Investigating the Influence of Fresh and Aged Garlic Extracts on the Biosynthesis of Trimethylamine N-Oxide

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ABSTRACT

Introduction: Garlic-derived organosulfur compounds are associated with physiological benefits, including the reduction of cardiovascular disease (CVD) risk, possibly by reducing the risk marker trimethylamine-N-oxide (TMAO). TMAO production in humans is largely influenced by the metabolic activity of the intestinal bacteria on dietary precursors including L-carnitine. Dietary supplementation of bioactive garlic phytochemical allicin has recently been suggested to reduce the formation of TMAO precursor molecule trimethylamine (TMA) from L-carnitine through impact on the intestinal bacteria, thereby limiting the formation of TMAO by the host.

Purpose: The objective of this research was to evaluate and compare the efficacy of fresh and aged garlic extracts (rich in alliin and allicin, respectively) in the reduction of circulating TMAO levels produced from L-carnitine metabolism and identify shifts in the abundances of gastrointestinal bacterial genes that may contribute to reduction in circulating TMA levels, which may, in turn, influence the levels of circulating TMAO.

Methods: Five-week old female C57BL/6 mice (n = 12) were challenged with L-carnitine to assess the animal's capacity for TMAO production. Animals were gavaged daily with fresh or aged garlic extract dissolved in L-carnitine for 13 days, then challenged with L-carnitine post-treatment to evaluate changes in TMAO production. Whole blood samples were evaluated for TMAO content using UPLC-MS/MS and compared to non-extract consuming control groups. Post-mortem hepatic tissues were collected and analyzed for TMA-oxidizing flavin monooxygenase 3 (*Fmo3*) gene abundance and protein expression using quantitative real-time PCR (qPCR) and ELISA. Fecal samples collected prior to and following treatment were analyzed using qPCR to quantify shifts in the abundance of L-carnitine metabolizing genes *cntAB* and *grdH*.

Results: Postprandial and circulating TMAO levels were not significantly affected ($p < 0.05$) by inclusion of garlic extract in the diet. Dietary intervention with extracts significantly increased L-carnitine-derived proatherogenic CVD risk marker γ-butyrobetaine levels ~28% higher than the increased levels observed in the positive control group supplemented with L-carnitine only. Mice administered garlic extracts had significant increases of, γ-butyrobetaine, relative to negative control mice and mice supplemented with broad-spectrum antibiotics. Mice supplemented fresh garlic extract saw a 25-fold increase in circulating γ-butyrobetaine levels after intervention; mice

supplemented aged garlic extract saw a 23-fold increase in circulating γ-butyrobetaine levels after intervention. Furthermore, FMO3 protein expression levels in either extract treatment group were not significantly different ($p < 0.05$) from controls. Abundances of L-carnitine metabolizing genes in fecal samples of mice fed either garlic extract were not significantly higher than levels observed in positive or negative controls. Interestingly, treatment with broad-spectrum antibiotics significantly increased abundances of L-carnitine metabolizing genes *cntAB* and *grdH* when compared with controls. Abundances of hepatic *Fmo3* mRNA transcript in mice supplemented garlic extracts were not significantly different from the positive control group when data were normalized to mg of liver used. Mice supplemented aged garlic extracts significantly lowered *Fmo3* mRNA transcript levels relative to the negative control.

Significance: This research suggests that garlic extract supplementation in conjunction with excess L-carnitine consumption may not be an appropriate dietary intervention strategy to reduce CVD risk. As it stands, garlic extract supplementation may increase CVD risk by promoting the biosynthesis of proatherogenic γ-butyrobetaine. The impact of garlic extract mediated increases in γ-butyrobetaine should be further investigated in tandem with CVD outcomes to confirm the findings presented in this study.

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GENERAL AUDIENCE ABSTRACT

Garlic compounds that contain sulfur are associated with many health benefits, including the reduction of heart disease risk, possibly by lowering the amount of risk marker trimethylamine-N-oxide (TMAO) in the body. TMAO is produced when the gut bacteria break down L-carnitine into trimethylamine (TMA), which is then absorbed and converted to TMAO in the liver. Garlic supplementation has recently been suggested to reduce TMAO formation, which may, in turn, reduce heart disease risk. The objective of this research was to evaluate the potential of fresh and aged garlic extracts (which have different sulfur compounds in them) to reduce TMAO levels and identify changes in the gut bacteria that may contribute to this lowering effect. Mice were fed daily with either fresh or aged garlic extract for 13 days, then given L-carnitine to evaluate changes in TMAO levels in the blood. These levels were then compared to mice that did not consume any garlic extract. Liver samples were tested for their ability to turn TMA into TMAO. Fecal samples were tested to determine if there were any changes to gut bacteria caused by the garlic extracts. TMAO levels in the mice were not significantly affected by consuming garlic extracts. Consuming garlic extracts did, however, increase another risk marker of heart disease known as γ-butyrobetaine. Feeding mice garlic extracts did not affect the ability of mice to turn TMA into TMAO, nor did it affect the gut bacteria. This research suggests that garlic extracts may not be an appropriate strategy to reduce heart disease risk. As it stands, garlic extract supplementation may increase heart disease risk by promoting the γ-butyrobetaine formation. The means that garlic extracts increase γ-butyrobetaine levels should be further investigated

DEDICATION

To my mother, Michelle Hughes, and my sister Marissa Hughes, without your love and unwavering support, none of this work would have been possible, and for that I am eternally grateful.

To my late father, Michael Hughes Sr., despite our tumultuous relationship, you provided me with the motivation to strive for excellence and settle for nothing less. For that I am grateful.

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ATTRIBUTION

Andrew P. Neilson, PhD is the co-chair of this doctoral dissertation committee and has been crucial in the conceptualization and experimental design of this research. He has served in an advisory capacity and instructed me in the various analytical techniques conducted in this study.

Monica A. Ponder, PhD is the co-chair of this doctoral dissertation committee and has been instrumental in understanding the microbiological aspects of this research. She has served in an advisory capacity and provided endless hours of mentorship that have been invaluable in the completion of this research.

Laura E. Griffin, PhD is a former doctoral student of the Department of Food Science and Technology at Virginia Tech and assisted in the animal portion of the study, including gavage during the L-carnitine challenge, daily treatment gavage, whole blood collection, fecal collection, and animal husbandry.

Kathryn C. Racine, MS is a current doctoral student of the Department of Food, Bioprocessing and Nutrition Sciences at North Carolina State University and assisted in the animal portion of the study, including gavage during the L-carnitine challenge, daily treatment gavage, whole blood collection, fecal collection and animal husbandry.

Lauren Essenmacher is a former graduate student of the Department of Food Science and Technology at Virginia Tech and assisted in the animal portion of the study, including gavage during the L-carnitine challenge, daily treatment gavage, whole blood collection, fecal collection, and animal husbandry.

David Raines is a former lab technician of the Plants for Human Health Institute at North Carolina State University and assisted with animal husbandry and various laboratory procedures performed during this study.

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CHAPTER 1: INTRODUCTION

1.1 Introduction

Cardiovascular disease (CVD) or heart disease is a consistent and significant health and economic burden both in the United States and globally[1–4]. It manifests as several life-threatening phenotypes such as increased arterial atherosclerotic plaque buildup (blocked arteries), heart attack, heart failure, and stroke. CVD development can be reduced by employing different strategies such as consuming diets rich in fruits and vegetables, regular physical activity, and smoking avoidance[5]. Despite the efforts of organizations like the American Heart Association in disseminating knowledge on preventing CVD development, many are still not meeting the ideal metrics designed to decrease CVD risk[1]. Many clinical treatments aim to reduce the associated symptoms, instead of prevention altogether. It is imperative that preventative, cost-effective intervention strategies be employed to prevent initial CVD development, thereby reducing the associated disease and economic impact.

The gastrointestinal microbiome, the collection of native microorganisms (mostly bacteria) within the gastrointestinal tract, have been highlighted as major contributors to human health in recent years[6–14]. Increased circulating levels of proatherogenic intestinal bacterial metabolite trimethylamine-N-oxide (TMAO) positively correlate with increased risk of CVD, major adverse cardiovascular event (MACE), arterial thrombosis risk, and increased atherosclerotic plaque development in mouse and human models[15–24]. New evidence suggests that the production of TMAO precursor molecule trimethylamine (TMA) by the intestinal bacteria may represent a link between diet, intestinal bacteria and CVD risk[15]. Dietary substrates such as L-carnitine and choline are metabolized by specific bacterial members of the gastrointestinal microbiota, releasing TMA to be absorbed into circulation and transported to the liver, where it is oxidized by the host hepatic flavin monooxygenase 3 (FMO3) into trimethylamine-N-oxide (TMAO)[15,16].

Garlic has long been associated with significant human health benefits[25,26]. Bioactive garlic compounds are implicated in the reduction of inflammation, improvement of vasodilation, are described as free radical scavengers, and are associated with reduction in CVD risk and atherosclerosis development; these benefits extend to various garlic products, including powders and extracts[27–30]. Many of garlic's healthy attributes are believed to stem primarily from the activity of its predominant bioactive component, allicin[29,31–33]. Allicin specifically has been suggested to reduce the production of TMAO in mice supplemented with dietary L-carnitine, a TMAO substrate[34]. Elucidating the inhibitory capacity of dietary allicin on TMAO formation and its underlying mechanisms is necessary for the development of an allicin-based dietary intervention strategy capable of modulating CVD risk.

The overall objective of this research was to evaluate the efficacy of dietary garlic extracts in the reduction of TMAO production and clarify the metabolic pathway in which TMAO production is reduced. The central hypothesis was that supplementation with fresh or aged garlic extracts would significantly lower TMA production by reducing quantities of intestinal bacterial genes encoding for the release of TMA from dietary L-carnitine substrate (*cntAB and grdH*), thus reducing the quantity of TMA available to be

oxidized into TMAO by host hepatic FMO3. We assessed this by completing the following sub-objectives:

- Determine the impact of fresh (rich in alliin) and aged (rich in allicin) garlic extract supplementation on circulating fasting and postprandial TMAO levels during high carnitine challenge conditions and compare differences in the circulating TMAO levels of each group.
    - H_0: Garlic extract supplementation will not cause a significant difference in circulating levels of TMAO in mice relative to non-extract controls.
    - H_a: Mice supplemented either garlic extract will have lower circulating TMAO levels when compared with non-garlic extract supplemented controls.
- Identify the effects of fresh and aged extracts on the ability of host intestinal bacteria to catalyze production of TMA by quantifying differences in the abundances of genes implicated in the biosynthetic pathway concerned with the release of TMA (*cntAB* and *grdH*).
    - $H_{0:}$ Mice supplemented with garlic extracts will have abundances of *cntAB* and *grdH* that are statistically similar to control groups.
    - H_a: Mice supplemented with garlic extract will have significantly lower *cntAB* and *grdH* gene abundances when compared to non-extract supplemented controls.
- Evaluate the impact of fresh and aged garlic extracts on the abundance of hepatic *Fmo3* mRNA expression and hepatic FMO3 protein expression by quantifying abundances of mRNA transcript encoding *Fmo3* and FMO3 protein abundance relative to total hepatic protein.
    - H_0: Mice supplemented with fresh or aged garlic extracts will have mRNA and protein expression similar to that of control groups.
    - H_a: Mice supplemented either fresh or aged extracts will have lower mRNA and protein expression of *Fmo3* when compared to non-extract supplemented controls.

The rationale behind this research is that the use of garlic organosulfur components has been linked to decreased TMA production by the gastrointestinal bacteria in prior studies, which may ultimately contribute to a reduction in circulating TMAO levels when consuming a high L-carnitine diet, thereby serving as a potential dietary intervention strategy to reduce overall CVD risk.

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CHAPTER 2: REVIEW OF LITERATURE

2.1 Cardiovascular Disease

Cardiovascular disease (CVD) (or heart disease) is broadly defined as all diseases of the heart, vascular diseases of the brain, and diseases of blood vessels[1]. A number of heart-related conditions and atherosclerotic events fall under the umbrella term of CVD, including, but not limited to myocardial infarction (MI), stroke, hypertension and heart failure (HF) [1,2]. According to the U.S. Centers for Disease Control and Prevention (CDC), the most common type of CVD is coronary artery disease (CAD) which involves the deposition of atherosclerotic plaque onto the walls of the arteries responsible for circulating blood throughout the body; this causes a decrease in the blood flow and increases risk of MI[3]. In the U.S., CVD is the leading cause of death for both men and women, killing over 600,000 people each year[4]. It is also the leading cause of death for non-Hispanic Blacks and non-Hispanic Whites and one of the leading causes of death for Native Americans and Asian/Pacific Islanders[5]. Each year, over 700,000 Americans have a MI. The annual healthcare costs in the US of patients with CVD is $320 billion[6]. Due to the high economic burden and mortality rate, it is safe to state that CVD is one of the greatest health challenges we face in this modern age. It is imperative that strategies be implemented to alleviate the harsh burden of CVD, not only through pharmacological technologies, but also through lifestyle changes.

Though CVD refers to any disease of the heart or blood vessels, the term is commonly used to describe actual damage to the heart caused by atherosclerosis[7]. Atherosclerosis is the deposition of plaque into the arteries, leading to thickening of the arterial walls, reduced blood, oxygen and nutrient flow to the heart and the rest of the body[8]. The plaque itself is made of cholesterol, fatty substances, cellular waste products, calcium, and fibrin[8]. A common risk factor associated with atherosclerosis is elevated serum cholesterol levels. Damaged arterial endothelial tissues in atherosclerotic lesion-prone areas retain low-density lipoproteins (LDL), which are then oxidized through unknown methods[9,10]. As the LDL become oxidized, it triggers an inflammatory response causing arterial endothelial cells to express adhesion molecules which recruit monocytes (later differentiated into macrophages) to enter the artery[9,10]. The macrophages engulf the oxidized LDL and become foam cells that eventually accumulate and potentially die, releasing oxidized LDL and recruiting more macrophages, causing a feedback loop, and creating the fatty streaks that become plaque[10]. The increased number of foam cells promote the proliferation of smooth muscle cells over the plaque receptors, enlarging the plaque and decreasing arterial diameter [9,10]. Eventually the growth of plaque and stiffening of the endothelial wall results in diminished blood oxygen into the heart[10].

With damage to the endothelium being the initial trigger of plaque formation, researchers began searching for potential causes of this damage. Symptoms that have been tapped as precursors for plaque formation include autoimmune dysfunction, the metabolic syndrome (including hypertension), hypercholesterolemia, and dyslipidemia[11]. Body mass index and serum concentration of triglycerides have also been shown to correlate with atherosclerosis[12]. Older studies have suggested that an increase in saturated fat consumption could increase risk of atherosclerosis and/or artery thickness[13,14],

however newer research suggests that progression of atherosclerosis has less to do with the degree of fat saturation and more to do with the dietary source of the fat. One such study suggests that substituting 2% of daily energy (~50 kcal in this study) normally obtained from meat saturated fat with energy from dairy saturated fat (contained in foods such as whole milk and yogurt) results in a reduction of CVD risk[15]. Another study in postmenopausal women diagnosed with coronary heart disease found that coronary atherosclerotic progression was inversely related to saturated fat intake[16]. Some research suggests that reducing saturated fats below 9% could be detrimental and induce further atherogenic dyslipidemia and recommends reevaluating saturated fats and their effect on CVD risk[17]. A meta-analysis of epidemiologic studies estimating risk of CVD and its correlation with dietary saturated fat intake stated that there is insufficient evidence to suggest that saturated fat intake increases risk of CVD, citing publication bias as the reason for the common belief[18].

2.2 TMA and TMAO

When it was revealed that saturated fat intake was not correlated with an increased risk of CVD, researchers began searching for other potential causes. In more recent metabolomics studies aiming to identify small molecules serving as CVD markers, it was discovered that a molecule known as trimethylamine n-oxide (TMAO) and its precursors (choline, betaine, and phosphatidylcholine) were elevated in subjects with CVD[19]; these molecules were found to exist at higher levels in fasted subjects with CVD and its corresponding phenotypes (peripheral artery disease, coronary artery disease, and history of MI). Phosphatidylcholine (PC) and its metabolites were also found to upregulate atherosclerosis development in mice independent of cholesterol, triglyceride, lipoprotein, and glucose levels[19]. With TMAO and its precursors having been tapped as markers for CVD, research on these compounds has rapidly increased.

TMAO is a small organic compound (molecular mass ~75.1 Daltons) that was first identified as an osmolyte in the cells of marine eukaryotes, particularly elasmobranches such as sharks and rays[20]. In these organisms, TMAO levels are maintained at approximately 2:1 ratio with urea in the muscle cells [21]. TMAO (as well as other methylamines) counter the toxic effects of urea on enzyme function; TMAO molecules lower K_m when added to a solution containing enzymes and inhibitory urea when compared to K_m of non-TMAO controls[22]. These K_m values persist so long as the [urea]:[TMAO] 2:1 ratio is maintained[22]. The denaturation of enzymes by urea is a two-stage process with urea disturbing water-water H bonding and allowing for solvation of the hydrophobic portions of the protein, and urea molecules utilizing the opened protein structure to bind to polar components on the peptide backbone[23]. TMAO prevents that denaturation by disrupting urea-protein H bonds, enhancing water-water H bonding, and encouraging urea-water bonding, preventing solvation of the hydrophobic core and overall denaturation of the enzyme[24].

Studies in human and murine models demonstrated that TMAO is synthesized through a series of metabolic reactions (Figure 2.1). When trimethylamine (TMA)-containing compounds such as L-carnitine and phosphatidylcholine (lecithin) are ingested, they are metabolized by gastrointestinal microbiota containing genes that

encode for enzymes capable of cleaving TMA from the substrate[19,25–29]; betaine and creatinine are also precursors but were shown to be less efficient precursors (when measuring urinary concentrations of TMA and TMAO)[30,31]. This release of TMA from large dietary precursors occurs naturally and is dependent on the presence of intestinal microbiota[19,26,32]. After TMA is released, it is absorbed into circulation and transported to the liver where it is then oxidized by hepatic flavin-containing monooxygenase-3 (FMO3)[29,30]. The oxygenation of TMA by FMO3 is a physiological necessity, as it prevents the accumulation of the volatile, toxic, and foul-smelling compound in the tissues and instead allows it to be harmlessly excreted in the urine[35–38]

The body of research concerned with the relationship between TMAO, CVD and its associated phenotypes is quite substantial and is currently growing. Following its discovery as a CVD marker, increased levels of TMAO were shown to have a dose-dependent relationship with CVD presence and associated CVD phenotypes (e.g. peripheral artery disease, coronary artery disease, MI)[19]. Human patients who have experienced a major adverse cardiac event (MACE) have significantly higher baseline TMAO levels when compared with healthy controls, thus TMAO serves as a predictor of CVD and MACE risk[19,26,39–41]. HF patients have significantly elevated plasma TMAO levels when compared with healthy controls; these levels are also associated with disease severity as measured by the New York Heart Association[32,42]. Elevated plasma TMAO levels in HF patients also predict mortality risk[41,43]. In mice with elevated TMAO levels, atherosclerotic plaque area was significantly increased[19,26].

The mechanisms through which TMAO promotes CVD have been studied and significantly expanded upon but are still unclear. One proposed method of TMAO-mediated atherosclerosis (and thus CVD) is through the inhibition of reverse cholesterol transport (RCT)[26]. Excess cholesterol in circulation is thought to contribute to the development of atherosclerotic plaque buildup within the arteries[10]. RCT is the process in which excess cholesterol is moved out of arterial foam cells, enters circulation and is excreted in feces; this process is largely dependent on the ability of cholesterol receptors to successfully remove cholesterol from macrophages and foam cells within the arteries[44]. In mice with elevated circulating TMAO levels, reverse cholesterol transport is reduced by 35% when compared to mice on a control diet[26]. Excess circulating cholesterol is also used as a substrate in the biosynthesis of bile acids[45]. When the mechanisms behind inhibited RCT were further investigated, it was revealed that hepatic expression of bile acid synthesizing enzymes Cyp7a1 and Cyp27a1 was greatly reduced; hepatic bile acid transporters also showed significantly lower expression in groups with higher TMAO levels[26]. Furthermore, these mice saw significant decreases in total bile acid pool size and reduced expression of intestinal cholesterol transporters[26]. Collectively, these data suggest that TMAO promotes atherosclerotic CVD by inhibiting the clearance of cholesterol from circulation, causing an increase in the rate of atherosclerotic plaque development. TMAO can also contribute to CVD development by directly upregulating platelet activation, platelet hyperreactivity, aggregation of platelets in humans, and increasing thrombosis risk in mice[40]. Higher TMAO levels have also been shown to increase interstitial fibrosis and inflammation in the heart and increase endothelial dysfunction in aged rats by increasing vascular inflammation and oxidative stress[46,47]. TMAO has been shown to activate the cardiac autonomous nervous system, thus

inducing atrial fibrillation in canines[48]. In rat models, TMAO induces cardiac hypertrophy and fibrosis and pro-inflammatory cytokines[47,49,50].

The evidence linking TMAO to CVD has prompted researchers to further investigate the TMAO precursor molecules choline, betaine, L-carnitine, and γ-butyrobetaine. Understanding their presence in the human diet and metabolism into TMAO, will help clarify their role in TMAO-mediated CVD development.

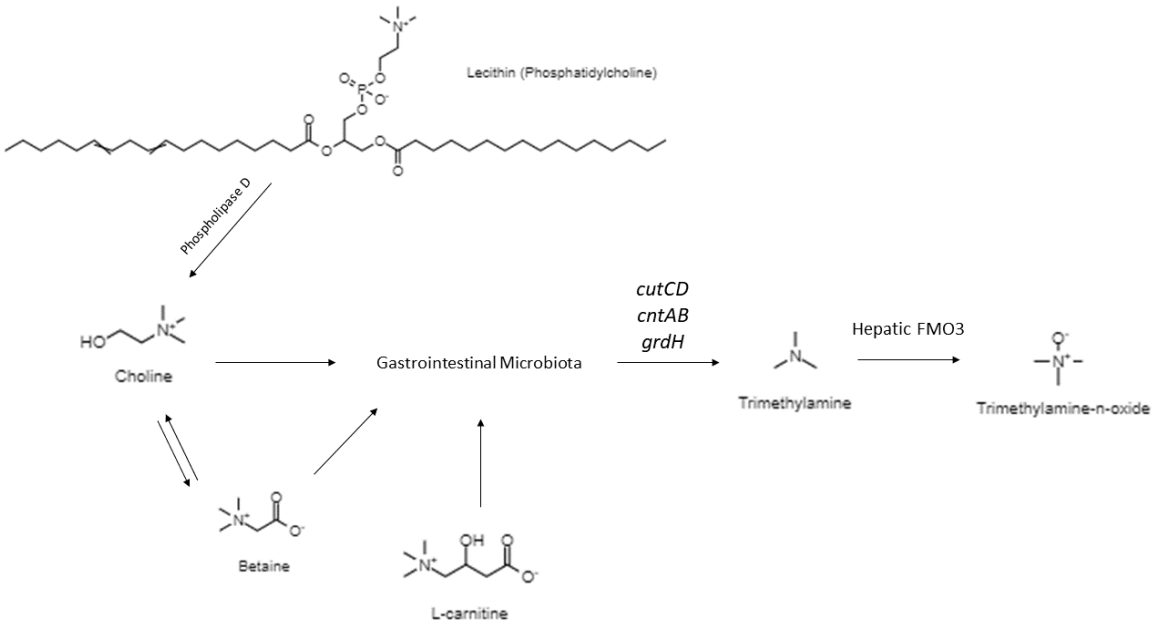

Figure 2.1: *Metabolic formation of TMAO from phosphatidylcholine, choline, L-carnitine, and betaine.*

2.3 Dietary Precursors to TMAO

TMAO is primarily formed from dietary nutrients containing a nitrogen atom with three methyl groups attached (TMA). This group is enzymatically cleaved by members of the intestinal microbiota, releasing the TMA molecule. TMA is then absorbed into circulation until it reaches the liver, where it is oxidized by host hepatic FMO3 into TMAO; other FMO isoforms (*Fmo1* and *Fmo2*) are able to oxidize TMA to TMAO at significantly lower levels[19,26,51]. The major TMA(O) precursor compounds are choline, betaine, and L-carnitine; γ-butyrobetaine, can also be metabolized to TMA but is not typically consumed in the diet and is instead formed as an intermediate during L-carnitine metabolism[52,53].

2.3.1 Choline and Betaine

Choline is a metabolite of phosphatidylcholine (or lecithin) and a major precursor for TMAO[25]. Choline is considered to be an essential vitamin for humans; humans can

readily synthesize it but must consume it within the diet to maintain adequate health[54]. Adequate food sources of choline include eggs, liver, peanuts, red meats, poultry, fish[55,56]. The adequate daily intake level of choline is 550 mg for males and 425 mg for females; female intake is recommended to be increased to 450 mg when pregnant, and 550 mg when breast-feeding[55,56]. Choline is a key structural component of acetylcholine, a vital neurotransmitter, and plays a role in many other critical human body functions such as growth and development[57,58]. Choline deficiency has been shown to damage myocytes, impair fetal brain development, dysregulate triglyceride release in the liver, and increase homocysteine levels in the body[59–62]. Choline is metabolized to TMA by intestinal microbes containing genes within the choline utilization gene cluster[63]. These genes encode for a glycyl radical TMA-lyase *cutC* and a glycyl radical activating protein *cutD* collectively referred to as known as *cutCD*[29,63]. *CutCD* genes are fairly ubiquitous in human and mammalian intestinal microbiota, detected in over 95% of bacteria found within samples of human intestinal isolates in multiple studies[64–66]. The genes are found in ~0.1-1% of human intestinal microbiota, with an average of 0.73% of microbiota containing the gene in mammalian intestines and 0.16% of human intestinal microbiota containing the gene[64,66,67]. The genes are most commonly observed in *Proteobacteria* and *Firmicutes* while noticeably absent from *Bacteroidetes*[64,65,67,68].

Betaine, also known as trimethylglycine or glycine betaine is a molecule similar in structure to choline and is commonly found in spinach, invertebrate seafood, wheat germ, and sugar beets[69,70]. Humans obtain much of their betaine through the metabolism of choline to betaine aldehyde by choline dehydrogenase, which is then oxidized to betaine by betaine aldehyde dehydrogenase[69]. In the body, betaine acts as a methyl group donor and can act as an osmolyte to protect cells from damage caused by osmotic pressure[71,72]. Betaine has been shown to decrease homocysteine levels in a manner similar to that of choline[73]. Though betaine is not an essential nutrient for humans, consumption of betaine at standard dietary intake levels (~1g/d via choline intake) is sufficient for lowering plasma homocysteine levels, a known CVD risk factor[69,74–77]. Betaine is metabolized into TMA in a few different metabolic pathways. Betaine can be directly catabolized by betaine reductase (*grdH*), or indirectly by conversion to dimethyl glycine and subsequent carboxylation to TMA[31,67,78,79]. The *grdH* gene is detected less frequently than *cutCD* in human and intestinal metagenome analyses, appearing in only 55% of mammalian fecal samples and ~80% in human samples[65,66,80]. The abundance of *grdH* in the gastrointestinal microbiome is typically lower than *cutCD*, with an average abundance of ~0.03%[66]. *GrdH* is most commonly observed in *Firmicutes*, with some detection in *Spirochaetes* as well[66,67].

Although these substrates has been proven to play many vital roles in the body, newer research shows that high intake of dietary choline (and its metabolites phosphatidylcholine and betaine) have been linked with markers associated with increased risk of CVD (such as TMAO or N-terminal pro-B-type natriuretic peptide (NT-proBNP), a common indicator of heart failure and coronary artery disease)[32,81]. Higher betaine intake is associated with higher curvilinear risk of coronary heart disease (CHD) incidence in males, and higher curvilinear risk of CHD and CVD incidence in females[81]. Patients living with chronic (>6 months) stable heart failure have elevated plasma levels of choline, betaine, and TMAO when compared with healthy controls[32]. Elevated plasma choline and betaine levels are associated with an increased risk of MACE[39]. Furthermore,

choline, betaine and TMAO are all independently associated with CVD development, but elevated plasma choline and betaine levels can only contribute to CVD risk when accompanied by elevated TMAO levels; in other words, the production of PC metabolite TMAO is the driving force behind CVD risk and development, rather than the substrates choline and betaine[39].

2.3.2 L-carnitine and γ-butyrobetaine

L-carnitine is a conditionally essential nutrient in humans, found in the tissues of most animals and at low concentrations in plants; it can be consumed in the diet or biosynthesized in mammals from lysine and methionine[82–85]. L-carnitine is found in substantial quantities in meats, poultry, fish, and dairy products[86]. L-carnitine plays a critical role in the metabolism of fatty acids; L-carnitine molecules esterified to coenzyme A (CoA) transport long chain fatty acids across the mitochondrial membrane into the mitochondrial matrix, where they are allowed access to enzymes involved in β-oxidation; these functions are essential for energy production in various body tissues[82,83,87,88]. Muscle carnitine deficiency and systemic carnitine deficiency are syndromes resulting from insufficient intake or biosynthesis of dietary L-carnitine[86,89]. Both syndromes are characterized with insufficient β-oxidation of long-chain fatty acids, resulting in excessive quantities of lipid deposits within muscle tissue[86,89,90]. While L-carnitine is vital for cellular processes, endogenous biosynthesis and a standard western diet provides sufficient L-carnitine to avoid deficiency[88]; differences in L-carnitine consumption caused by dietary restrictions (i.e. vegetarianism or veganism) are insignificant and unlikely to contribute to disease[91]. Metabolism of L-carnitine is initiated by a gene cluster *cntAB* coding for a two component Rieske-type oxygenase (*cntA*) and reductase (*cntB*)[28]. Of the TMA-forming genes discussed thus far, *cntAB* has the lowest distribution in mammalian intestinal microbiomes, with detection in ~42% of mammalian samples and 14-33% of human samples[64–66] . Like *cutCD*, the *cntAB* gene cluster is most observed in members of the *Proteobacteria* (particularly *Gamma-* and *Betaproteobacteria*) with some representation in *Firmicutes*[28,64]. Copies of *cntAB* tend to originate from *Escherichia coli* in the human gastrointestinal tract, while in other mammalian gastrointestinal tractss the sequences may originate from *Acinetobacter* and *Citrobacter*. *CntAB* also has the lowest reported abundances in the intestinal microbiome at 0.03-2.17%, with an average abundance of ~0.42%[64,66,67].

γ-butyrobetaine is an organic compound metabolically related to L-carnitine, formed during its biosynthesis and catabolism[52,85]. Biosynthesis begins when proteins containing lysine and methionine residues are hydrolyzed, releasing trimethyllysine (TML), which is then hydroxylated and cleaved to form γ-trimethylaminobutyraldehyde, then dehydrogenated to γ-butyrobetaine[27,85,92,93]. The γ-butyrobetaine is later hydroxylated to form L-carnitine. Alternatively, γ-butyrobetaine is formed when L-carnitine is dehydrogenated by the intestinal microbiota[52,53,94,95]. Given that γ-butyrobetaine is a metabolic intermediate in L-carnitine metabolism, there are no dietary recommendations for intake to date. γ-butyrobetaine is metabolized to TMA by a TMA-lyase encoded for by the *yeaWX* complex[52]. This enzyme demonstrates significant substrate promiscuity, capable of producing TMA from L-carnitine, choline, and

betaine[52]. *YeaWX* also shares 74% of its sequence identity with *cntAB* and metabolizes L-carnitine and γ-butyrobetaine at similar rates, prompting some researchers to consider the genes to be part of the same genetic cluster cluster[52,64]. The distribution and abundance of *yeaWX* independent of *cntAB* in the intestinal microbiome has yet been reported.

Increased dietary L-carnitine consumption has been shown to have a dose-dependent relationship with increased risk of CAD, peripheral artery disease (PAD), and overall CVD[26]. Dietary L-carnitine has also been shown to promote intestinal microbiota-dependent atherosclerosis in mice by increasing atherosclerotic plaque burden; when antibiotics are used to suppress intestinal microbiota, L-carnitine-dependent atherosclerosis is markedly reduced and TMA formation is inhibited[26]. In mice supplemented γ-butyrobetaine, atherosclerotic plaque area increases by 50% when compared with mice with suppressed intestinal microbiota[52]. High serum levels of γ-butyrobetaine are also associated with carotid atherosclerosis and cardiovascular death[53]. Like choline and betaine, CVD/MACE risk and atherosclerotic plaque development are not directly due to L-carnitine or γ-butyrobetaine consumption, but their conversion to TMA and TMAO[26,52].

A consistent theme in the studies described above is that TMAO precursors phosphatidylcholine, choline, betaine, L-carnitine, and γ-butyrobetaine contribute to CVD pathogenesis, but only in organisms with an intact intestinal microbiome. It is only through the metabolic activity of these microbes that TMAO substrates can be catabolized into TMAO and contribute to the development of CVD. As a result, the role of the intestinal microbiome must be explored.

2.4 The Gastrointestinal Microbiome, CVD, and TMAO

The gastrointestinal microbiome is a large, extremely complex ecosystem comprised of the collective genomes of trillions of microbiota including (but not limited to) bacteria, viruses, fungi, and archaea living within the human gastrointestinal tract[96–98]. These organisms outnumber human cells at approximately 10 bacterial cells for every human cell; the combined genome pool of the gastrointestinal microbiome is vastly larger than the human genome at an estimated ≥100 times the human genome size [96–99]. The human gastrointestinal tract has long been thought to be sterile until birth, with microbes from the birth canal serving as the initial inoculum[100,101]. Conversely, there is emerging evidence that suggest that prior to birth, the amniotic fluid and placenta may provide the initial inoculum for the human intestinal microbiome[102,103]. The initial intestinal composition of neonates is also influenced by vaginal exposure; infants born through Caesarean section often have intestinal microbiota similar to the skin microbiome of the mother while vaginally-delivered infants have a intestinal microbiota composition similar to the mother's vaginal microbiota[104]. As infants age and begin feeding, their diets begin to influence the structure of their intestinal during the first week of life, with their intestines dominated by *Bifidobacteria* (if breast-fed)[105]. Within the next few weeks of life up until the end of the infant's first year, *Bacterioides* spp., *Enterobacteria*, *Enterococci*, *Clostridia*, and anaerobic gram-positive bacteria (*Peptostreptococcus* and *Peptococcus)* have all been found[106]. This development and shift in species and diversity of the intestinal microbiota is known as microbial succession and is driven by

environmental selection for species[107]. As humans grow and age, the native taxa found in the intestinal microbiome change in response to host genetics and a number of environmental stimuli such as host dietary habits[108].

Despite the large amount of diversity and dynamic nature of the intestinal microbiota, some researchers suggest the existence of a healthy "core microbiota", a group of microbiota associated with "good health"[109]. The most abundant taxa in healthy adult humans are members of *Firmicutes* and *Bacteroidetes*; within *Firmicutes*, ~ 95% of the population are *Clostridium*[110–112]. Certain phyla are less abundant in the healthy gastrointestinal tract, including *Proteobacteria*, *Actinobacteria*, *Fusobacteria*, and *Verrucomicrobia*[110,113]. Recently, some researchers have rejected the idea of the presence of core taxa indicating "healthy" status, postulating that the presence of common bacterial genes that contribute to human health or "core metagenome" is more appropriate, though this too proves to be difficult to define[114–116].

The intestinal microbiome plays a critical role in the maintenance of adequate human health, often described as the "forgotten organ" due to its importance[117]. The microbiome in healthy adults provides several functions crucial for life, including protection from pathogens, nutrient metabolism, membrane transport, immune regulation and development, vitamin synthesis, fermentation of non-digestible substrates, modulation of insulin sensitivity, cholesterol metabolism and cellular signaling[99,112,116–121]. The intestinal microbiota can carry these functions and their associated phenotypes when transferred to a new host[122].

A number of various diseases and conditions have been determined to be at least partially influenced by the intestinal microbiome, including irritable bowel syndrome (IBS)[123], obesity[108–110], and CVD[19,124,125]. The differences in community membership in the intestinal microbiome of CVD-afflicted individuals versus healthy individuals suggests that the present taxa of the intestinal microbiome contribute to or result from disease onset. Analysis of stool samples representative of intestinal microbiota in patients with atherosclerotic cardiovascular disease (ACVD) compared against healthy controls are significantly different, with diseased subjects having reduced abundances of *Bacteroides* and *Prevotella* and enriched abundances of *Streptococcus, Escherichia, Klebsiella, and Enterobacter*[125]. Stroke/Transient Ischemic Attack (TIA) patient intestinal microbiota have been characterized by increased *Proteobacteria* abundance and reduced *Bacteroides*, *Prevotella*, and *Faecalibacterium* abundances[126]. The intestinal microbiota of patients with acute decompensated or stable heart failure (HF), show significantly decreased abundances of *Coriobacteriaceae*, *Erysipelotrichaceae*, and *Ruminococcaceae* when compared to healthy controls[127]. Patients diagnosed with severe stroke (as classified by the National Institutes of Health Stroke Scale) possess greater *Proteobacteria* levels than patients that experience a mild stroke (who had greater *Bacteroides* abundances)[126]. HF patients possess a greater α-diversity (phylogenetic diversity within one sample) and β-diversity (phylogenetic diversity from host to host) when compared with healthy controls[127]. This provides evidence for a shift in the intestinal microbial community in patients who have experienced HF which may be indicative of dysbiosis, a disturbance in the microbial community in which dominant species are depleted and lesser found species increase in number. These changes are further reflected in the patient intestinal metagenome, which is enriched with genes associated with the biosynthesis of peptidoglycan, a suggested inflammatory agent that primes the innate immune system

and can cause an influx of neutrophils, contributors to the development of atherosclerotic plaque[128].

A mechanistic link between cardiovascular disease and the intestinal microbiome has been discovered, but research regarding the community representation of the intestinal microbiome in subjects with CVD is novel and ongoing. While it is known that TMA is synthesized by the intestinal microbiota, the diversity of species that contribute to TMA production is currently under investigation. By studying the microbiota in individuals with elevated TMAO levels and CVD phenotypes, researchers illustrate the relationship between representative intestinal bacterial taxa, TMA production, host TMAO production, and CVD. In mice with elevated thrombosis potential and TMAO levels (after chronic choline consumption), distinct community differences were observed when compared with controls, including increased abundances of *Coriobacteriaceae*, *Erysipelotrichaceae*, and *Allobaculum*[40]. Elevated TMAO levels induced by chronic L-carnitine supplementation in mice are associated with an altered intestinal microbiome; the intestines of these mice are enriched with microbiota associated with elevated plasma levels of TMA (*Bacteroidetes* and *Deferribacteres*) and TMAO (*Tenericutes* and *Verrucomicrobia*[26]. As mentioned earlier, recent studies have indicated that an intact intestinal microbiome must be present to facilitate the catabolism of substrate to TMAO, resulting in CVD development[19,26,46,94,124,124,129]. Atherosclerosis and atherosclerotic plaques are known to be associated with increased TMAO production, but any associations between atherosclerosis and the composition of the intestinal microbiome have only recently been explored[19,26].

The identification of taxa involved in TMAO production and thrombosis development further show the role of the intestinal microbiota in CVD. The intestinal microbiota has been shown to play major roles in maintaining homeostasis from birth to death, specifically being involved in the development of CVD through metabolism of TMAO substrates. Through several *in vitro* and *in vivo* studies, meal challenges, germ-free comparisons, and metagenomic analysis, it is clear that CVD and its associated phenotypes are associated with deviations or shifts in the present taxa of the intestinal microbial community. The research on these imbalances (or dysbiosis) in the "normal" intestinal microbes represented in healthy humans is up for debate on whether they are induced by or result from CVD development. Regardless, it is imperative that intervention strategies be developed and implemented to correct the disrupted intestinal microbiome and potentially alleviate the burden of CVD.

2.5 Potential Strategies to Inhibit TMAO Formation

The intestinal microbiota and their production of TMA have a very profound influence on the production of TMAO pathogenesis of CVD. So much so, that many researchers are targeting intestinal microbes when developing strategies and techniques to prevent the synthesis of TMA, thus inhibiting TMAO formation (and by association, lower risk of CVD). By modulating the intestinal microbiome with these intervention strategies, researchers hope to reduce the abundances of microbiota associated with diseases (e.g. CVD) and improve patient health while minimizing the need for invasive surgeries and excessive use of pharmaceutical drugs.

2.5.1 Broad-Spectrum Antibiotics

When dealing with health conditions and diseases induced or exacerbated by bacteria, one of the first solutions proposed is the use of broad-spectrum antibiotics to significantly reduce the presence of microbes that may or may not be the root of the issue. Broad-spectrum antibiotics are a category of antibiotics that are effective at eliminating both Gram-positive and Gram-negative organisms, unlike single antibiotics like streptomycin or penicillin which target organisms based on Gram stain[130]. The ability of these antibiotics to target organisms over a wide range of taxa is particularly useful when applied to the intestinal microbiome, which can contain several different taxa. Several different studies have shown that when intestinal microbiota abundances are reduced significantly *in vivo* by broad-spectrum antibiotics, circulating plasma TMAO levels also drop. In three studies, the use of broad-spectrum antibiotics has been associated with reduced TMAO to nearly undetectable levels[19,26,124]. Furthermore, the suppression of intestinal microbes via broad-spectrum antibiotics is associated with reduced atherosclerosis development in atherosclerosis-prone mice[26,124,129].

Considering these results, one may wonder why broad-spectrum antibiotics are not used to reduce intestinal microbiota-mediated TMA production and alleviate risk of CVD. The simple fact of the matter is that maintaining a good quality of life without a fully intact intestinal microbiome is implausible. As mentioned earlier, the intestinal microbiome is responsible for a myriad of health benefits such as digestion and immune system function; removing the majority of the population of intestinal microbiota could cause severe metabolic consequences[131]. Another reason to rule out antibiotics as a TMAO intervention strategy is the fact that antibiotics are already used excessively in modern society. One study found that antimicrobial agents were over-prescribed, excessively administered to patients, or used unnecessarily[132]. Excessive antibiotic treatments are not only costly, but they can also pose a risk to health in the form of antibiotic resistant bacterial growth and the elimination of commensal bacteria. Repeated use of antimicrobial agents permanently alters the intestinal microbiome community, preventing a full recovery of all members of the population[133]. The use of antimicrobial therapies also promotes the growth of antibiotic resistant intestinal microbiota such as *Helicobacter pylori* (a native intestinal microbe implicated in nosocomial infections) that can persist for years[134,135] and prevent future antibiotic treatments from being effective. Use of antibiotics has contributed to the emergence of new highly virulent, antibiotic resistant strains of *Clostridium difficile* in clinical environments[136,137]. The continued overuse of antibiotics also creates a societal cost of antibiotic resistance, causing an increase in antibiotic pricing, and hospitalization as antibiotic resistance grows[138]. As of 2018, the cost of treating antibiotic-resistant infection exceeds $2 billion annually[139].

Using broad-spectrum antibiotics as a therapeutic strategy to reduce circulating TMAO levels is simply not practical. Between drug costs, damage to the native intestinal microbiota, and promotion of antibiotic resistant bacterial growth, employing antibiotics as a tool to alleviate CVD burden would be, at best, counterproductive, and at worst detrimental.

2.5.2 Exercise

Regular exercise has been shown time and time again to provide a multitude of benefits to human health, including obesity reduction[140], reduced risk of hypertension[141,142], improved insulin sensitivity[143], and improved cardiovascular function[144]. Because regular exercise can influence health aspects that are also linked to the intestinal microbiome and the human intestinal microbiome is so susceptible to external stimuli, it is reasonable to suggest that a regular exercise regimen can alter the community structure of the intestinal microbiome and potentially reduce circulating TMAO levels.

Some studies have already conducted preliminary analysis on the effects of exercise on the intestinal microbiome community. In mice fed a low fat or high fat diet, regular exercise is associated within an alteration in the ratio of *Bacteroidetes* to *Firmicutes* by increasing the percentage of *Bacteroidetes* present and decreasing the percentage of *Firmicutes* present[145]. In obese and hypertensive rat models from three different genotypes, controlled moderate exercise was associated with increased fecal microbial diversity and altered abundance levels of bacterial taxa associated with elevated CVD risk, e.g. *Proteobacteria*[146]. Similar findings have also been demonstrated in humans. The intestinal microbiome α-diversity was significantly higher in exercising individuals when compared to non-exercise controls with major flux in abundance of *Firmicutes*[147]. In a human study seeking to explore the effects of various fitness levels on diversity of intestinal microbiota, researchers found that α-diversity increased significantly with VO_2peak (considered the "gold standard" of cardiorespiratory fitness evaluation) with several taxa observed to correlate with positively with fitness level[148].

There is substantial evidence that exercise can modulate intestinal microbiota composition in murine models (and appears to be evident in humans as well, though more research is needed), using exercise as a means of alleviating TMAO production does have a few drawbacks. Many adults have trouble adhering to an intense exercise regimen largely due to self-efficacy issues among other demographic factors such as access to an environment such as a gym[149–151]. In addition, it appears that forcing (or prescribing) mandatory exercise can cause the composition of the intestinal microbiota to vary more than if the exercise were voluntary[152]. While exercise can change the diversity of the intestinal and improve overall human health, its effects are not fully understood and should be researched further before it is implicated as a treatment for TMAO production.

2.5.3 Prebiotics and Probiotics

The human large intestine is the area of the digestive tract that is the most heavily colonized by bacteria, inhabited by massive quantities of varying species both known and unknown[153,154]. Intestinal microbiota are able to ferment nondigested materials (e.g. resistant starch, non-starch polysaccharides) to produce a variety of compounds that are absorbed by the host and exert a variety of effects on the body[155]. Seeing this interaction between microbes and host prompted researchers to explore manipulation of intestinal microbes. By controlling the growth of beneficial microbes and restricting the growth of

pathogens, researchers hope to improve overall human health. One such method of manipulating intestinal microbiota growth is the utilization of prebiotic and probiotic dietary supplements. Prebiotics were originally defined as "nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus, improving host health"[156]. This definition was further expanded upon with the establishment of the following criteria to be considered a prebiotic: resistance to gastric acidity and hydrolysis by mammalian enzymes and gastrointestinal absorption, fermentable by the intestinal microbiota, and selectively stimulating of the growth/activity of intestinal bacteria associated with health and well-being[157]. Prebiotics act as a sort of dietary substrate, catalyzing the growth of beneficial organisms within the host. Probiotics differ, as they consist of actual living organisms. They are "live microbial feed supplements which beneficially affect the host animal by improving its intestinal microbial balance"[158].

Prebiotics and probiotics are effective at increasing the abundance of "healthy" bacterial genera such as *Bifidobacteria* in the intestinal microbiome, which are commonly depleted in individuals afflicted with metabolic disorders. Higher levels of these groups are inversely associated with markers of poor metabolic health (e.g. glucose tolerance, insulin sensitivity) are improved[159]. Fecal transplants of intestinal microbiota from obese mice into gnotobiotic mice have been associated with mice that rapidly gain body fat and become obese[121,122,160]. Specifically, the transplanted microbiota are associated with upregulated hepatic synthesis of triglycerides and triglyceride storage in adipocytes through enzymatic means[122]. It is hypothesized that susceptibility to atherosclerosis can also be transferred to a new host in a similar fashion[161].

Although the literature available on the pre/probiotic influence on TMAO synthesis is scarce, there is some evidence that probiotic supplementation can lower TMA and TMAO levels in the liver of mice[162]. Unfortunately, these results do not extend to circulating TMAO levels. In adult male humans fed a hypercaloric, high fat diet, a multi-strain dietary probiotic did not reduce or reduce fasting plasma TMAO levels diet despite successful alteration of the intestinal microbiome[163]. Furthermore, the probiotic treatment did not significantly alter the plasma levels of l-carnitine, betaine, or choline[163]. The information provided by this study suggests that probiotics cannot effectively alter the intestinal microbiome to prevent biosynthesis of TMAO, with researchers believing the probiotic of choice could not outcompete TMA-producing bacteria. A similar study conducted to observe the effect of probiotic supplementation on TMAO levels in adults with metabolic syndrome showed that while probiotics did lower TMAO levels, they were not significantly different from non-probiotic groups controls[164]. In patients at risk for Type 2 diabetes mellitus, a phenotype associated with elevated TMAO levels and increased CVD risk, researchers found that there were no significant changes in fasting or postprandial plasma TMAO levels when supplemented prebiotic inulin [165,166]. The use of inulin also did not alter fasting levels of choline, betaine, l-carnitine, or γ-butyrobataine[165]. These results suggest that dietary prebiotic and probiotic intervention cannot disrupt the ability of the intestinal microbiota to produce TMA.

Although prebiotics and probiotics are known to change the composition of the human intestinal, their administration does not seem to alter or halt the production of TMAO. Prebiotic and probiotic supplementation are associated with modest increases in the abundances of various genera of fecal microbiota when compared to a placebo control,

with some studies showing slight shifts in phyla, but there is limited evidence available to suggest that these shifts are large enough to alter the overall structure of the microbial community or the metagenomic function[163,167–172]. These changes in genera seem unlikely to significantly affect TMA-lyase presence in the intestinal metagenome, as these enzymes are typically distributed widely across phyla rather than exclusively at the genera level[66]. Until more evidence is available, we cannot definitively conclude that supplementation with prebiotics or probiotics will effectively reduce the abundance of TMA-producing bacteria and subsequently, reduce the formation of TMAO.

2.5.4 3,3-Dimethyl-1-Butanol

The reduction of TMAO production via the intestinal microbiome has remained elusive; traditional means of altering the intestinal microbial community such as probiotic administration have proven unsuccessful. A novel non-lethal treatment does, however, show promise in modulating the intestinal microbiome and inhibiting TMA formation, thus reducing TMAO production: the use of 3,3-dimethyl-1-butanol (DMB)[173]. DMB is a structural analog of choline and is commonly found in fermented liquids, oils, and distilled products (e.g. balsamic vinegar, red wines and cold pressed olive/grapeseed oils at concentrations up to 25 mM, though these data are not reported within the study[173]. Its structure is remarkably similar to choline, differing only at one atom (carbon in DMB, nitrogen in choline), making the two molecules structural analogs. It is for this reason that DMB was identified by researchers as a potential inhibitor of the microbial choline TMA lyase (*cutCD*)[173]. *In vivo* studies showed that DMB delivered at 1% v/v in drinking water successfully inhibits TMA production from choline by ~30% while still allowing choline uptake by microbial cells cloned with *cutCD* genes[173]. When these genes were cloned into other microbes, DMB did successfully inhibit TMA lyase activity, although with varying degrees of effectiveness dependent on microbial species[173]. When the inhibition potential of DMB was tested on bacteria cloned with L-carnitine metabolizing enzyme complex *cntAB*, there was no reported inhibition of TMA production when supplemented with L-carnitine[173]. DMB inhibition of L-carnitine metabolizing enzyme complex *yeaWX* showed inhibition of TMA release from L-carnitine, and crotonobetaine, but not γ-butyrobetaine *in vitro*[173]. DMB displayed broad inhibitory activity on TMA formation in mice and human fecal cultures for a variety of TMA-containing substrates (albeit, at varying degrees), including choline, phosphatidylcholine, glycerophosphocholine (GPC) (in mice only), and phosphocholine (a phosphatidylcholine intermediate)[173]. In mice fed a choline/carnitine-supplemented diet and provided DMB *ad libitum*, plasma TMAO levels were significantly lower than their non-DMB controls when DMB was administered orally[173]. DMB is suggested to be a non-lethal inhibitor of TMA metabolism; when DMB was added to a nutrient-rich broth containing human intestinal commensals capable of metabolizing choline to TMA, no significant differences in cell growth were observed[173]. DMB supplementation is also correlated with reductions in abundance of microbial taxa positively associated with TMA/TMAO levels[173]. DMB is successful in reducing TMAO levels in mice with overload-induced HF and reduces cardiac remodeling induced by HF[174]. DMB is associated with reduced TMAO production,

cardiac dysfunction, pro-inflammatory cytokines, and cardiac interstitial fibrosis in western diet-induced obese mice aged rats[46,47].

The ability of DMB to successfully inhibit TMA production from a wide range of substrates and microbes suggest that structural analogs of choline can serve as a preventative tool for patients afflicted with CVD. While the use of DMB is promising, the association between DMB supplementation and change in intestinal microbial composition is evidence of the potential development of bacterial resistance[173]. Furthermore, the toxic effects of DMB *in vivo* have not been reported in the last two decades, suggesting that any toxic capacity is widely underreported. Studies involving DMB inhibition of TMA-lyases have not been performed in humans, and thus have the potential to cause undesired side effects. The results of these studies have paved the way for further investigation of DMB and other non-lethal inhibitors as a means of preventing TMAO synthesis by the intestinal microbiome but must be further investigated.

2.5.5 Other Strategies to Control TMAO

Several other strategies have been suggested and tested in regard to the reduction of circulating TMAO levels and subsequent CVD risk. One proposed method is the reduced dietary intake of TMA-containing substrates (e.g. choline and L-carnitine). While non-substrate consuming controls are shown to have lower circulating TMAO levels when compared with L-carnitine/choline-fed groups, choline and L-carnitine deficiency have severe health consequences in the human body, some of which include increased CVD risk[19,26,61,62,92,175]. Genetic knockdown of *Fmo3* in mice is associated with significant reduction (~87%) in enzymatic oxidation of TMA, 50% reduction in circulating TMAO levels, and reduction in atherosclerotic lesion size[36]. This approach is problematic, as the knockdown or inhibition of *Fmo3* is associated with an increase in circulating TMA levels, causing trimethylaminuria, a condition characterized by an undesirable foul-smelling fishy odor emanating from the host[37,176]. Acute co-administration of meldonium (an aza-analog of γ-butyrobetaine) and L-carnitine reduces *in vitro* production of TMA in enteric *Klebsiella pneumoniae* without affecting L-carnitine uptake of L-carnitine, but does not produce the same effect when provided choline substrate, limiting its applications[177]. Treatment with meldonium and L-carnitine also increases postprandial levels of proatherogenic γ-butyrobetaine[177].

Phytochemicals are particularly attractive in their potential for reduced production of TMAO. Phytochemicals are readily available in many common fruits and vegetables and are generally considered to be non-toxic, though the toxicological aspects of higher phytochemical doses must be investigated further[178,179]. Although phytochemicals are poorly absorbed by the human gastrointestinal tract, they are rapidly metabolized by the intestinal microbiota; they are also associated with significant shifts in intestinal microbiota community[180–182]. A diet rich in phytochemicals has also been suggested to significantly alter the collective functions of the intestinal metagenome[183]. Increased consumption of fruits and vegetables rich in phytochemicals is inversely associated with CVD risk and risk of MACE[184–187]. Recently, supplementation of 0.4% grape polyphenolic compound resveratrol in a 1.0% choline diet has been suggested to reduce

TMAO formation in mice[188]. Collectively, this information suggests that phytochemicals could potentially reduce TMAO-mediated CVD risk via interaction with the intestinal microbiome.

2.6 Garlic and Allicin

Garlic (*Allium sativum*) is of particular interest when considering phytochemical means of reducing TMAO levels, largely due to its historical associations with human health, its rich phytochemical content, and multiple associations with positive health outcomes[189–191]. Garlic and its metabolites have long been associated with their many health benefits, including antioxidant potential, antimicrobial properties, anticarcinogenic properties, and cardioprotective properties for generations[191–197]. Many researchers attribute these abilities to the bioactive compounds associated with garlic bulb, particularly allicin[194,198–200]. Allicin is an oxygenated thiosulfinate compound that is not found in intact garlic bulbs, but is formed through enzymatic hydrolysis of its endogenous precursor alliin upon damage to the plant tissue such as crushing or cutting of the cloves[196,201]. This reaction releases two molecules of allyl sulfenic acid that spontaneously condense, forming allicin[196,202]. Allicin's cardioprotective and antimicrobial effects are of particular interest.

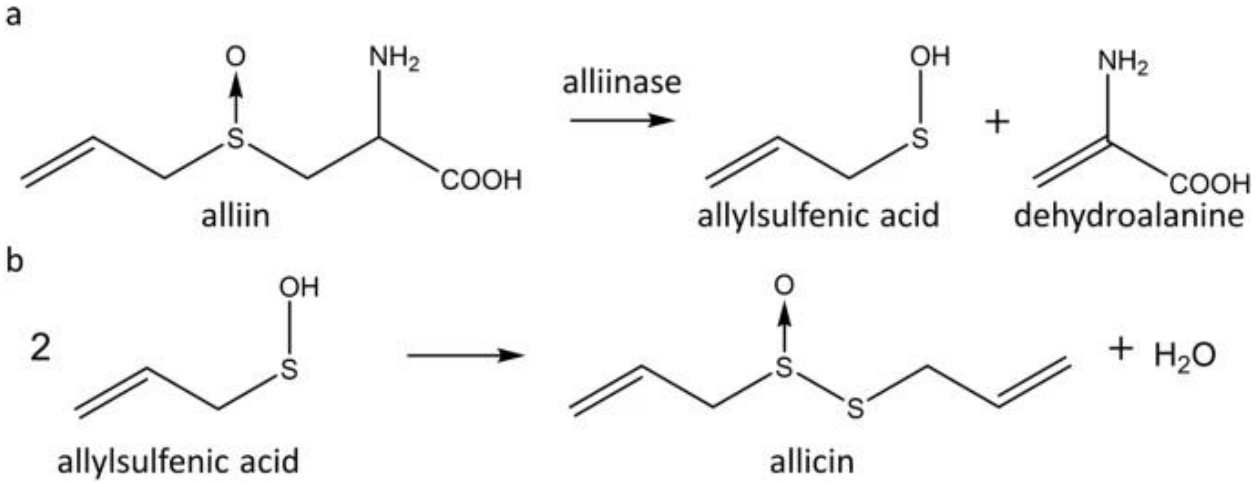

Figure 2.2: *Biosynthesis of allicin from metabolic precursor alliin*[203]

The antimicrobial effects of allicin and garlic have been heavily researched and tested. Garlic products (i.e. oils, powders, extracts) rich in allicin have been shown to exert a significant antimicrobial effect on a wide variety of bacteria[204,205]. The antimicrobial activity of garlic extract is reported to directly correlate with the allicin content[206]. Crushed garlic containing allicin has been shown to exert antibacterial effects on both Gram negative and Gram positive organisms, multi-drug resistant microbes, enteric bacteria (e.g. *Escherichia*, *Klebsiella*), and pathogens (e.g. *Salmonella* and *Staphylococcus*[198,207–210]. Garlic compounds (including allicin) can also inhibit the production of some bacterial enterotoxins[198]. Allicin and garlic extracts have also been

shown to modulate the intestinal microbiome, and administration is associated with reduced abundances of cecal microbiota and is suggested to inhibit quorum sensing gene expression in the intestinal microbiome[211]. Garlic supplementation also improves α-diversity, reduces disruptions in the community structure of the intestinal, and increases bacterial richness and diversity in the cecal contents of pigs (when supplemented alongside α-ketoglutarate)[212,213].

The cardioprotective effects of garlic and allicin have been extensively reported on. Garlic and garlic oils in the diet are associated with reduced triacylglycerol, total and LDL cholesterol reduction[214–216]. Allicin can suppress cholesterol biosynthesis via inhibition of squalene-monooxygenase and acetyl-CoA synthetase enzymes[217,218]. Allicin can also inhibit platelet aggregation with greater efficacy than aspirin and lower blood pressure through release of H_2S, a compound signaling relaxation of smooth muscle cells surrounding blood vessels[219,220]. Mice supplemented with L-carnitine in conjunction with allicin in the diet saw significantly lower circulating plasma TMAO levels when compared with mice who consumed L-carnitine only[129]. Furthermore, mice with allicin supplemented into their diet had circulating plasma TMAO levels significantly lower than those maintained on L-carnitine diets; mice fed allicin and L-carnitine had plasma TMAO levels that were comparable to control mice fed chow diet with no significant differences between the two groups[129]. Though this study shows promising results for the use of dietary allicin in the modulation of the intestinal microbiome to reduce formation of TMA (and thus lower TMAO production), there are some limitations associated with its experimental design. For one, the study supplemented synthetic allicin as opposed to allicin obtained from raw garlic. The use of synthetic allicin may not exhibit the same bioavailability during host metabolism as allicin extracted from raw garlic, thus limiting the practical applications of the data presented. Furthermore, the use of synthetic allicin does not allow for the observation of potential matrix effects that could alter the impact of the compound on the intestinal microbiota-mediated TMA production. The study suggests that allicin supplementation can alter the intestinal microbiome in mice, but human microbiota can vastly differ from mice and may not have similar responses to the compound[129].

2.7 Major Unknowns Regarding TMAO-mediated CVD risk

Despite the increase in available evidence regarding TMAO and its relationship with CVD, there are still several gaps and inconsistencies in our understanding of this molecule, its metabolism in the body, and the mechanisms in which it associates with increased CVD risk. It has been shown that increased TMAO levels are associated with shifts in abundances of microbial taxa, however, it is unknown whether increased circulating TMAO causes shifts in the intestinal microbial community or if the shifts in the community result in increased circulating TMAO levels[26,40,221]. Further investigation must be done in regard to the associations between increased TMAO levels and intestinal taxa if the intestinal microbiome is to be targeted in mediation of TMAO induced CVD risk. Many studies show that TMAO levels are positively associated with an increased risk of CVD, but some studies suggest the inverse. One study showed that plasma levels of TMAO were not significantly associated with coronary heart disease presence,

incidence, or MACE in patients undergoing coronary angiography[222]. Another study in stroke/transient ischemic attack (TIA) patients showed that subjects had significantly lower circulating TMAO levels when compared to the asymptomatic atherosclerotic control group; furthermore, asymptomatic atherosclerotic subjects did not have elevated levels of TMAO when compared with non-atherosclerotic controls [126]. Higher dietary intake of fish is positively associated with increased plasma TMAO levels, but is inversely associated with risk of CVD[223–226]. These inconsistencies in the relationship between TMAO and CVD risk suggest that other unknown factors may influence the mechanisms by which TMAO increases risk of CVD and require further research. Plant-derived phytochemicals have been promoted as a potential means to regulate TMAO production, in part due to their native ability to modulate intestinal microbiome composition and promote positive health outcomes[227]. Dietary supplementation of grape polyphenol resveratrol or garlic phytochemical allicin are associated with reduced TMAO formation *in vivo*, with researchers suggesting modulation of the intestinal microbiome as the primary mechanism[129,188]. It is unclear, however, how this is achieved; whether these phytochemicals promote the growth of organisms lacking the ability to produce TMA or if these genes are somehow inhibited by the phytochemicals is unknown. The lack of information on the mechanisms behind phytochemical-induced shifts in the intestinal microbiome community suggests the need for further research.

2.8 Conclusion

It is clear from this review of literature that the quaternary-ammonium compound TMAO is highly and significantly associated with the development of atherosclerosis and CVD risk. TMAO production from phosphatidylcholine, choline, L-carnitine, betaine, and γ-butyrobetaine is mediated by the presence of an intact intestinal microbiome containing genes coding for the enzymes that catalyze the cleavage of TMA. Inhibition of these genes and/or reduction of intestinal taxa that contain these genes are proven methods of reducing TMAO production and overall CVD risk. Garlic and garlic compounds, specifically allicin, bestow numerous benefits to its host including CVD amelioration, antimicrobial pressure, and probiotic modulation of the intestinal microbiome. Because allicin is a naturally-occurring compound, is readily obtained from garlic, is associated with shifts in intestinal microbial taxa, and can act in CVD pathways, it has the potential to serve as a therapeutic method for reducing circulating TMAO levels and consequently, alleviating CVD risk. The study described in the following chapter was designed to examine the efficacy of garlic extracts on the reduction of TMAO and CVD.

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CHAPTER 3: RESEARCH

3.1 Introduction

Cardiovascular disease (CVD) is a class of diseases affecting the heart or blood vessels that still burdens millions globally despite advances in modern medicine[1,2]. In the U.S. alone, billions of dollars are spent annually treating adverse cardiovascular events such as myocardial infarction, stroke, and heart failure[3]. CVD is the leading cause of death in the U.S., killing over 600,000 Americans annually regardless of sex or race/ethnicity[4–6]. Risk factors of CVD are extremely prevalent in Americans, with nearly half the population possessing at least one (e.g. elevated blood pressure, elevated LDL cholesterol levels, and habitual smoking)[7]. Although many treatments and prevention strategies are employed, CVD continues to persist among individuals within developed countries, prompting researchers to investigate dietary treatments and preventative changes.

In recent years, the gastrointestinal microbiome has been implicated in the development of CVD. The gastrointestinal microbiome is a complex microbial ecosystem within the human gastrointestinal tract capable of greatly influencing host health via metabolite biosynthesis and community representation [7–12]. The intestinal microbiota within the host microbiome are so impactful, that transplantation of a conventional host's microbiota into a gnotobiotic host can induce aspects of the donor's phenotype such as obesity.[13–15]. Within organisms afflicted with CVD, an altered intestinal microbiome community structure is often observed (i.e., dysbiosis); humans with heart failure or heart attack history often have reduced microbial diversity and distinct differences in community abundances when compared to the microbiome of their healthy counterparts[16–18]. The link between the intestinal microbiome and CVD development remains poorly defined, warranting further investigation into the root of the problem.

Organic metabolite trimethylamine-N-oxide (TMAO) is strongly associated with CVD risk[19]. TMAO is ultimately formed by the action of host hepatic enzymes on circulating TMAO precursor molecule trimethylamine (TMA); it is hypothesized that a reduction in circulating TMA levels will result in lower circulating TMAO levels. When substrates such as phosphatidylcholine (PC), choline, betaine, and L-carnitine are consumed in the diet, they are catabolized by the intestinal microbiota in different metabolic pathways dependent on the substrate. PC and choline are metabolized by intestinal bacteria possessing one or more functional copies of the choline utilization gene cluster (*cutCD*); these genes code for a glycyl radical enzyme that cleaves the C-N bond and releases TMA[20–22]. TMA is, in turn, absorbed into circulation and oxidized by the host's hepatic flavin monooxygenase 3 (FMO3) into TMAO[19,23]. In the intestinal compartment, betaine is reduced by bacterial betaine reductase (*grdH*), releasing TMA to be absorbed and oxidized into TMAO by FMO3[24,25]. L-carnitine is metabolized in the gastrointestinal tract by bacteria through activities encoded by the *cntAB* gene cluster, which codes for a Rieske-type oxygenase/reductase releasing TMA to be absorbed and converted into TMAO[26]. Interestingly, bacterial metabolism of L-carnitine produces γ-butyrobetaine (γBB), a metabolic intermediate in the L-carnitine to TMA pathway, in significant quantities. γBB is subsequently converted to TMA by enzymes coded for by the *yeaWX* gene cluster[27]. The *yeaWX* gene cluster shows substrate promiscuity, also catalyzing production of TMA directly from L-carnitine, choline, and betaine[27]. Like

TMAO, γBB is highly associated with the development of atherosclerosis; in mice fed diets supplemented with γBB, atherosclerotic plaque area was increased when compared to controls. In mice consuming a high L-carnitine diet for at least 10 weeks, plasma concentrations of γBB exceed that of TMA/TMAO by approximately 2-fold[27].

In both humans and animal models, TMAO levels are positively associated with adverse clinical outcomes, disease severity of heart failure patients, incidence of major adverse cardiovascular event, increased atherosclerotic plaque burden, and incidence of thrombotic event [15,23,28–30]. The correlations between severe cardiac conditions and increased TMAO levels supports the idea that CVD risk is significantly tied to TMAO, prompting researchers to investigate intervention strategies.

Garlic has long been associated with antioxidant activity, antimicrobial properties and other human health benefits are thought to be provided by its naturally occurring sulfur-containing compounds such as allicin[31–35]. Allicin is not found in intact garlic bulbs but formed through an enzymatic reaction post-harvest(Figure 3.1)[35]. In fresh bulbs, amino acids cysteine or serine are given an allyl group (from an unidentified source) forming S-allyl cysteine, which is later oxidized into the allicin precursor molecule alliin; glutathione has also been suggested to form S-allyl cysteine and later be converted into alliin [35].

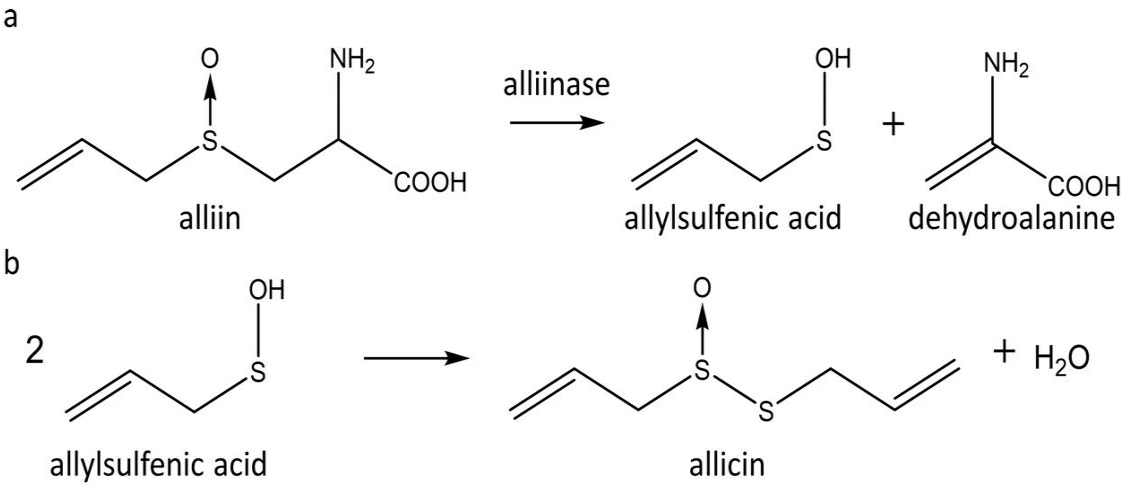

Figure 3.1**:** *Biosynthesis of allicin from metabolic precursor alliin*[36].

When the garlic tissue is damaged by crushing garlic cloves, the enzyme alliinase is released, hydrolyzing alliin and releasing molecules of allyl sulfenic acid, which are rapidly condensed into the sulfur-containing garlic component allicin[34,35]. Allicin has been suggested to inhibit TMAO biosynthesis by exerting an antimicrobial effect on the intestinal tract microbiome[37]. When mice were supplemented L-carnitine in drinking water in conjunction with allicin supplementation via daily gastric gavage for six weeks, the animal's postprandial TMAO levels (following an L-carnitine challenge) were severely reduced in comparison to animals that were supplemented L-carnitine alone[37]. Allicin also acts as a bacteriostatic agent *in vitro* when dosed at 32-64 μg/mL, inhibiting the growth of some Gram-positive and Gram-negative human enteric organisms like *Escherichia coli* (*E. coli* [32,38–40]. Garlic and allicin supplementation have also shown to associate with increases in intestinal tract microbial α-diversity, defined in study as

increases in Chao1 value, observed species, phylogenetic diversity whole tree index, and Shannon value[41,42]. In the context of CVD, allicin is associated with the reduction of some of the burden of CVD-related conditions such as platelet aggregation and hypertension[43,44]. Because of the apparent links between bioactive garlic compounds and the community structure of the intestinal tract microbiome, we believe allicin may hold some potential as a dietary intervention strategy for reducing TMAO production.

The main objective of this study was to evaluate the efficacy of dietary garlic extracts to reduce TMAO production in a mouse model. We hypothesized that supplementation with aged and/or fresh garlic extracts would reduce TMAO production, and that this would be accompanied by reduced copies of bacterial genes encoding for the release of TMA from dietary L-carnitine substrate (*cntAB* and *grdH)*. We assessed this by determining the impact of extract supplementation on circulating fasting and postprandial TMAO levels during high carnitine challenge conditions. We compared differences in the inhibitory effects of aged extracts rich in allicin against fresh extracts rich in its precursor alliin on TMAO production rates, and identified effects on pathways of TMAO synthesis by quantifying changes in genes implicated in the biosynthetic pathway: *cntAB, yeaWX, grdH,* and *Fmo3*.

3.2 Materials and Methods

3.2.1 Preparation of Garlic Extract

Garlic extracts were prepared from whole bulbs purchased from a local supermarket. Bulbs were peeled and separated into cloves. Garlic cloves (~500 g) were weighed out and separated into two groups designated as two of the experimental treatments tested: alliin-rich (fresh) garlic and allicin-rich (aged) extracts. The sample to serve as the allicin-rich treatment was blended using 500 mL of distilled water. The extract was then incubated at room temperature for 30 min to allow for enzymatic conversion of alliin to allicin (confirmed through UPLC-MS/MS characterization of garlic extracts in section 3.2.2). After incubation, the extract was blended with 500 mL of absolute ethanol to extract the bioactive compound, then centrifuged for 5 min at 2046 x g at 20°C to separate solid matter from the desired extract. The supernatant was collected, the pellet was resuspended in ethanol, then blended again to achieve a second extraction.

The sample designated as the alliin-rich treatment was prepared by blending the extract with 500 mL of absolute ethanol (Fisher Scientific, Hampton, NH) instead of water to inhibit alliinase, preventing the enzymatic conversion of alliin to allicin then immediately centrifuged at the same parameters as previously described. The supernatants of both extracts were collected and pooled (separate from each other) while the pellet was discarded. The garlic extracts were separately funneled into round bottom flasks and placed on a Hei-VAP Value rotary evaporator from Heidolph Instruments© (Schwabach, Germany) at 90 rpm and ~65°C until the ethanol was removed. The flask containing the sample was then moved to a FisherbrandTM CPX1800 ultrasonic bath (Fisher Scientific, Hampton, NH) for 20 min to remove any extract adhered to the wall of

the flask. Alliin and allicin content of extracts was evaluated using UPLC-MS/MS (see 2.2 below). The final extract solutions were collected and stored at −80° VirTis GPFD 24DX48 freeze dryer C. When extracts were completely frozen, they were placed in a from SP Scientific (St. Louis, MO). Extracts were freeze-dried for 72 h to remove all water and reduce the extract to a powder-like solid.

3.2.2 Characterization and Quantification of Garlic Standards

Alliin and allicin-rich garlic extracts were quantified using UPLC-MS/MS. Freeze-dried extracts were dissolved in ethanol at 1 mg/mL, then used to prepare 5, 10, and 20-fold dilutions in triplicate. The mass spectrometer (MS) was tuned to maximize response signal for allicin and alliin. A corresponding standard curve was built using authentic standards to quantify these compounds in the extracts. Sulfur-containing standards were combined and quantified at their respective concentrations: alliin (1 mg/mL), allicin (0.2 mg/mL), S-allyl-L-cysteine (1 mg/mL), allyl sulfide (1 mg/mL) (Sigma-Aldrich, St. Louis, MO) E/Z ajoene (0.5 mg/mL), and L-glutamyl-S-allyl-cysteine (1 mg/mL) (Santa Cruz Biotechnology Inc., Dallas, TX). The standards were combined at 200 µL each into a 1X stock solution serving as a mixed standard. The standard solution was then diluted 15-fold.

Samples were analyzed by UPLC-MS/MS; each tested sample was prepared from either freeze-dried extract. Samples were separated on a Waters BEH C18 column (2.1 x 50 mm 1.7µm particle size). Column and sample temperatures were set at 30 and 10°C, respectively. The mobile phases were 0.1% Formic Acid in water (phase A) and 0.1% Formic Acid in acetonitrile (phase B). The flow rate was set at 0.4 mL/min, and isocratic elution was achieved for mobile phase A (100% A) over 2 min. Following separation, analytes and internal standards were quantified using electrospray ionization (ESI) in (+)-mode. Source and capillary temperatures were 150 and 400°C, respectively. Capillary voltage and cone voltage were 0.6 kV and 25 V respectively, and desolvation and cone gas (both N_2) flow rates were 800 and 20 L/h, respectively. Syringe draw rate was set 22 µL/min and needle placement from the bottom of the vial was 2.0 mm.

Compounds were quantified using optimized multi-reaction monitoring (MRM) functions. MRMs were optimized to achieve 12 points/peak, and the detection span was ±0.2 Da. Quantification was performed using ratio of the target analyte and respective IS peak areas, based on authentic external standard curves prepared using a wide range of target analyte concentrations and the same IS concentrations used to prepare the garlic extract samples. Parent and daughter ions, cone voltages, and collision energies for all compounds are listed in Table 3.1. Results of the garlic extract quantification are presented in Figure 3.2.

3.2.3 Mice and Treatment Protocol

IACUC approval was obtained from the David H. Murdock Research Institute Institutional Animal Care and Use Committee (IACUC) (Protocol #19-011). The animals

selected for this study were Female C57BL/6J mice (*Mus musculus*) (The Jackson Laboratory, Bar Harbor, ME) and were 5 weeks old upon arrival to the lab. Female animals were selected over males for this study because they have been reported to have significantly higher hepatic *Fmo3* mRNA levels and FMO3 protein expression when compared to males of the same species[45–47]. *Fmo3* expression is crucial for TMAO production and thus, is critical for evaluating treatment efficacy. Animals were group housed at 4 per cage and conditioned at 23 ± 2°C with humidity at 50-70% on a 12 hr light/dark cycle for 1 week. The animals were fed the AIN-93G Growing Rodent Diet from Research Diets (New Brunswick, NJ). This diet does not contain L-carnitine but does contain choline. The complete formulation of the diet is listed in Table 3.2. The cages were then randomized into 5 groups depending on assigned treatment: water only (NegC), carnitine only (PosC), allicin-rich (aged) extract + carnitine (AC), alliin-rich (fresh) extract + carnitine (AL), antibiotics + carnitine (ABX). Each treatment group contained 3 cages, each housing four animals. Prior to experimental treatments, mice were weighed using a digital scale.

An acute carnitine challenge was administered to each mouse within every treatment group before and after the chronic experimental treatments to assess baseline and post-intervention circulating TMAO concentration. Chronic treatments were given to the animal by gavage daily for the duration of the study (see 3.2.4 below). For the 13 days between the carnitine challenges, all mice were on the AIN-93G Growing Rodent Diet and allowed access to food and water ad libitum throughout the study except where explicitly stated. Following the final carnitine challenge, the mice were euthanized. Animal livers, cecum, and colon contents were collected post-mortem. Fecal samples were collected during the initial and final carnitine challenges, specifically when the animal was gavaged and at each timepoint the animal was sampled for blood.

Table 3.1: *MRM settings for UPLC-MS/MS characterization of garlic* ***extracts***

Compound	Parent $[M+H]^+$ (m/z)	Daughter (m/z)	Cone voltage (V)	Collision energy (eV)
Allyl Sulfide	88.94	60.89	28	6
E/Z Ajoene	235.05	144.93	18	6
(+)-L-Alliin	178.01	87.92	22	8
Allicin	162.99	72.94	16	10
L-Gamma-Glutamyl-(S)-Allyl-Cysteine	291.19	72.93	24	26
S-Allyl-L-Cysteine	162.02	144.96	20	8

Table 3.2: *AIN-93G Growing Rodent Diet Formulation*

Class Description	Ingredient	Mass (g)
Protein	Casein, Lactic, 30 Mesh	200
Protein	L-cysteine	3
Carbohydrate	Corn Starch	397.49
Carbohydrate	Lodex 10	132
Carbohydrate	Fine Granulated Sucrose	100
Fiber	Solka Floc, FCC200	50
Fat	Soybean Oil, USP	70
Mineral	S10022G Blend by ResearchDiets.com	35
Vitamin	V10037 Blend by ResearchDiets.com	10
Vitamin	Choline Bitartrate	2.5
Antioxidant	Tert-Butylhydroquinone (tBHQ)	0.01
	Total:	**1000g**

3.2.4 Treatment Dose Determination

Previous studies[37] used 1.25-1.3% L-carnitine dissolved in drinking water to elevate L-carnitine levels in C57BL/6 mouse models; we aimed to deliver 1.25% L-carnitine (0.0125g L-carnitine/mL) dissolved in water to mice daily. A 25g adult mouse will consume roughly 4 mL of water daily[48,49]. This would thus equal a dose of 0.05 g of L-carnitine per day. To deliver the same L-carnitine dose, we prepared a solution of L-carnitine at 0.25 g/mL (to provide 0.05 g from a 0.2 mL gavage). The stock carnitine solution was stored at −80°C. This L-carnitine solution served as the solvent for the garlic extracts and antibiotics.

The garlic extracts were administered to provide similar doses of garlic via acute gavage as would be obtained from adding 2% extract in the animal's diet. Based on mouse reported daily food consumption of ~0.10 kg/kg bodyweight/d and an approximate bodyweight of 25 g, each animal was expected to consume 2.5 g of food daily, resulting in a desired garlic extract dose of 0.05 g. Extracts were prepared at 0.25 g extract/mL to provide 0.05 g from a 0.2 mL gavage. Antibiotics were also administered via gavage in order to match doses achieved when the following concentrations are administered via drinking water: 0.5 g/L vancomycin, 1 g/L neomycin sulfate, 1 g/L metronidazole and 1 g/L ampicillin. Assuming the animals consume 4 mL water/d, the masses of antibiotics administered would be 0.002 g Vancomycin, 0.004 g of neomycin sulfate, 0.004 g metronidazole, and 0.004 g of ampicillin. To deliver this in a 0.2 mL gavage, antibiotics were prepared at the following concentrations: 0.01 g/mL of vancomycin, 0.02 g/mL neomycin sulfate, 0.02 g/mL metronidazole, and 0.02 g/mL of ampicillin. Each aliquot of experimental solution was stored at −80°C until use.

3.2.5 L-Carnitine Challenge and Sample Collection

All animals were subjected to an acute L-carnitine challenge before and after receiving their respective experimental treatments for 13 days to assess post-prandial levels of TMAO. All animals received the same treatment during the acute carnitine challenges regardless of assigned intervention treatment group. Each animal was weighed to determine the volume of the initial L-carnitine dose and labeled using the ear punch method. After a 12 h fast, the 0.25 g/mL L-carnitine solution was gavaged to each animal (0.2 mL/25 g body weight). The average animal mass was 17.9 g, resulting in an average gavage volume was 0.14 mL and carnitine dose of ~35 mg per animal. The animals received 2000 mg of L-carnitine/kg of bodyweight followed by a whole blood collection from the saphenous vein using 11 x 40 mm MiniCollect® Serum and Plasma Tubes (Greiner Bio-One, Kremsmünster, Austria). This blood sample established baseline TMAO concentration. Subsequent blood samples were collected at 2, 4, 6, and 8 h to determine circulating postprandial TMAO levels over time. These timepoints were selected based on a previous study evaluating dietary allicin supplementation on TMAO production[37]. Fecal samples were also collected for subsequent quantitative PCR to identify shifts in the expression of genes releasing TMA from L-carnitine in the mouse gastrointestinal tract. All blood and fecal samples were stored at −80°C until analysis. The animals were then returned to their cages and monitored. For the next 13 days, the animals were gavaged their assigned experimental treatment once daily. The animals were then subjected to a final L-carnitine challenge (as described above), with euthanasia and post-mortem liver and cecum content, immediately following. Euthanasia was performed in a closed vessel (home cage) with CO_2 from a compressed gas cylinder. CO_2 flow was controlled to displace 10-30% of the cage volume/min. Animals were exposed to CO_2 until > 1 minute following apparent clinical death (cessation of respiration). Following euthanasia, the abdominal cavity was opened ventrally from the rectum to the neck, and bilateral pneumothorax was performed to ensure death. All collected blood samples were centrifuged at 500 x g for 2 min (with capillary tube still in vial) to ensure all blood has been transferred to tube. Animal livers were cut into segments, stored in RNAlater™ (Invitrogen), PBS (Sigma-Aldrich) or RIPA buffer (Sigma-Aldrich) and stored at −80°C. Samples were stored at −80°C until analysis.

3.2.6 TMAO analysis (UPLC-MS/MS)

All samples to be analyzed were prepared with an internal standard (IS) to account for variability that may occur during sample preparation and analysis. A 100X IS stock solution in water was prepared containing TMAO-d_9 (Cambridge Isotope Laboratories), at 25 μM, choline chloride-d_9 (Sigma-Aldrich) at 25 μM, carnitine-d_9 at 120 μM (Cambridge Isotope Laboratories), and betaine-d_9 (Sigma-Aldrich) at 25 μM. 1 mL of stock IS was diluted with acetonitrile (ACN) to prepare the 1X IS/extraction solution and vortexed to mix. A standard curve of target analytes was created using TMAO, betaine, choline chloride, L-carnitine HCL, and γ-butyrobetaine HCl external standards from 500 μM to $1.05x10^{-4}$ μM. The 15 standard dilutions were analyzed by the TMAO UPLC method as if they were blood samples (described below).

100 μL of 1X IS:ACN solution was pipetted into each well of a clear, plastic 96-well plate using a multi-channel pipettor. 10 μL of 5% zinc sulfate in water was pipetted

into each well. 10 μL of each whole blood sample were individually pipetted into each well. The 96-well plates were sealed, placed into a sonicator (FisherbrandTM CPX1800 ultrasonic bath), and sonicated for 5 min. Plates were then removed and shaken for 10 min. Using a multi-channel pipette, the contents of each well were transferred from the clear plate to an AcroPrep™ Advance 96-Well Filter Plate for Solvent Filtration with wwPTFE filter membrane, 0.25 cm^2 well bottom area and 1 μL centrifuge hold-up volume (Pall Laboratory, NY, USA). Filter plates were covered with a clear lid and taped down to a 96-well Sample Collection Plate with Round Wells (Waters, MA, USA). The filter and collection plates were centrifuged at 2000 x g for 10 minutes. The collected analyte was then quantified using a UPLC-MS/MS method validated from previous studies[50].

Samples from all analyses were separated on a Waters BEH HILIC column (2.1 x 100 mm; 1.7 μm particle size) with a BEH HILIC VanGuard pre-column (2.1 x 5 mm; 1.7 μm). Column and sample temperatures were at 30 and 10°C, respectively. The mobile phases were 15 mM ammonium formate, pH 3.5 (phase A) and ACN (phase B). The flow rate was set at 0.65 mL/min, and isocratic elution was achieved (20% A/80% B) over 3 min. Following separation, analytes and internal standards were quantified using electrospray ionization (ESI) in (+)-mode. Source and capillary temperatures were 150 and 400°C, respectively. Capillary voltage was 0.60 kV, and desolvation and cone gas (both N2) flow rates were 800 and 20 L/h, respectively. Compounds were quantified using optimized multi-reaction monitoring (MRM) functions. MRMs were optimized to achieve 12 points/10 s peak, and the detection span was ±0.2 amu. Parent and daughter ions, cone voltages, and collision energies for all compounds are listed in Table 3.3. Quantification was performed using ratio of the target analyte and respective IS peak areas, based on authentic external standard curves prepared using a wide range of target analyte concentrations (bracketing the peak areas observed in the blood samples) and the same IS concentrations used to prepare the blood samples. Blood was prepped for analysis using a previously tested method.

Table 3.3: *MRM settings for UPLC-MS/MS characterization of blood TMAO*

Compound	Parent $[M+H]^+$ (m/z)	Daughter (m/z)	Cone voltage (V)	Collision energy (eV)
betaine	118.24	59.42	59	44
betaine-d_9	127.30	68.10	68	46
TMAO	76.16	58.91	59	40
TMAO-d_9	85.22	68.10	68	40
L-Carnitine	162.26	84.99	34	20
L-Carnitine-d_9	171.28	84.96	34	20
choline	104.20	60.02	60	38
choline-d_9	113.32	69.08	69	40
γ-butyrobetaine	146.27	87.00	26	16

3.2.7 Quantification of FMO3Protein Levels

Total FMO3 liver protein was quantified using an enzyme linked immunosorbent assay (ELISA) and a bicinchoninic acid (BCA) assay. The ELISA kit used was the Mouse Dimethylaniline Monooxygenase [N-Oxide-Forming] 3 (FMO3) ELISA Kit

(Catalog # MBS9327471, MyBioSource.com). The BCA kit used was the Pierce™ BCA Protein Assay Kit (Catalog # 23227, ThermoFisher Scientific). Each liver was homogenized by bead-beating in PBS at 14000 rpm for 5 min. Homogenates were centrifuged at 17,000 x g for 3 min to separate homogenate from any remaining solid matter. Homogenates used in the BCA kit were diluted 4-fold to obtain samples in the readable range. Sample volumes per well for the ELISA and BCA assay were 50 µL and 25 µL respectively. Optical Density (OD) of samples subjected to ELISA was read at 450 nm; OD of samples subjected to BCA assay was read at 562 nm. Results of both assays were analyzed using a PowerWave™ XS Microplate Reader by BioTek Instruments, Inc. (Winooski, VT, USA). The results of each assay were presented in a ratio to show hepatic FMO3:total hepatic protein.

3.2.8 Nucleic Acid Extraction and Real-Time PCR Quantification of TMA associated genes

All collected fecal samples (40-100 mg/cage) for each animal were separated by time (pre-treatment administration vs post-treatment administration) and pooled by cage. DNA was extracted from pooled fecal samples using QIAamp PowerFecal DNA Kit from QIAGEN (Germantown, MD, USA). Extracted DNA samples were stored at -80°C until use in the assay. Two genes were targeted in the assay: *cntAB* and *grdH*. Targeted quantitative real-time PCR (qPCR analysis) was conducted on all extracted DNA samples using SsoAdvanced™ Universal Inhibitor-Tolerant SYBR® Green Supermix from Bio-Rad (Hercules, CA, USA) in accordance manufacturer instructions and a Bio-Rad CFX connect thermal cycler (Hercules, CA, USA) to quantify targeted gene abundance. *CntAB* and *grdH*, gene amplification was performed using 100 ng of template DNA. The *cntAB* and *grdH* gene amplification consisted of an initial activation cycle held at 95°C for 15 min and 40 cycles of the following: denaturation at 95°C for 45 sec, annealing at 53°C for 45 sec, and extension at 72°C for 45 sec, deploying final primer concentrations of 1.5 µM. Primers are listed in Table 3.4. The qPCR results of *cntAB* and *grdH*
genes were normalized to the total 16S rDNA gene abundance using previously verified primers targeting the V4 region of the 16s rDNA gene listed in Table 3.4. Amplification was performed with 100ng of template DNA. Primers were amplified using an initial activation cycle held at 95°C for 3 minutes and 40 cycles of the following: denaturation 95°C for 30 s, annealing at 60.2°C for 40 s, and 72°C for 1 min. The melt curve was established by heating at 0.5°C increments from 62 to 95°C.

mRNA was extracted from homogenized liver samples (~10-20mg per sample) using the RNeasy Mini Kit according to manufacturer's instructions (QIAGEN, Germantown, MD, USA). Extracted mRNA was stored at −80°C until use in the assay. Using the USB® First-Strand cDNA Synthesis Kit for Real-Time PCR from ThermoFisher Scientific (Waltham, MA, USA), mRNA samples were converted to cDNA via reverse transcription deploying primer concentrations of 0.5 uM and using the following method: 25 °C for 5 min, 42 °C for 1 h, and 70 °C 15 min. Samples were then quantified using SsoAdvanced™ Universal Inhibitor-Tolerant SYBR® Green Supermix from Bio-Rad (Hercules, CA, USA) using the following method: initial denaturation at 95 °C for 10 min, 35 cycles of denaturation at 94°C for 10 s, annealing at 58°C for 20 s,

and extension at 72°C for 18 s, then a second extension step at 72 °C for 7 min, a melt curve from 50-93°C at 0.2 °C intervals, and a final extension for 10 min at 72 °C.

Table 3. 4: *Primers used in Real-Time PCR assay*

Primer	Annealing Temperature (°C)	Reference
[*cntAB*] -F 5-TAYCAYGCITGGRCITTYAARCT-3’	53	[51]
[*cntAB*] -R 5’-RCAGTGRTARCAYTCSAKRTAGTTRTCRAC-3’	53	[51]
[*grdH*] -F 5’-ATWCARTCIGCWTCIGCNAC -3’	53	[25]
[*grdH*] -R 5’-GTIGTWCCNGTWCCIACIGT -3’	53	[25]
[*FMO3*mRNA] -F 5’-CACCACTGAAAAGCACGGTA-3’	58	[52]
[*FMO3*mRNA] -R 5’-GTTTAAAGGCACCAAACCATAG -3’	58	[52]
[16s rDNA] -F 5’ – GGGTGGTAATGCCGGATG -3’	60.2	[53]
[16s rDNA] -R 5’-CCACCGTTACACCGGGAA -3’	60.2	[53]

3.2.9 Data Analysis and Statistics

Data analysis and figure generation were done using Prism version 8.4.2 (679) (GraphPad, San Diego, CA, USA). All data are presented as mean ± SEM of concentrations of detected compounds (in the case of garlic extract content), all points within individual treatment groups (in the case of bar graphs) or timepoints (in the case of time series). Independent t-tests were used to compare individual compound concentrations present in the aged vs. fresh extracts. Two-way ANOVA with repeated measures analysis was used to compare pre- and post-intervention Tmax, Cmax, AUC, postprandial concentrations of TMAO, L-carnitine, and γ-butyrobetaine, and pre-and post-intervention log DNA copy numbers of *cntAB* and *grdH*. Log DNA copies of *cntAB* and *grdH* were normalized to log 16s DNA copies detected. These values were later further normalized to ng of DNA used and mg of feces from which the DNA was extracted. Ordinary one-way ANOVA was used to compare *Fmo3* mRNA log copy numbers and FMO3:total protein ratios between treatment groups. See figure captions for details on statistical analysis performed for specific datasets.

3.3 Results

3.3.1 Quantification of Aged and Fresh Garlic Extracts.

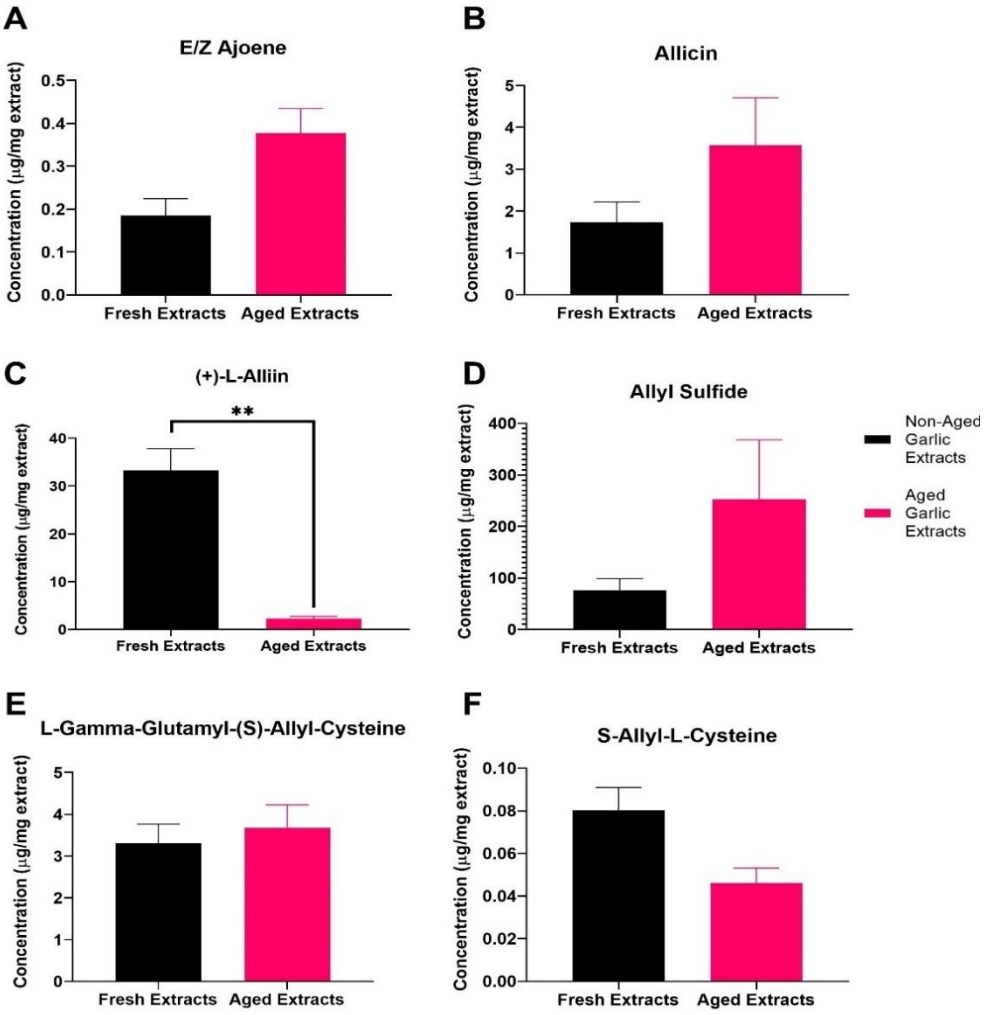

Figure 3.2**:** *Results of the characterization and quantification of fresh and aged garlic extracts as prepared in section 2.1. Concentrations of the following organosulfur species found within the extracts are shown: (A) (E/Z) ajoene, (B) Allicin, (C) (+)-L-Alliin, (D) Allyl Sulfide, (E) L-Gamma-Glutamyl-(S)-Allyl-Cysteine, and (F) S-Allyl-L-Cysteine. All values represent mean ± SEM of triplicate analyte concentrations quantified by UPLC-MS/MS. Bars linked by a bracket have significantly different concentrations between fresh and aged extracts. Statistical analysis (α=0.05): Independent t-test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

The aged and fresh garlic extracts were prepared using an original method developed for this experiment described in section 3.2.1. Allicin content in extracts increased by ~105% after aging, though this difference was not statistically significant (Figure 3.2 B). 93% of alliin content in fresh extracts was lost during aging (Figure 3.2 C). Interestingly, allyl sulfide content increased (but not significantly) following aging, with content increasing by ~234% (Figure 3.2 D). The alliin and allicin content of aged and fresh extract is influenced by strain, native region, maturation of the bulb, method of extraction, and the time the extract is aged[34,54,55]. Whole garlic cloves are reported to contain ~3.4-8 µg alliin /mg whole garlic bulb (13–17 mg alliin/garlic clove)[56], while freshly prepared garlic powders average 10 µg alliin/mg powder, indicating loss during the dehydration process[34]. Our prepared fresh extracts exceeded the reported average

alliin content in garlic powder by ~232%. Fresh aqueous extracts yield only about 0.66-3.98 μg allicin/mg extract whole garlic[54]. Aged (30 min) aqueous garlic extract is reported to contain ~0.16-13.0 μg allicin/mg[57]. Aged (2h) ethanol-based garlic extracts yield 7.88-17.13 μg allicin/mg extract[55]. Our aged extract allicin content is within the range of aged (30 min) aqueous garlic extracts but falls short of the range of 2hr aged extracts. It is possible that the aged garlic extracts thought to be rich in allicin were aged for too long a time period, as allyl sulfides can be rapidly formed through the degradation of allicin[34,58–60].

3.3.2 Postprandial Concentrations and Pharmacokinetics of TMAO

To measure the inhibitory effect of garlic extracts on TMAO production, we gavaged mice with water (NegC), L-carnitine (PosC), an antibiotic cocktail + L-carnitine (ABX), alliin-rich garlic extract + L-carnitine (AL), or allicin-rich garlic extract + L-carnitine (AC) for 13 days. We performed an acute carnitine challenge pre- and post-treatment and quantified the subsequent pharmacokinetics of TMAO, L-carnitine, and γ-butyrobetaine.

After 13 days, the area of TMAO peaks (AUC) in the ABX group was significantly lowered by 92%, (Figure 3.3 A), no other group experienced a reduction); this group also saw a 94% decrease in max postprandial TMAO levels (Figure 3.3 B, no other group experienced a reduction). Max TMAO levels of the ABX group saw a significant delay in the time they were observed (~2 hrs) (Figure 3.3 C), no other group experienced such a change).

In all groups, we identified a significant treatment effect on postprandial TMAO kinetics at some time points. The TMAO levels of mice in the NegC group (Figure 3.3 D) significantly increased (~2-fold) at 6 hrs after the carnitine gavage. Within that same group 8 hrs after gavage, TMAO levels were 54% lower than levels observed prior to dietary intervention. However, we re-emphasize that Cmax and AUCs for NegC and the other treatment groups were not significantly different from each other post-treatment (with the exception of ABX). The PosC, AL, and AC groups (Figure 3.3 E, 3.3 G, and 3.3 H respectively) all saw significant decreases in postprandial TMAO levels 8 hrs post-treatment; TMAO levels of each group decreased 64%, 55%, and 58% respectively. Interestingly, the significantly lower postprandial TMAO levels of the PosC treatment group (L-carnitine only) following dietary intervention (at 8 hrs) are similar to that of the AC and AL treatment groups, suggesting that TMAO elimination may be enhanced rather than the reduction of TMAO formation. The differences in TMAO concentrations would likely have been greater if we sampled blood at timepoints beyond 8 h; the TMAO content at the 8 h time point does appear to be decreasing when compared to pre-treatment levels for all treatment groups. The ABX treatment group (Figure 3.3 F) saw a significant decrease of 88% in postprandial TMAO levels at 2 hrs, 94% at 4 hrs, 93% at 6 hrs, and 89% at 8 hrs, suggesting (along with AUC results) that ABX treatment reduced TMAO production.

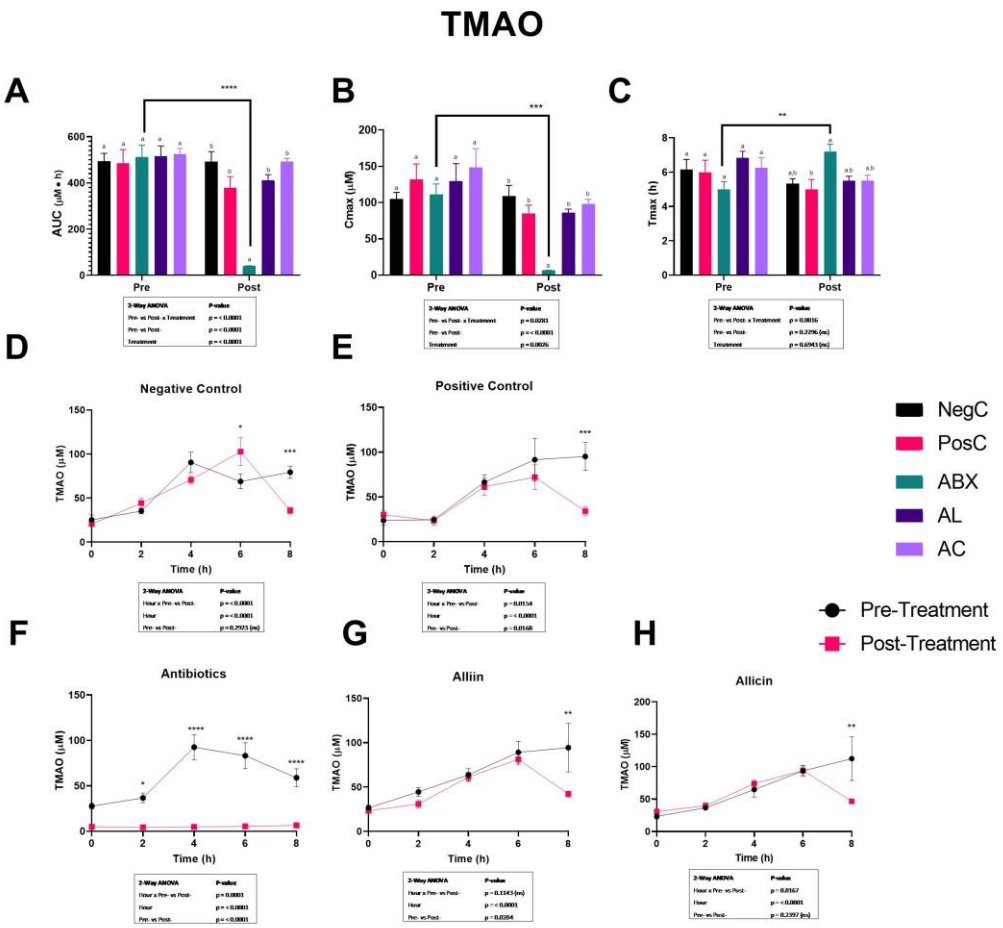

Figure 3.3**:** *Impact of 13-day treatment on postprandial levels and pharmacokinetics of TMAO. (A) Area under the curve of TMAO chromatograph peaks. (B) Maximum postprandial concentrations of TMAO (Cmax). (C) Time that max TMAO levels were observed (Tmax). For panels A-C, bars linked by a bracket are significantly different between pre- and post-treatment administration; bars not sharing a common superscript letter within the same time point are significantly different from each other. (D) NegC postprandial TMAO levels before and after treatment. (E) PosC postprandial TMAO levels before and after treatment. (F) ABX postprandial TMAO levels before and after treatment. (G) AL postprandial TMAO levels before and after treatment. (H) AC postprandial TMAO levels before and after treatment. For panels D-H, asterisks indicate a significant difference between pre- and post- treatment analyte concentrations at the specified hour post-carnitine gavage. All values represent mean±SEM. Statistical analysis (α=0.05): 2-way ANOVA with repeated measures; Sidak's post-hoc. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

3.3.3 Postprandial Concentrations and Pharmacokinetics of L-carnitine

Although not significantly different, the AUC and Cmax for circulating L-carnitine appeared to be lower following intervention (Figures 3.4 A and 3.4 B respectively) suggesting that the host's ability to clear L-carnitine has been enhanced. Dietary intervention increased the time that max postprandial L-carnitine levels (Figure 3.4 C) were observed (~2.5 h) for all groups except PosC and AC. The only treatment group to experience a significant difference in postprandial L-carnitine levels at any of

the observed timepoints was the PosC group (Figure 3.4 E) at 6 hrs, with a 94% decrease in L-carnitine levels. These data suggest that postprandial L-carnitine levels and pharmacokinetics were generally not significantly changed by treatment with garlic extracts or ABX.

3.3.4 Postprandial Concentrations and Pharmacokinetics of γ-butyrobetaine

Postprandial levels of γ-butyrobetaine (the pro-atherogenic intermediate of L-carnitine to TMAO metabolism) were greatly impacted by 13-day treatment with garlic extracts. The areas under the curve of γ-butyrobetaine significantly increased for the PosC, AL and AC groups after treatment (Figure 3.5 A, ~15-25-fold). AL and AC treatment significantly increased the area under the curve when compared to the PosC group (~24-25% higher). The AUCs of AL and AC were not significantly different from each other. Max postprandial γ-butyrobetaine levels (Figure 3.5 B) increased significantly following treatment in all groups (~18-24-fold) except NegC and ABX. The max concentrations of γ-butyrobetaine in the groups that increased post-treatment were not significantly different from each other. The time that max γ-butyrobetaine levels were observed (Figure 3.5 C) significantly increased from the times observed prior to treatment in all groups (~2-3 h) except AC; these times were not significantly different across treatment groups.

Postprandial concentrations of γ-butyrobetaine increased at each timepoint in all groups (except ABX) after chronic treatments. Postprandial γ-butyrobetaine levels of the NegC group (Figure 3.4 D) significantly increased at the 6 h (73%) and 8 h (42%) timepoints. The PosC group (Figure 3.5 E) saw varying increases in γ-butyrobetaine at all timepoints, with the greatest increase at 8 h. Concentrations increased ~16-fold at 0 h, ~12-fold at 2 h, ~18-fold at 4 h, ~10-fold at 6 h, and ~29-fold at 8 h. The ABX group (Figure 3.4 F) saw a decrease in γ-butyrobetaine levels only immediately after carnitine gavage (0 h). The AL and AC (Figure 3.5 G and 3.5 H) groups both saw a significant increase in γ-butyrobetaine levels at all observed timepoints. The AL group increased ~16-fold at 0 h, ~17-fold at 2 h, ~35-fold at 4 h, ~20-fold at 6 h, and ~35-fold at 8 h. The AC group increased from ~22-fold at 0h, ~19-fold at 2h, ~34-fold at 4 h, ~18-fold at 6 h, and ~23-fold at 8 h. Collectively, these data suggest that AC and AL treatment actually increase levels of γ-butyrobetaine more so than L-carnitine supplementation alone.

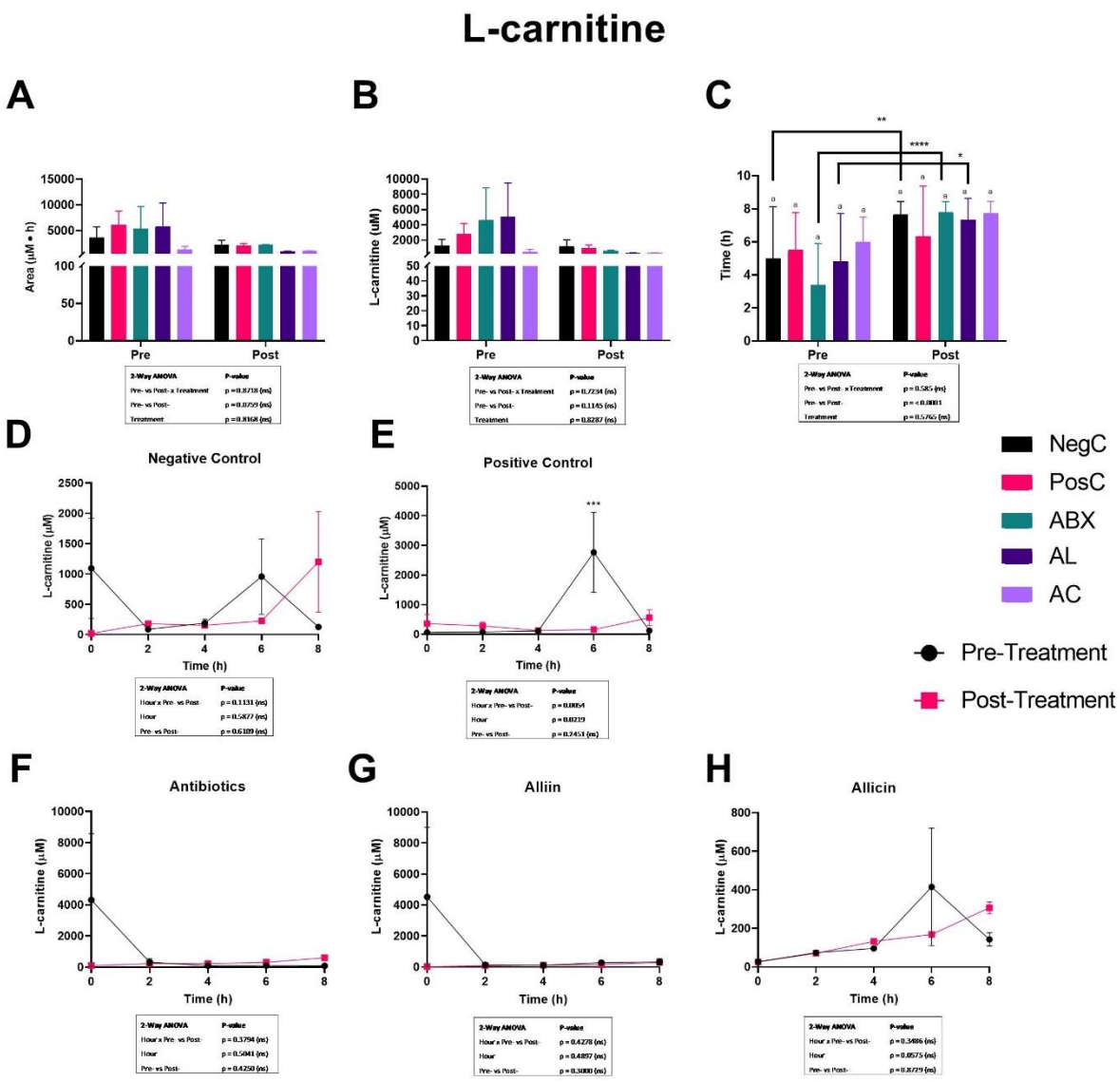

Figure 3.4**:** *Impact of 13-day treatment on postprandial levels and pharmacokinetics of L-carnitine. (A) L-carnitine levels observed (Tmax). (B) Maximum postprandial concentrations of L-carnitine (Cmax). (C) Area under the curve of L-carnitine chromatograph peaks. For panels A-C, bars linked by a bracket are significantly different between pre- and post-treatment administration; bars not sharing a common superscript letter within the same time point are significantly different from each other. (D) NegC postprandial L-carnitine levels before and after treatment. (E) PosC postprandial L-carnitine levels before and after treatment. (F) ABX postprandial L-carnitine levels before and after treatment. (G) AL postprandial L-carnitine levels before and after treatment. (H) AC postprandial L-carnitine levels before and after treatment. For panels D-H, asterisks indicate a significant difference between pre- and post-treatment analyte concentrations at the specified hour post-carnitine gavage. All values represent mean±SEM. Statistical analysis (α=0.05): 2-way ANOVA with repeated measures; Sidak's post-hoc. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

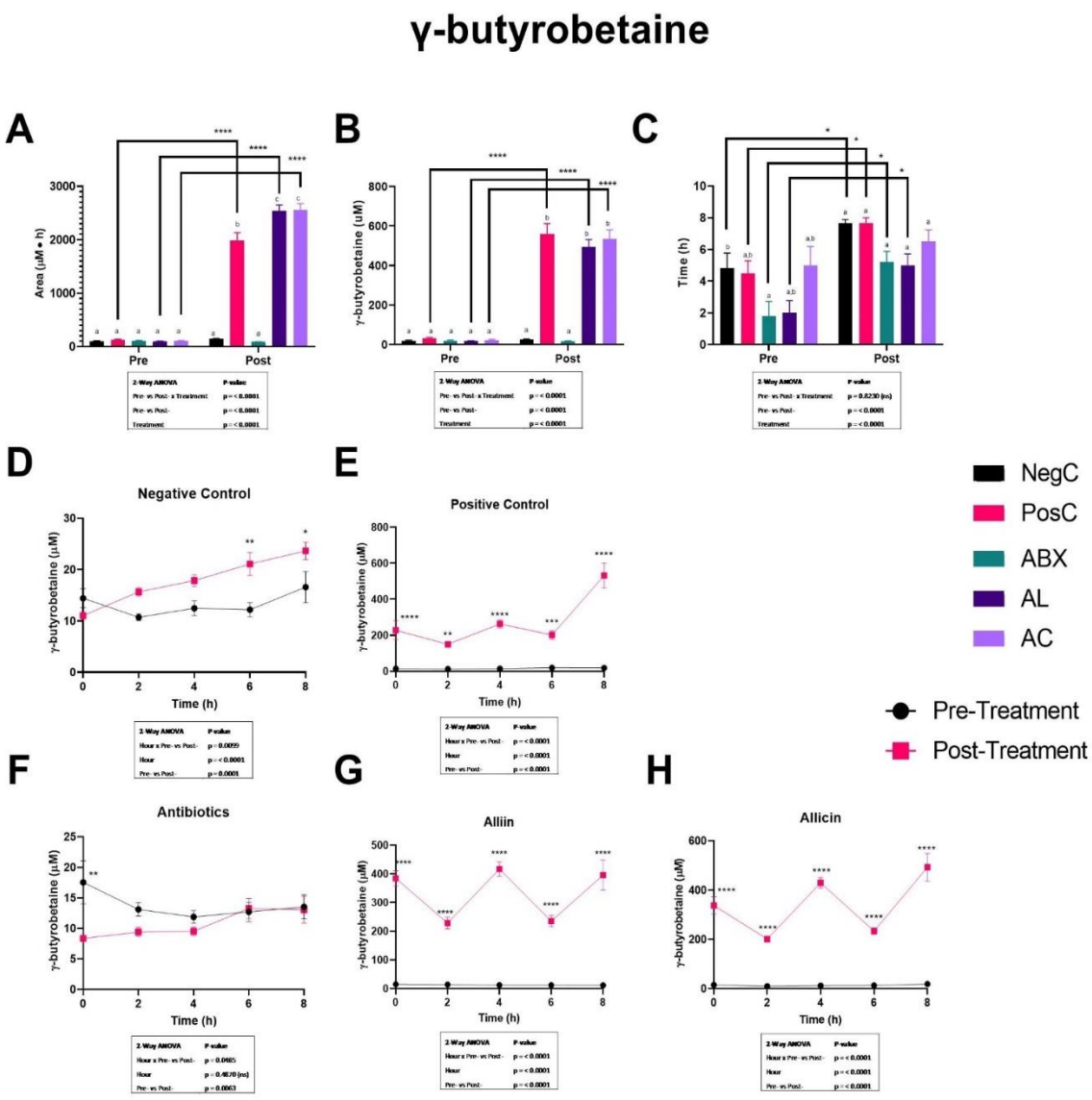

Figure 3.5**:** *Impact of 13-day treatment on postprandial levels and pharmacokinetics of γ-butyrobetaine. (A) γ-butyrobetaine levels observed (Tmax). (B) Maximum postprandial concentrations of γ-butyrobetaine (Cmax). (C) Area under the curve of γ-butyrobetaine chromatograph peaks. For panels A-C, bars linked by a bracket are significantly different between pre- and post-treatment administration; bars not sharing a common superscript letter within the same time point are significantly different from each other. (D) NegC postprandial γ-butyrobetaine levels before and after treatment. (E) PosC postprandial γ-butyrobetaine levels before and after treatment. (F) ABX postprandial γ-butyrobetaine levels before and after treatment. (G) AL postprandial γ-butyrobetaine levels before and after treatment. (H) AC postprandial γ-butyrobetaine levels before and after treatment. For panels D-H, asterisks indicate a significant difference between pre- and post- treatment analyte concentrations at the specified hour post-carnitine gavage. All values represent mean±SEM. Statistical analysis (α=0.05): 2-way ANOVA with repeated measures; Sidak's post-hoc. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

3.3.5 Fmo3 Transcript Quantification and Gene Expression

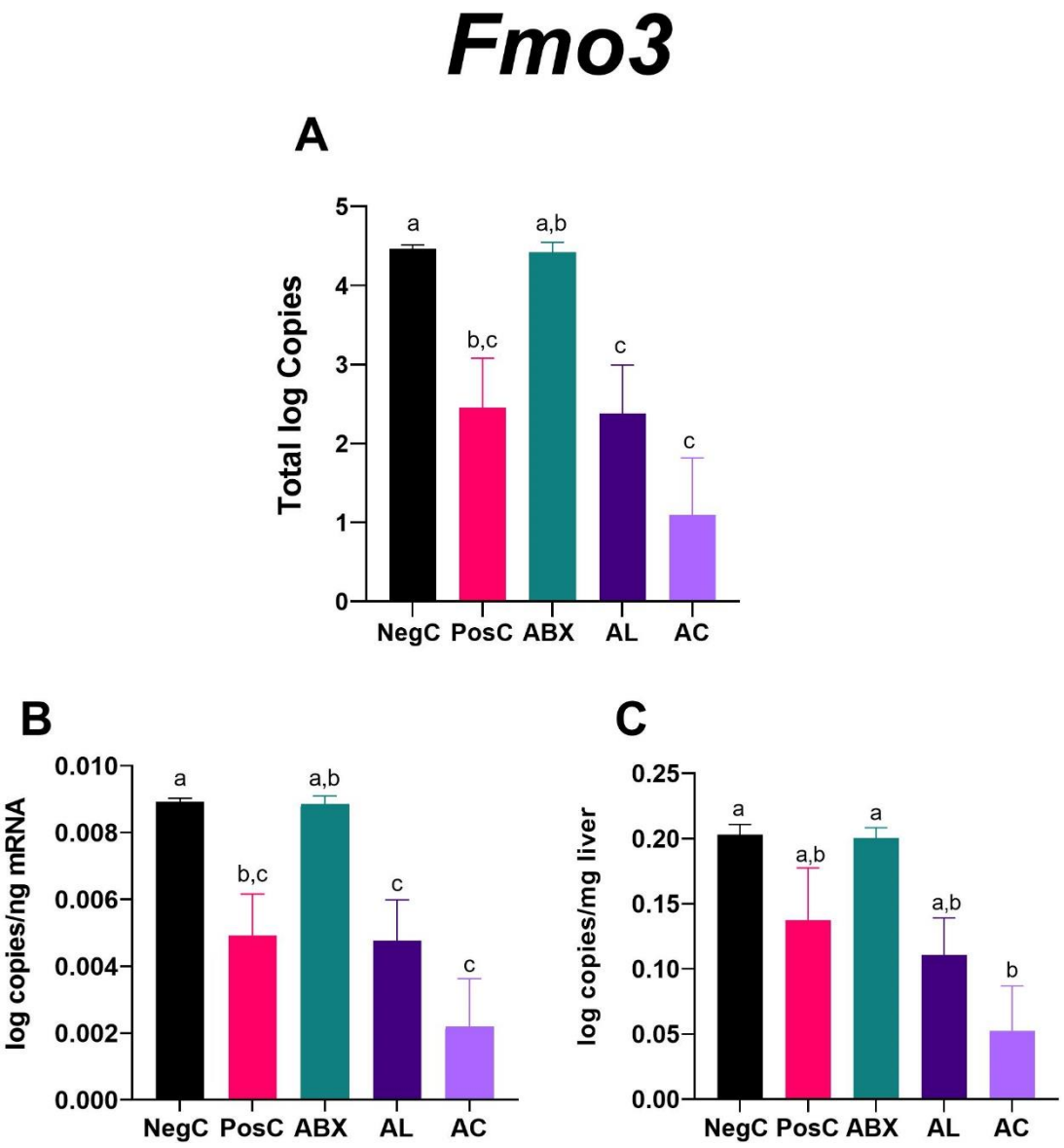

Figure 3.6: *Quantity of hepatic Fmo3 detected from liver samples. (A) Total log copies of Fmo3detected. (B) Total log copies of Fmo3 normalized to ng mRNA used in the assay. (C) Total log copies of Fmo3normalized to total mg liver used in the assay. Each bar represents the mean±SEM of Fmo3 of each treatment group. Statistical analysis (α = 0.05): Ordinary One-Way ANOVA with Tukey's multiple comparisons post-hoc test comparing mean log FMO3gene copies of each treatment group to the other treatments. Overall treatment effect: p < 0.0001. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Bars not sharing a common superscript letter are significantly different from each other.*

Figure 3.6 shows the abundance of hepatic *Fmo3* gene transcripts, determined using reverse transcription PCR (RT-PCR) to generate cDNA followed by quantitative real-time PCR analysis. mRNA was extracted from liver samples collected following euthanasia of animals. *Fmo3* gene copies were quantified to identify any potential shifts in *Fmo3* transcript abundance induced by garlic extract treatment. After 13-day dietary

intervention with garlic extracts, the total quantity of *Fmo3* gene copies detected in the AL and AC groups was significantly lower than those observed in the ABX or NegC groups (Figure 3.6 A) (~2 and 3.3 logs, respectively). These results persisted when data were normalized to ng of mRNA used (Figure 3.6 B). When data were normalized to mg of liver used, however, only AC significantly differed from the ABX and NegC groups (Figure 3.6 C). The quantity of *Fmo3* gene copies within the AL and AC groups did not significantly differ from each other, nor the PosC group (Figure 3.6 A, 3.6 B, 3.6 C). The ABX group saw high levels of *Fmo3* but was not significantly different from those of the NegC or PosC group in any instance (Figure 3.6 A, 3.6 B, 3.6 C). The NegC group saw significantly higher levels of *Fmo3* than the PosC group (Figure 3.6 A and 3.6 B) until normalized to mg of liver used (Figure 3.6 C). Collectively, these data suggest that dietary intervention using garlic extracts significantly lowers the abundance of *Fmo3* gene transcript in host liver cells relative to the levels observed in negative controls supplemented water only. These statistically significant differences, however, no longer hold true for the group supplemented fresh extract (AL) after normalization to mg of liver used, suggesting that these differences are largely due to the mass of liver used rather than the effect of dietary intervention. The levels of *Fmo3* transcript in mice supplemented aged extract remained significantly lower than the negative control after normalization to mg of liver used, suggesting that dietary intervention with aged garlic extract may have an association with lower levels of hepatic *Fmo3* gene expression. Interestingly, all treatment groups that saw increased circulating levels of γ-butyrobetaine (PosC, AL, and AC) (Figure 3.5 A and 3.5 B) saw lower (but not significantly different) levels of *Fmo3* mRNA transcript when compared with the negative control and ABX groups. This may suggest that L-carnitine supplementation may associate with lower transcription of *Fmo3* and may prove to be a worthy of future investigation.

To determine liver FMO3 protein content of the animals relative to total protein content, animal livers were analyzed by ELISA (Figure 3.7). The AL group had significantly higher observed levels of FMO3 than those observed in ABX and AC groups (~40% and ~49% respectively). These levels were not, however, significantly different from those observed in groups supplemented water (NegC) or L-carnitine only (PosC). The animals supplemented aged extract did not see any significant differences in FMO3 protein expression relative to the water or L-carnitine only groups. These data suggest that intervention with fresh or aged garlic extracts do not significantly alter FMO3 protein expression from levels observed in both negative and positive control groups. Because dosing with L-carnitine did not appear to alter FMO3 protein expression (as evidenced by lack of difference between the PosC and NegC groups), it is unlikely that supplementation with L-carnitine affects the rate of transcription nor does it influence the translation of *Fmo3* mRNA to FMO3 protein.

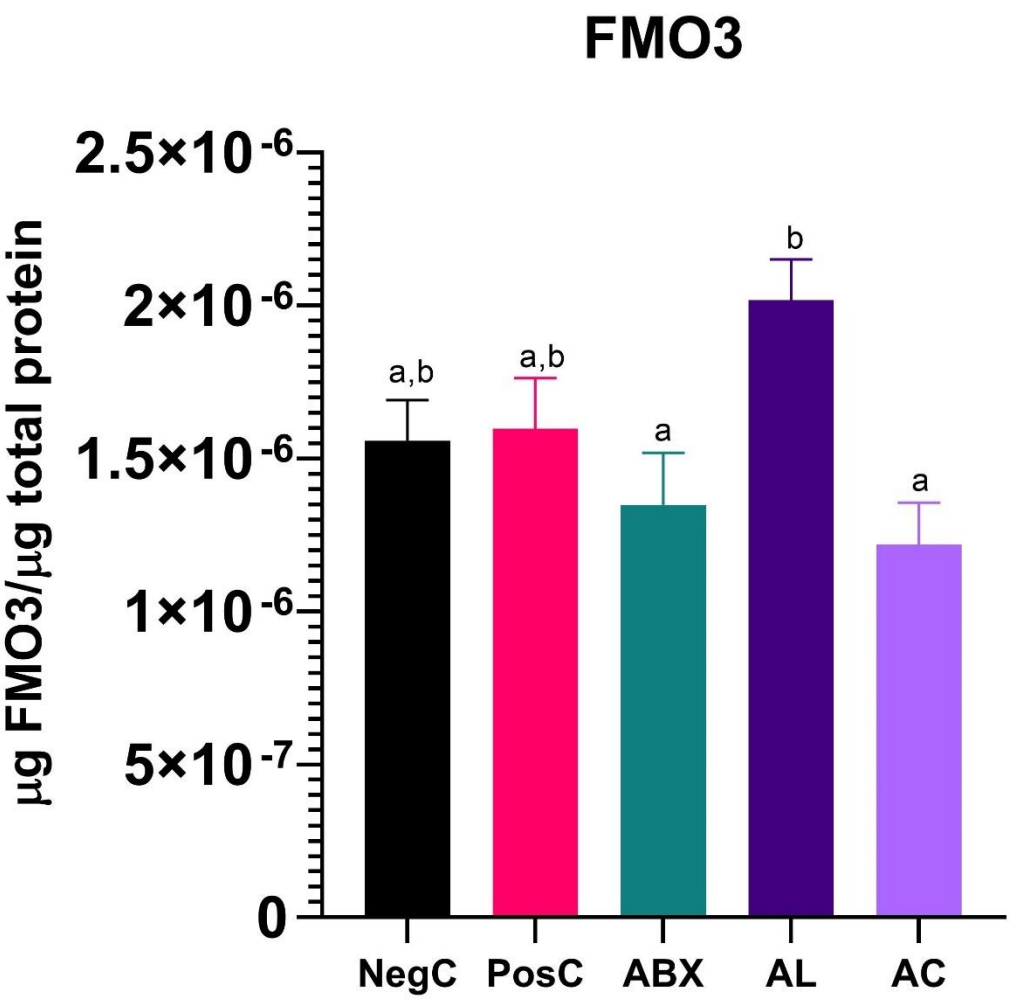

Figure 3.7**:** *FMO3 enzyme content in relation to total hepatic protein in animal livers. Each bar represents the mean±SEM of FMO3:total protein of each treatment group. Statistical analysis (α = 0.05): Ordinary One-Way ANOVA with Tukey's multiple comparisons post-hoc test comparing mean FMO3:Total Protein ratios of each treatment group to the other treatments. Overall treatment effect: p = 0.0073. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Bars not sharing a common superscript letter are significantly different from each other.*

3.3.6 Quantification of L-carnitine metabolizing genes using real-time PCR

Gene abundances of L-carnitine metabolizing *cntAB* and *grdH* were determined using quantitative real time PCR (qPCR) on DNA extracted from fecal samples prior to and following 13-day dietary intervention. The effect of garlic extract supplementation on log copies of genes before and after the intervention were compared to each other as well as across treatment groups.

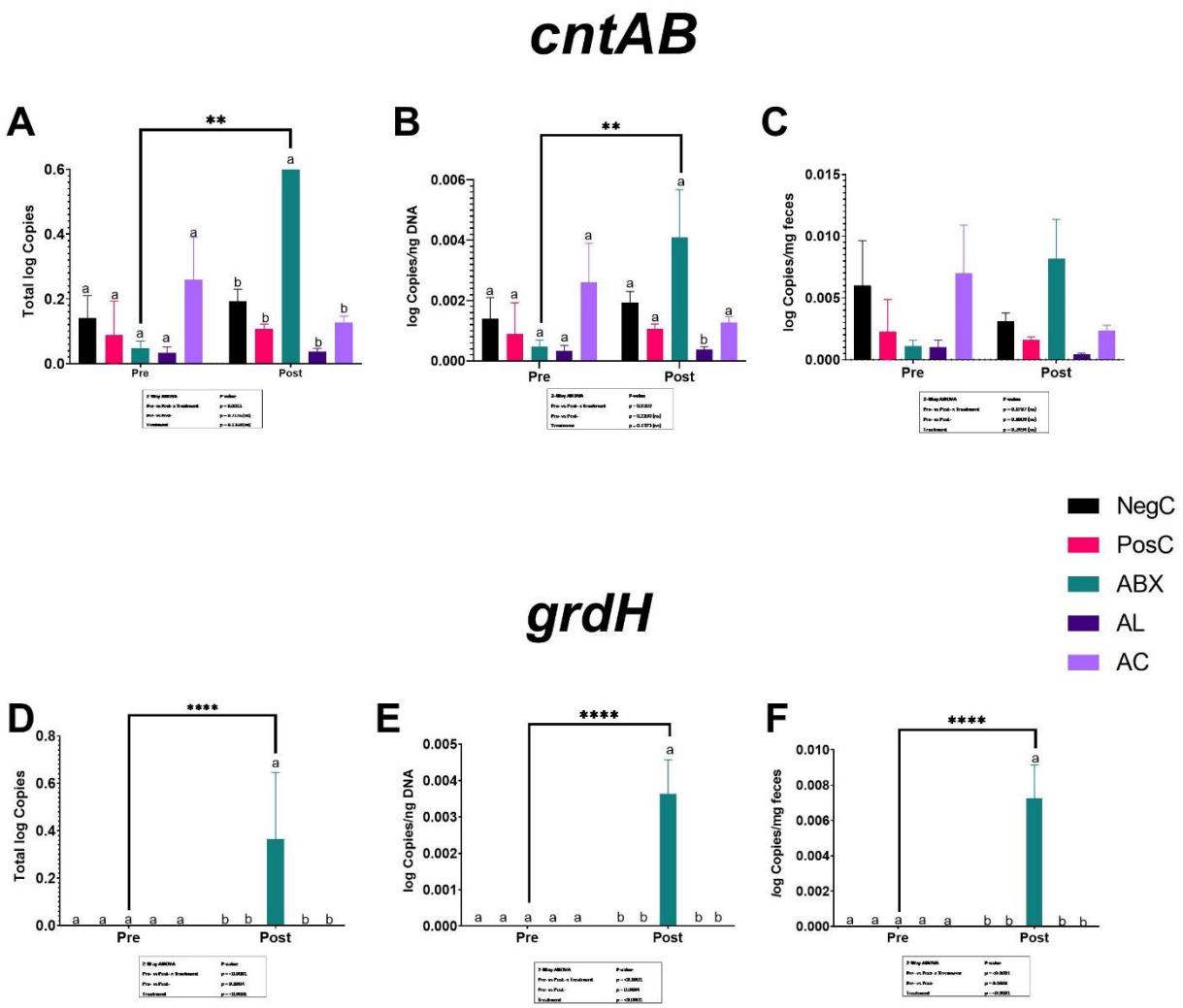

Figure 3.8**:** *Impact of 13-day garlic extract treatment on log quantity of L-carnitine metabolizing gene copies. All log gene quantities have been normalized to 16s rDNA values. (A) Total log copies of cntAB detected in fecal samples before and after treatment. (B) Log copies of cntAB normalized to ng of DNA used in the assay. (C) Log copies of cntAB normalized to mg of feces used in the assay. (D) Total log copies of grdH detected in fecal samples before and after treatment. (E) Log copies of grdH normalized to ng of DNA used in the assay. (F) Log copies of grdH normalized to mg of feces used in the assay. Bars linked by a bracket are significantly different between pre- and post-treatment administration; bars not sharing a common superscript letter within the same time point are significantly different from each other. All values represent mean±SEM. Statistical analysis (α = 0.05): 2-way ANOVA with repeated measures; Sidak's post−hoc. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

The *cntAB* gene was detected in all treatment groups prior to dietary intervention (Figure 3.8 A, 3.8 B, and 3.8 C). The group supplemented aged extract had the greatest number of total log copies prior to the intervention but were not significantly different from any other group. Post-intervention, the ABX group was the only group shown to have a significant change in total log gene copies, increasing by ~6.7 logs. This statistically significant increase is not observed when data were normalized to mg of

feces used in analysis (Figure 8C), suggesting a greater mass of feces may have been used in DNA extraction assays for that group. These data are unusual in that the ABX group saw significant decreases in observed TMAO peak area and max concentrations of TMAO (Figures 3.3 A and 3.3 B respectively) following the intervention. These contrasting results may have implications for the metabolic fate of the TMA produced by the *cntAB* gene-containing bacteria. leading to higher, not lower TMAO levels. These data suggest that garlic extract supplementation (as prepared in this study) does not reduce the quantity of microbiota that metabolize L-carnitine via the enzyme coded for by the *cntAB* gene. The *grdH* gene was virtually undetected in all treatment groups prior to dietary intervention (Figure 3.8 D, 3.8 E, and 3.8 F). Post-intervention, only the ABX group was shown to have any sort of significant *grdH* gene content with an increase ~6 logs. These data mirror that of the *cntAB* gene: extract supplementation did not appear to alter the abundance of *grdH*-containing microbes while treatment with broad-spectrum antibiotics appeared to select for organisms containing the *grdH* gene.

Collectively, these data suggest that the broad-spectrum antibiotic suppression of the intestinal microbiome may exert a selective pressure on the TMA-producing bacteria in the intestinal microbiome. We theorize that intervention with broad-spectrum antibiotics (ABX) (dissolved in L-carnitine solution as described in section 3.2.4) select for *cntAB* bacteria by limiting the growth of other bacteria which preferentially utilize choline (another TMA-containing nutrient present in the mouse chow diet). Bacteria that produce TMA from choline do so by metabolizing it using the TMA-lyase coded for by the *cutCD* gene and is a more widely detected/abundant TMA-producing gene in the gastrointestinal tract[51]. The use of ABX may have reduced the abundance of organisms that utilize choline (said to exist in larger quantities in the gastrointestinal tract), allowing the organisms that utilize L-carnitine (suggested to exist in lower abundances in the gastrointestinal tract) to thrive without competition from bacteria that preferentially metabolize choline over L-carnitine, although further studies are necessary to validate this theory.

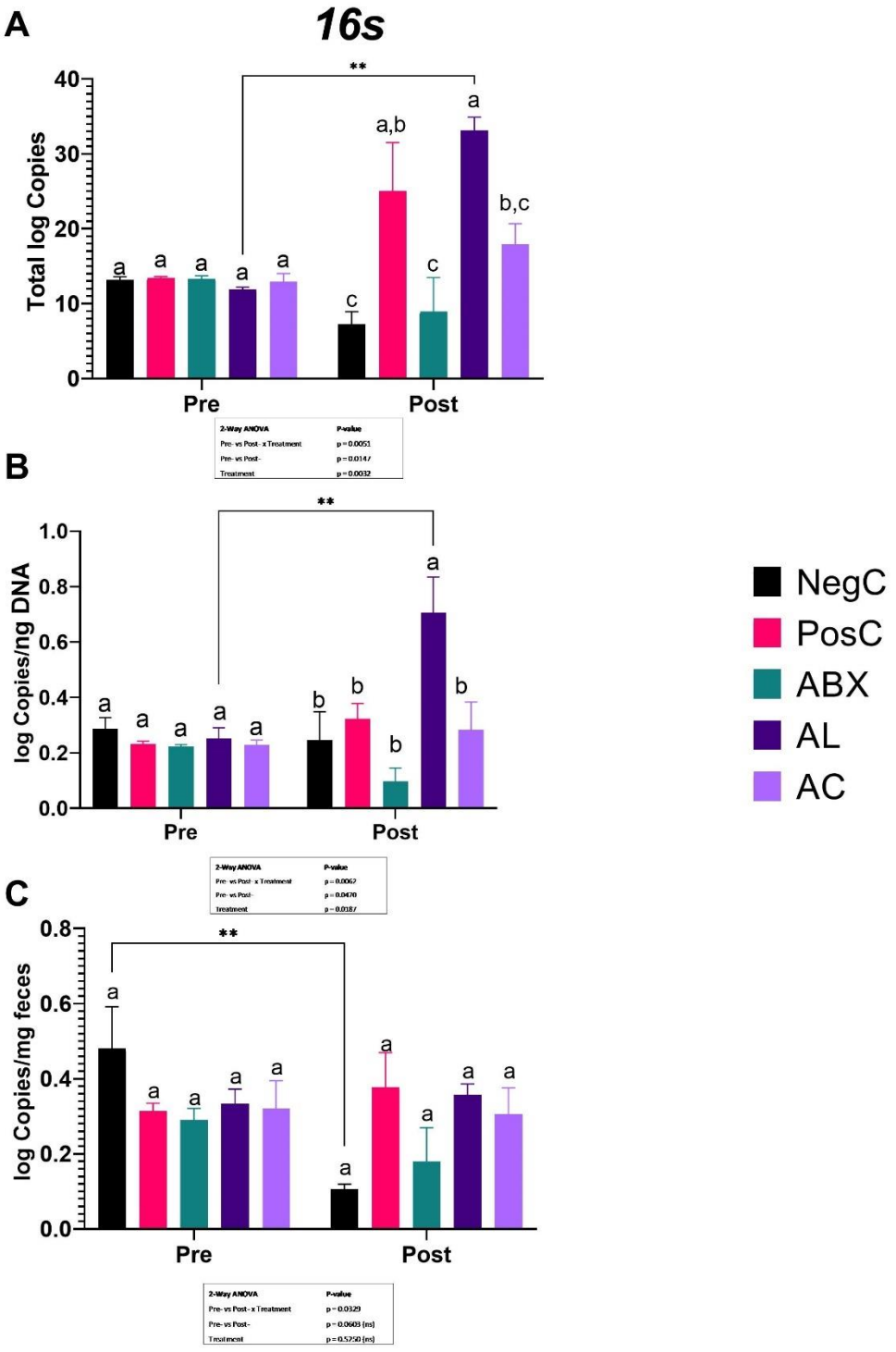

Figure 3. 9**:** *Impact of 13-day garlic extract treatment on log quantity of 16s rDNA gene copies. (A) Total log copies of 16s detected in fecal samples before and after treatment. (B) Log copies of 16s normalized to ng of DNA used in the assay. (C) Log copies of 16s normalized to mg of feces used in the assay. Bars linked by a bracket are significantly different between pre- and post-treatment administration; bars not sharing a common superscript letter within the same time point are significantly different from each other. All values*

*represent mean±SEM. Statistical analysis (α = 0.05): 2-way ANOVA with repeated measures; Sidak's post−hoc. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

3.4 Discussion

Previous studies have demonstrated that chronic (6-week) dietary supplementation of garlic phytochemical allicin can reduce levels of TMAO in mice[37]. Other studies have suggested that allicin exerts cardioprotective effects and alleviate pathological conditions associated with CVD[34,43,61]. In addition, some studies have shown that freshly prepared garlic extracts and aged garlic extracts can lower risk factors associated with CVD development, including atherosclerotic plaque development and platelet aggregation[62,63]. The goal of the present study was to evaluate and compare the efficacy of aged and fresh garlic extracts as a dietary intervention strategy in the attenuation of pro-atherogenic TMAO formation by the intestinal microbiome.

We failed to observe a significant increase in TMAO levels following L-carnitine supplementation (as evidenced by our positive control in Figures 3.1 B and 3.1 C). This may be due to the length of the intervention study. Most studies see an increase in TMAO levels after a chronic substrate supplementation of $\geq$ 4 weeks [15,28,37,64]; a period of 13 days may not have been sufficient to elevate TMAO levels (although 13 d ABX was sufficient to significantly reduce TMAO production). There was no significant reduction in maximum TMAO levels after 13-day supplementation with aged or fresh garlic extracts (Figures 3.1 B and 3.1 C). There were, however, notable decreases in observed postprandial TMAO levels 8 hours after L-carnitine oral gavage for both fresh (AL) and aged (AC) garlic extract treatment groups (Figures 3.1 G and 3.1 H). As mentioned earlier, larger reductions in postprandial TMAO levels may have been observed had we sampled blood at timepoints beyond 8 h, given the downward trend in Figures 3.1 D-3.1 H. These findings suggest that treatment with aged and fresh garlic extracts can prevent acute increases in postprandial TMAO levels, but not overall increases in max TMAO levels observed. Furthermore, the use of garlic extracts may be shunting L-carnitine into γ-butyrobetaine production, as evidenced by the increase in concentration in AL and AC groups (Figures 3.3 B and 3.3 C). It also appears that the increased γ-butyrobetaine being formed is not being fully metabolized further into TMAO, suggesting that γ-butyrobetaine can serve as a CVD risk marker independently of circulating TMAO levels[27,65].

L-carnitine concentrations decreased, but not significantly, after garlic extract treatment. Garlic extract treatment did not significantly alter postprandial L-carnitine levels at any timepoint. These findings are similar to those of a previous study, in which treatment with dietary allicin did not alter postprandial L-carnitine levels after treatment at any timepoint[37].

Pro-atherogenic γ-butyrobetaine is formed as an intermediate metabolite in the L-carnitine to TMAO pathway; this compound has been shown to be associated with atherosclerotic plaque development and cardiovascular mortality independent of TMAO levels[27,65]. Chronic L-carnitine administration significantly upregulated γ-butyrobetaine production, whereas TMAO saw no such increase; ABX administration eliminated this effect. Interestingly, both garlic extract treatments significantly increased γ-butyrobetaine

levels (Figure 3.3 B and 3.3 C) beyond those of PosC; γ-butyrobetaine levels post-treatment exceeded that of TMAO. This is in accordance with a previous study that reported increased γ-butyrobetaine production when L-carnitine is provided as a dietary substrate[27]. Moreover, garlic extract supplementation for 13 days also increased postprandial γ-butyrobetaine levels at all observed timepoints following L-carnitine gavage. Interestingly, antibiotic supplementation inhibited the formation of γ-butyrobetaine at all timepoints, confirming the obligatory role of the intestinal microbiome in its production[27,65]. Taken together, this information suggests that increased dietary intake of L-carnitine in combination with garlic extract supplementation does not inhibit the intestinal microbiota-dependent metabolism of L-carnitine to TMA, and therefore, does not serve as an appropriate dietary intervention to reduce circulating TMAO levels as evidenced by the unchanged TMAO levels and increased levels of γ-butyrobetaine (which occur when L-carnitine metabolism by the intestinal microbiota has increased). However, garlic compounds appear to actually stimulate γ-butyrobetaine production. These results are problematic, as higher circulating levels of γ-butyrobetaine are reported to be more highly associated with carotid atherosclerosis and cardiovascular death than TMAO[65]. Taken together, this information suggests that using garlic extracts to modulate TMAO production and alleviate CVD risk may be counterproductive. These data also suggest that in the L-carnitine induced CVD pathway, γ-butyrobetaine may also serve as a valuable CVD risk marker alongside (but independent of) TMAO. Despite our findings, several studies on CVD outcomes suggest a protective effect of garlic, which may imply that these increases in GBB are not physiologically significant.

The results presented in this study are not in line with those reported in the Wu et al. 2015 study, which suggest that further research must be conducted regarding the activity of garlic organosulfur compounds such as allicin in the transformation of L-carnitine to TMA in the intestinal microbiome, and the subsequent oxidation of TMA in the host liver[37]. One thing to note is that while Wu et al. did observe decreases in circulating TMAO levels, they did not quantify levels of γ-butyrobetaine, the proatherogenic intermediate formed during L-carnitine metabolism to TMA. It is within the realm of possibility to suggest that the group saw lower TMAO levels because the L-carnitine supplemented was converted to γ-butyrobetaine and was not further metabolized to TMA. Our significantly increased levels of γ-butyrobetaine (Figure 5A and 5B) did not associate with significantly increased levels of TMAO (Figure 3A and 3B), which suggests that the circulating γ-butyrobetaine is not immediately metabolized into TMA (and subsequently TMAO) and may have a different metabolic fate. It would better prove the impact of dietary allicin intervention if a future study measured circulating γ-butyrobetaine levels and CVD outcomes alongside TMAO levels. An observed increase in root atherosclerotic plaque area and increased levels of proatherogenic γ-butyrobetaine despite decreases in circulating TMAO levels would put the two studies in agreement.

It is also worth noting that while we did achieve conversion to allicin in our aged garlic extract preparation, our aged extracts contained much higher concentrations of allyl sulfide, a downstream metabolite of allicin[66]. This conversion to allyl sulfide may explain why our aged extracts did not achieve results comparable to the study on which our research was based.

FMO3 is a flavin-containing monooxygenase enzyme that rapidly oxidizes TMA into TMAO and is largely responsible for the metabolic clearance of TMA *in vivo* in

humans[19,29,67]. Female C57BL/6J mice were selected for the experiments since hepatic FMO3 is expressed at significantly higher levels in females than in males[45–47]. This allowed us to properly evaluate changes in expression without being burden by naturally low endogenous protein levels. In *Fmo3* knockdown mice, plasma TMAO, atherosclerotic lesion size, rate of cholesterol absorption, and plasma lipids are lowered[68,69]. The major role of FMO3 in the biosynthesis of TMAO and atherosclerotic plaque growth prompted us to evaluate whether garlic extract as a dietary intervention strategy could alter *Fmo3* gene or protein expression, thus reducing TMAO levels in a manner independent of the intestinal microbiome. Mice treated with fresh extracts had higher (but not significantly different) levels of hepatic FMO3 expression when compared with positive and negative controls; protein expression in mice treated with aged extracts were lower but not significantly different from levels in both control groups. FMO3 expression in the positive and negative control groups did not significantly differ, meaning that L-carnitine gavage did not alter FMO3 expression.

From the data, we were able to draw a few conclusions regarding *Fmo3* gene expression and FMO3 protein expression. Firstly, after normalization to mg of liver used, mice supplemented aged garlic extract groups (AC) have significantly lower levels of gene expression when compared to mice supplemented water only (NegC) but have statistically comparable gene expression to both negative and positive controls (NegC and PosC respectively), suggesting that treatment with garlic extracts as prepared in this study do not influence FMO3 protein turnover. Secondly, we can conclude that treatment with L-carnitine only (PosC) does not influence gene expression indicated by the fact that the PosC group does not have significantly lower gene expression than the L-carnitine-free group (NegC) when normalized to mg of liver used, nor does it have a significant effect on hepatic FMO3 protein expression levels. The use of broad-spectrum antibiotics (ABX) also does not seem to alter host gene expression, as the ABX group does not significantly differ from NegC or PosC groups in terms of *Fmo3* log copy number or protein level. The ABX group and NegC (water only) groups saw the highest (but not significantly different) levels of gene expression. Collectively, we can conclude that *Fmo3* gene expression is not influenced by L-carnitine gavage, but may be associated with aged garlic extracts as prepared in this study. More research is required.

TMA-producing genes are ubiquitously detected in relatively low abundances in human and non-human mammalian intestinal microbiomes[25,51,70]. Rieske-type oxidoreductase *cntAB* was first identified in model organism *Acinetobacter baumannii* ATCC19606, whose carnitine to TMA degradation had been established[26,71]. The *cntAB* gene acts on L-carnitine by cleaving the C-N bond in L-carnitine molecules, releasing TMA and a four-carbon molecule (typically malate or succinate), the latter of which is used as a bacterial carbon source in downstream metabolic processes[26]. *CntAB* was shown to be located near the carnitine transporter gene *CaiT* and other genes involved in malate/succinate metabolism[26]. The gene cluster was first hypothesized (and later confirmed) to be found clustered in the genomes of representative phyla of the human intestinal microbiome, *Proteobacteria* (specifically *Betaproteobacteria* and *Gammaproteobacteria*) and *Firmicutes*[26]. A majority of *cntAB* in humans and non-human mammal intestines is primarily detected in *Proteobacteria*, with a large majority of sequences originating from *Escherichia* or *Shigella*; other sequences from non-human mammals contained sequences from *Acientobacter* and *Citrobacter*[25]. Functionality of

the gene has been shown in *A. baumannii* and *E. coli*[26]. In microbiota that degrade L-carnitine to TMA, it has been shown that when *cntAB* genes are deleted, the organisms lose the ability to grow and proliferate on L-carnitine as a sole carbon source[26]. *CntAB* is detected infrequently in human fecal samples and is found in low abundances (0.03-2.17%)[51], a statement that is in line with our data, as *cntAB* was detected in nearly all samples, but existed in small quantities, evidenced by low total log copy numbers observed during qPCR. Rath et al.' s 2017 study also states that *cntAB's* metabolism of L-carnitine to TMA represents a distinctive branch of TMA synthesis defined by the ecophysiologic traits of phyla associated with this gene (i.e. *Proteobacteria*); the authors suggest that features such as the facultative anaerobic nature of representative members like *E. coli* demonstrate that *cntAB*-containing organisms thrive in a niche distinct from that of organisms that produce TMA in a different metabolic pathway, such as the more abundant choline utilization gene cluster (*cutCD*)[51]. It is, therefore, reasonable to suggest that our use of broad-spectrum antibiotics generated environmental conditions that selected against *cutCD*-containing taxa, allowing *cntAB* containing taxa to occupy the ecological niche, explaining the increase in *cntAB* abundance post-treatment in the ABX group.

The *grdH* gene encodes for a betaine reductase that catalyzes TMA formation from betaine (specifically trimethylglycine)[24,25]. Detected sequences of *grdH* in human and non-human mammal intestines largely originate from *Firmicutes*; distribution of the gene within the phyla is dependent on host dietary patterns[25]. In a recent study aiming to quantify abundances of TMA producing genes across different mammalian intestinal microbiomes, *grdH* was detected in 55.1% of samples compared to the 42.7% of samples *cntAB* was detected in[25]. These data are in line with human microbiome data in which *grdH* was detected with greater frequency than *cntAB*[25,70]. While *grdH* is more likely to be detected in intestinal microbial samples than *cntAB*, its abundance is often lower than that of *cntAB;* one study (in non-human mammalian samples) showed *grdH* abundance at 0.03±0.01% compared to *cntAB* abundance at (0.42±0.13%)[25]. These abundances are reflected in human metagenomic data as well[70,72]. Our data indicate very little to no detection of *grdH* in the fecal pellets of animals prior to and after dietary intervention (apart from the ABX group post-treatment). Because *grdH* forms TMA primarily through the metabolism of betaine (which is produced as an intermediate in choline to TMA metabolism), it is possible that there simply was not enough betaine substrate available to stimulate *grdH*-containing microbiota proliferation to significant, detectable levels. L-carnitine, our experimental TMA-containing substrate and primary TMA source in this experiment, does not form betaine, but γ-butyrobetaine, as it is being metabolized. Neither L-carnitine nor γ-butyrobetaine have been shown to be metabolized by *grdH*. The scarcity of the substrate could cause microbiota containing the *grdH* gene to be outcompeted in the intestinal microbiome, and (in a similar fashion to the *cntAB* gene described above) were only able to proliferate when competing populations were greatly reduced by the broad-spectrum antibiotic use in the ABX group.

We originally planned on quantifying the abundance of the L-carnitine metabolizing gene *yeaWX* as well, but we were unable to successfully to target and amplify it. *YeaWX* is similar to *cntAB* in that the genes are located proximally to genes coding for choline/betaine/L-carnitine transporters; both genes are also located near other microbial genes associated with malate or succinate use (byproducts formed during TMA

formation from L-carnitine) and share 74% sequence identity[27]. Given the sequence similarities, close proximity to choline/betaine/L-carnitine transporter and malate/succinate metabolizing genes, some researchers consider *yeaWX* as part of the *cntAB* cluster of enzymes proposed by Zhu et al. [26,51]. The only taxa *yeaWX* have been found in within available literature have been *E. coli* strains[27]. Neither distribution nor abundance of gene have been reported in the literature. Due to time constraints, the complex nature of the primer design process, and difficulty associated with qPCR method development, we discontinued our efforts to quantify *yeaWX* gene abundances and decided to instead recommend it for future work.

Several studies have demonstrated that the TMA-forming communities in the intestinal microbiome are very subject to changes in dietary patterns[25,29,73]. These changes in community are accompanied by a shift in the pathway or enzyme in which TMA is produced. The variation in the dietary patterns of humans provide different TMA-containing substrates, leading to the simultaneous production of TMA from multiple enzymatic pathways, suggesting a single dietary intervention may not successfully lower TMA as intended.

While we were able to gather several data regarding the influence of garlic extract on TMAO production, some limitations must be acknowledged. Firstly, the dietary intervention was originally planned for 6 weeks but was reduced to only 13 days; we decided to end the study prematurely to preserve statistical power. It is possible that we may have seen a different effect if the mice were treated for the originally planned intervention length. Second, our animal diet, while free of any L-carnitine, did contain choline, which has no reported relationship with garlic extracts or their functional components, but is readily converted to TMA in the presence of an intact intestinal microbiome containing functional copies of the *cutCD* gene. It is possible that the presence of choline in the diet allowed *cutCD*-containing organisms to dominate the intestinal microbiome and skew the growth and proliferation of *cntAB*/*grdH*-containing organisms. Mice consuming the chow diet ingested 250 mg/kg/d of choline bitartrate in addition to 2000 mg/kg/d of L-carnitine. This dose of choline, while lower than the L-carnitine dose, could be a sufficient enough quantity of substrate to be metabolized to TMA by the intestinal bacteria and could potentially cause allow those organisms to outcompete those metabolizing L-carnitine.

Collectively, these findings indicate that garlic extracts rich in alliin or allicin do not significantly impact postprandial TMAO levels following an L-carnitine dose. However, these garlic compounds appear to enhance shunting of L-carnitine to γ-butyrobetaine production, which can in turn increase carotid atherosclerosis and cardiovascular disease risk.

3.5 References

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CHAPTER 4: CONCLUSIONS & FUTURE DIRECTIONS

4.1 Conclusions

The overall objective of this work was to evaluate the potential of garlic extracts rich in bioactive organosulfur compound allicin and its metabolic precursor molecule alliin in the inhibition of TMAO formation. We accomplished this through 13-day dietary intervention with garlic extracts (rich in alliin or rich in allicin), followed by quantitative analysis of whole blood TMAO, L-carnitine, and γ-butyrobetaine using UPLC-MS/MS. We also quantified shifts in abundances of L-carnitine metabolizing genes (*cntAB* and *grdH*), hepatic *Fmo3* mRNA transcript, and hepatic FMO3 protein expression using quantitative real-time PCR (qPCR), reverse transcription PCR (RT-PCR), and ELISA.

A previous study showed that mice provided chronically high (4 weeks) levels of dietary L-carnitine have significantly reduced TMAO production response when provided a daily dose of allicin via gastric gavage[1]. Our research was designed to determine if this compound delivered in a garlic extract containing other bioactive garlic compounds would achieve similar attenuation. Our results indicated that treatment with garlic extracts rich in either allicin or alliin did not significantly lower circulating TMAO levels. Treatment with extracts did appear to lower postprandial levels of TMAO, but only 8 hrs after L-carnitine dose. Furthermore, this reducing effect at 8 hrs was mirrored in the negative (NegC) and positive (PosC) control groups, which may suggest that the lower TMAO levels observed are unrelated to the use of garlic extracts.

Though our use of garlic extracts did not prevent TMAO formation when supplemented with L-carnitine, they did appear to be associated with higher circulating and postprandial levels of L-carnitine metabolic intermediate, γ-butyrobetaine. The subjects provided garlic extract (aged or fresh) with L-carnitine solution saw significantly higher γ-butyrobetaine levels than subjects provided water or broad-spectrum antibiotics. The AUC for γ-butyrobetaine in groups treated with garlic extracts were also significantly higher than in groups provided water, broad-spectrum antibiotics, or L-carnitine alone. These findings suggest that the use of garlic extracts to lower TMAO levels in diets high in L-carnitine may associate with increased cardiovascular risk by shunting L-carnitine metabolism into proatherogenic γ-butyrobetaine production.

Based on these data, we sought to further investigate the effects of garlic extracts on the gastrointestinal microbiome and its capacity to produce TMA from L-carnitine. We sought to quantify genes that metabolize L-carnitine to TMA in order to gain insight on the impact of garlic extracts (if any) on the abundances of microbiota possessing these genes and their expression. We also quantified the hepatic *FMO3* genes and translated FMO3 protein to determine if garlic extract treatment would lower TMAO production by reducing the abundance of bacteria containing these genes or inhibiting of oxidation of TMA in the host liver. Our results showed no indication that the use of garlic extracts prevented the growth of these organisms, as qPCR results did not show any significant changes in gene copies following treatment. Furthermore, use of garlic treatments did not appear to alter mouse FMO3 protein expression.

4.2 Future Work

Future studies in this field should be conducted in a similar fashion with modifications to some of the aspects of the experiment, including the garlic extract composition, treatment groups, and model organism.

A study utilizing similar experimental design with garlic extracts varying in alliin or allicin concentration should be performed in order to provide clarity on the impact of extract supplementation on circulating TMAO levels and propose appropriate extract concentrations of alliin/allicin that may associate with this effect. In this proposed study, three fresh extracts and three aged extracts will be produced using our method detailed in Chapter 3. Each fresh extract will have a 1X, 5X, or 20X concentration of alliin and each aged extract will have a 1X, 5X, or 20X concentration of allicin. These different treatments will be supplemented to mice in separate treatment groups (e.g. AL-1X, AL-5X, AL-20X) for 13 days in between L-carnitine challenges as previously described with 8 h blood collection, animal euthanasia, and UPLC-MS/MS analysis of samples following the second challenge. In our current work presented here, we did achieve higher allicin levels in our aged extracts than our fresh extracts, but they were not statistically significant differences. We believe that by producing and supplementing garlic extracts at varying concentrations, we may more accurately determine the effect of extract supplementation on circulating TMAO levels. If TMAO production is successfully lowered, the data collected should also clarify the concentration(s) of alliin or allicin within the extract that must be supplemented to observe that effect.

Another study could be performed with additional treatment groups in place. Such treatments should include garlic extracts without L-carnitine, broad-spectrum antibiotics without L-carnitine, a synthetic allicin, and a purified allicin extracted from garlic. This study would be conducted in a similar fashion, with an initial L-carnitine challenge, followed by 13-day supplementation of a single treatment, followed by a second L-carnitine challenge and subsequent 8 h blood collection. By including additional treatments groups, researchers can investigate multiple dietary intervention strategies at once and can provide more meaningful results, allowing researchers to compare the efficacy of garlic extracts more precisely with other validated TMAO lowering treatments. Using an aged/fresh extract without L-carnitine would serve as another type of control. This dietary intervention should allow us to isolate the effects of fresh/aged garlic extracts on γ-butyrobetaine levels and determine if the increased circulating γ-butyrobetaine levels that we associated with extract supplementation is produced from the gavaged L-carnitine, or another source. Similarly, the use of broad-spectrum antibiotics without the addition of L-carnitine would serve as a type of control group, allowing us to isolate the effect of broad-spectrum antibiotics on TMAO and confirm their role in lowering circulating TMAO levels.

The inclusion of a synthetic allicin as a treatment is of particular interest. The Wu et al. 2015 study supplemented mice with a purified synthetic allicin rather than a garlic extract rich in allicin[1]. The inclusion of a synthetic allicin allows for a more direct comparison of our findings here with that of the previous study and could provide clarity on extract properties that may have influenced our results, such as bioavailability of allicin within the extracts, whether or not the other extract compounds (i.e. allyl sulfide) play a role in the reducing of TMAO, or a matrix effect[1].Variable doses of fresh and aged

garlic extracts should be tested, as the cardioprotective, antioxidant and antimicrobial activity of allicin and garlic extracts is reported to be dose-dependent[2–4]. A similar study should also be conducted using human fecal samples as opposed to mouse feces. These studies would use a similar experimental design as ours, in which initial postprandial TMAO levels would be assessed using an L-carnitine challenge. During this time, human fecal samples should be collected and analyzed for the abundances of microbial DNA containing L-carnitine metabolizing genes *cntAB* and *grdH* prior to dietary intervention. Subjects would then be supplemented fresh or aged garlic extract, broad-spectrum antibiotics, a negative water control, or a positive L-carnitine control for 13 days, after which the subjects or animals would undergo a second L-carnitine challenge to assess the impact of the garlic extract treatment on the subject's circulating/postprandial TMAO levels. During this second challenge, human fecal samples should be collected a second time and tested for the abundances of the same L-carnitine metabolizing genes. While animal models can be useful for modeling human metabolism, the intestinal bacterial communities of humans and non-human animals can greatly differ. In human fecal samples, *cntAB* and *grdH* genes are detected in 26 and 79.2% of samples respectively, whereas in non-human mammalian samples, they are detected in 42.7 and 55.1% respectively; these differences are further reflected in the phyla detected within the samples[5,6]. The use of human fecal samples as opposed to murine samples would provide results that are more relevant to the human intestinal microbiome, thereby allowing more accurate determination of the influence (if any) on the composition and metabolic functions of the gastrointestinal microbiome.

The current study analyzed the abundance of genes involves in the metabolism of L-carnitine to TMA but did not associate those gene abundances with specific taxa or enzymatic function. Future experiments studying the garlic extract influence on the gastrointestinal microbiome should involve employ a high-throughput sequencing method targeting the 16s rDNA gene from gastrointestinal isolates of each individual animal in each treatment group following treatment. Gastrointestinal isolates serve as better representative samples of the intestinal microbial community than fecal samples, and this level of sequencing will allow researchers to identify any major shifts in taxa that are associated with treatment and/or higher circulating TMAO levels. Shotgun metagenomic sequencing should also be performed on intestinal contents to more accurately quantify the *cntAB*/*grdH* gene copies present in the gastrointestinal metagenome; it has been shown that both the microbiota composition and metabolic function of the intestinal microbiome can significantly differ when using fecal versus intestinal samples[7]. This analysis will further identify the taxa present and clarify the functionality of the microbiome, which cannot be determined using 16S rDNA amplicon reads or qPCR. In this case, it would allow us to determine if the treatments can shift the microbial community towards TMA production. Following these high-throughput analyses, α and β diversity should be assessed, allowing researchers to determine if garlic extracts improve host intestinal bacteria diversity and if they cause a shift in population across treatments. Correlating L-carnitine metabolizing gene abundances with the abundances of various microbial taxa and evaluating shifts in diversity of the intestinal microbiome following treatment with allicin/garlic extracts will shed light on the effects of sulfur-containing garlic compounds on the structure of the intestinal microbiome.

In summary, based on our data, the use of garlic extracts rich in allicin or alliin as a dietary intervention is not recommended for reducing TMAO production without further research. Our study has increased our understanding of the L-carnitine to TMAO metabolic pathway. We believe that these results can be used to develop new studies using garlic organosulfur compounds, TMAO, and the intestinal microbiome. While the results of our study did not agree with that of Wu et al., our findings do not necessarily invalidate theirs. If anything, these results highlight the complex interactions of phytochemicals (particularly organosulfur compounds) derived from garlic, the intestinal microbiome, and production of TMAO and suggest the need for clarification of this poorly understood pathway.

4.3 References

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