0.097	0.034	0.972

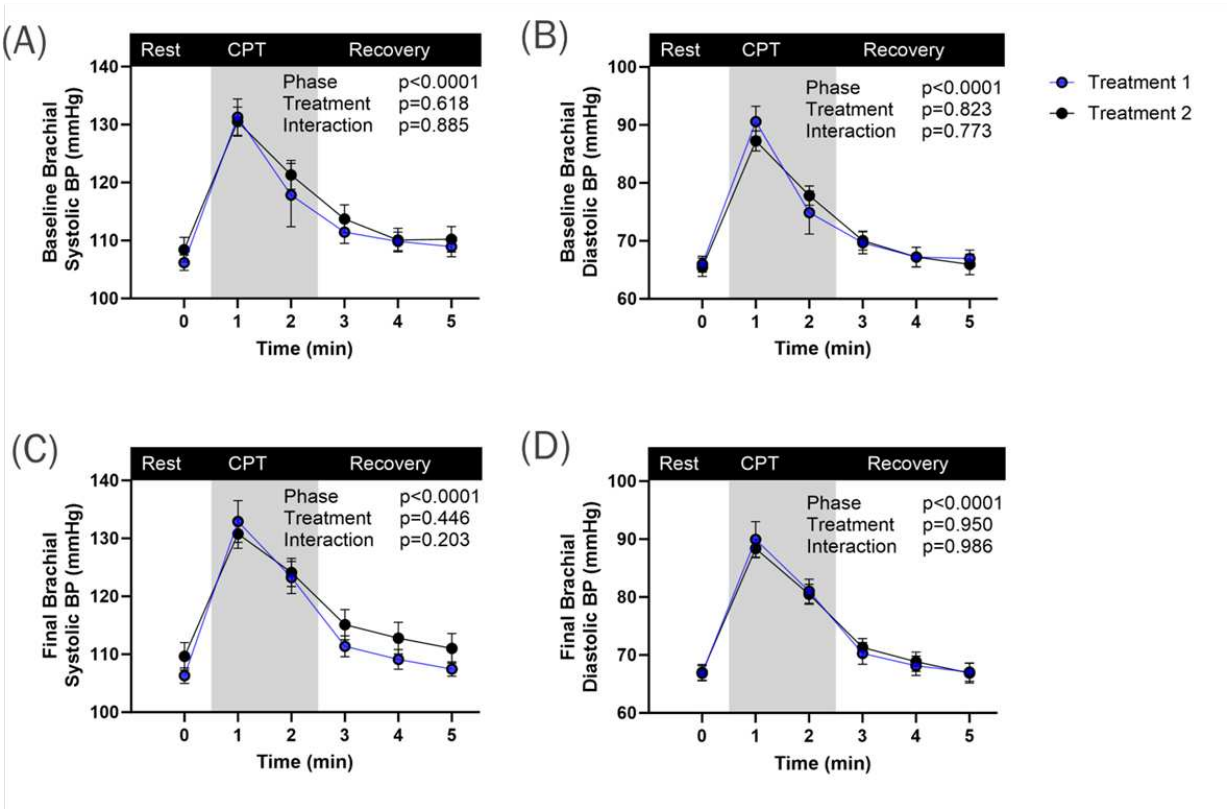

Figure 4: Brachial blood pressure responses to CPT. Baseline and final measure of baseline brachial systolic BP (A); baseline brachial diastolic BP (B); final brachial systolic BP (C); and final brachial diastolic BP (D).Data are based on mean scores at baseline and week 4 of supplementation and 95% confidence intervals. 2-way mixed model ANOVA were used for repeated measures and Tukey's post hoc analysis for multiple comparisons on scores changes.

DISCUSSION

This study assessed the impact of supplementation with *Tetradesmus obliquus* microalgae extract (TOME; 250 mg/day) for 4 weeks on subjective measures of gut health and subjective and physiological indicators related to stress and anxiety in healthy adults experiencing mild to moderate gastrointestinal complaints. To our knowledge, this is the first study to demonstrate the safety and tolerability, as well as impacts on gastrointestinal health, of TOME supplementation, as well as any microalgae supplementation in a human population. Our compliance data, metabolic panel, and adverse event reports suggest that 4 weeks of TOME supplementation was both safe and tolerable in a population with self-reported mild to moderate gastrointestinal distress. There was >90% compliance with both groups and none of the attrition was due to increases in symptoms or other study-related adverse events. The major side effect associated with TOME ingestion was increased flatulence, which is commonly associated with the initial adjustment period to gut-targeted supplements and frequently subsides with use[149]. Metabolic panels showed that liver enzymes remained within the references ranges during the four-week TOME supplementation period, and we even observed a significant decrease in AST between the baseline and Week 4. This may be due to compounds in the TOME such as the polyphenols and PUFAs, which can function as prebiotics and antioxidant compounds[150,151], which may reduce inflammation in the gut environment to improve digestion, ultimately reducing liver stress[147].

Stress, anxiety and other aspects of mental health can often trigger or exacerbate gastrointestinal symptoms[152,153]. Likewise, disruptions in the gut environment can impact the GBA to alter mood, behavior, and cardiovascular health. Therefore, we also explored the impact of TOME supplementation on aspects of mental health, particularly reductions in measures of anxiety and stress, and markers of sympathetic nervous system activation. Although this has not

been explored previously with TOME; astaxanthin, a potent antioxidant derived from the microalga *Haematococcus pluvialis*, has been shown to support emotional well-being and cognitive health[154]. In a study involving 28 healthy individuals aged 18-55 years, participants were supplemented with 12 mg/day of astaxanthin for 8 weeks. The results indicated significant improvements in mood, including a 57% reduction in depression scores and a 36% reduction in fatigue scores compared to the placebo. Additionally, there were notable improvements in overall mood (+11%) and vigor (+5%). Although this study used different assessment tools than the current study, the findings are similar. We showed that there were improvements in perceived stress in both the TOME and placebo groups, but only the TOME group also showed a significant decrease in negative affect scores from baseline to Week 2 and Week 4. Interestingly, there was also a significant decrease in positive affect in this group between baseline and week 4. However, the study population had relatively low levels of baseline stress and anxiety, so further study in a population specifically recruited for the purpose of establishing mental wellness benefits is warranted. To further support this, plasma levels of CgA were significantly reduced between baseline and week 4 in the TOME group and with TOME compared to placebo. CgA is associated with activation of the sympathetic nervous system and reductions in CgA are correlated with reduced stress responses[75]. CgA is also involved in the regulation of hormone secretion and vascular homeostasis and elevated levels have been associated with CVD[75,77,147].

Increased sympathetic nervous system activation is associated with increased risk for hypertension and has other adverse cardiovascular outcomes[48]. Factors such as chronic stress and anxiety activate the sympathetic nervous system and the HPA axis thus increasing the risk for developing hypertension through pathophysiological mechanisms such as inflammation[64]. Thus, targeting the gut-brain axis with dietary approaches could have benefits for cardiovascular health. The findings of the current study indicate no major effects of TOME on

brachial blood pressure at rest or in response to the CPT, nor for aortic parameters. Though there were differential effects in the responses of some parameters to the CPT, there were no differences between groups and the findings do not conclusively suggest a benefit or detriment to cardiovascular health. It is important to note that this was a relatively young, healthy population with low cardiovascular risk at baseline. Future studies are needed in populations with increased risk for CVD. Though previous studies have evaluated the influence of diet and nutrition on the GBA[146], as well as on blood pressure responses to sympathetic nervous system activation (e.g., though CPT), research evaluating the potential interactions of these systems in terms of diet and nutrition are lacking[44,147].

Some strengths of this study included recruitment of a population with pre-existing gastrointestinal complaints and the use of both subjective and objective assessments of gastrointestinal and mental health, as well as the randomized, double-blind, placebo-controlled study design. However, there were several limitations to interpretation of the data. This was a pilot study, so the sample size may not have been appropriately powered to detect changes in all outcomes. This is particularly true for some of the molecular markers of gut and immune function which were highly variable within treatment groups. A crossover design study would reduce some of the inter-individual variability and allow everyone to be compared back to their baseline and may be more able to detect subtle changes that were masked in this study. In addition, a better understanding of functional changes elicited by TOME in the gut microbiome is needed. Future studies should complement the 16s rRNA data with meta-transcriptome, meta-proteome or metabolome data that would provide greater insights into whether/how TOME interacts with the microbiome to elicit beneficial effects. Regrading the use of CPT, several strengths and limitations exist related to its use for measuring ANS reactivity and cardiovascular function. First, the strengths associated with CPT are its non-invasive characteristics and reliability for inducing sympathetic responses evidenced by significant increases in BP and HR.

Some limitations of the CPT may be the significant variability in individual responses to the CPT which has been consistent in previous studies. Variability in responses may limit reliability and require repeated measures for accuracy. Additionally, CPT causes pain and discomfort which may influence individual responses, particularly with cardiovascular responses. The association of CPT with pain may impact measurements following its initial use as anticipation of pain could influence ANS activity and hemodynamics. Furthermore, the use of the CPT alone may not be a reliable tool for predicting outcomes such as hypertension; however, it may prove to be a valuable complementary tool used alongside other measurements as it provides information regarding cardiovascular function and HRV before, during, and after stress stimulus. The use of the CPT should be further investigated for its use in predicting CVD[147].

In conclusion, after 4 weeks of microalgae supplementation, we found improved scores on PSS and negative affect questionnaires which may reflect improvements in mental stress but no significant treatment effects on blood pressure responses to acute stress. No major treatment effects were observed for blood pressure responses to CPT or salivary cortisol. Our results suggest microalgae supplementation may have implications to improved mental health; however, more evidence is needed to outline the mechanisms of microalgae on stress reduction and implications for cardiovascular health[147]. Further analyses are ongoing.

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