ABSTRACT

MCAMIS, ASHLEY MARIE. Inhibition of Gut Microbial Trimethylamine Production by Blueberries: The Potential Role of Chlorogenic Acid- (Under the direction of Dr. Andrew Neilson)

Cardiovascular diseases (CVDs) exert a large impact on populations worldwide, responsible for approximately 32% of all deaths, with atherosclerosis-related conditions being the leading cause of death in the United States. Elevated levels of trimethylamine N-oxide (TMAO), a bioactive gut microbiota-derived metabolite, in the blood have been linked to an increased risk of atherosclerosis and is produced when choline is metabolized to trimethylamine (TMA) by specific gut bacteria. With no FDA-approved drugs available to reduce TMAO, dietary interventions present a promising alternative. Chlorogenic acid (CGA), a phenolic compound abundant in blueberries, has shown potential to inhibit TMA production in preliminary studies. Due to the accumulation of phenolics such as CGA in the gut, they are considered promising compounds for inhibiting TMA production and subsequent TMAO levels. In an *ex vivo-in vitro* human fecal fermentation model it was previously demonstrated that whole blueberries significantly reduce TMA production compared to controls. The CGA content in blueberries varies depending on cultivar, growth conditions, post-harvest treatment, and storage conditions; however, it remains unclear whether these variations in CGA levels influence the TMAO-lowering activity of different blueberry varieties. The presented work investigated the effects of specific components of blueberries to further understand how phenolic compounds may influence CVDs and their potential as preventative agents. This thesis explores the optimization of fermentation conditions for sugar-rich fruits, as sugars are absorbed in the upper gastrointestinal tract and would not be present in the colon. The impact of CGA content on TMA inhibition was investigated using peel fractions from 20 genetically distinct blueberry cultivars,

chosen based on their high and low CGA content. This approach excluded the pulp, which contains most of the fruit's sugar, to better assess the role of CGA in TMA inhibition. No significant differences were observed in TMA inhibition between high and low CGA cultivars in kinetic curves or area under the curve (AUC) values for choline-d_9 and TMA-d_9 inhibition. However, significant differences were observed between peel fractions compared to controls, suggesting that the peel, where most phenolics are concentrated, provides similar TMA-lowering benefits across all blueberry varieties, suggesting that the CGA content of cultivars is not a crucial factor for lowering TMA. Future studies, including rodent and human studies, are needed to better understand why blueberries may inhibit TMA and potentially TMAO, as *in vitro* studies have limitations, and the mechanism of TMA production remains unclear. This work highlights the potential TMA-lowering and cardiovascular benefits of blueberries, identifying the source of their inhibitory effects and deepening our understanding of their interaction with gut bacteria.

Inhibition of Gut Microbial Trimethylamine Production by Blueberries: The Potential Role of Chlorogenic Acid

by
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BIOGRAPHY

I grew up in Colorado Springs, Colorado, and attended Lewis Palmer High School, where I spent much of my time involved in drama club, playing tennis, and enjoying outdoor activities. After graduating, I moved to Boise, Idaho, to attend Boise State University, where I worked as an undergraduate research assistant in the ISBL lab focusing on protein expression and purification. I earned a B.S. in Health Sciences with a concentration in science and a minor in biology. After graduation, I took a year off to work and spend time skiing, hiking, and backpacking before moving to North Carolina to pursue an M.S. in Food Science.

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CHAPTER 1 INTRODUCTION AND AIMS

1.1 Introduction

Cardiovascular diseases (CVDs) exert a large impact on populations worldwide, accounting for ~32% of all deaths[1]. This multifaceted group of disorders includes conditions such as heart attacks and strokes and represents a leading cause of morbidity and mortality[2]. The prevalence of cardiovascular diseases necessitates the need for comprehensive research on pharmaceuticals, surgical interventions, and complementary strategies such as dietary and lifestyle changes to mitigate its influence on global health. Atherosclerosis linked diseases are the leading causes of death in the United States[3]. Atherosclerosis develops when cholesterol, fat, blood cells, and other substances form plaque within the artery causing the walls to narrow and reducing blood flow[3]. Studies suggest that elevated concentrations of trimethylamine N-oxide (TMAO) in the blood are associated with increased risk of atherosclerosis[4,5]. TMAO, a bioactive gut microbiota-derived metabolite, is produced when choline is metabolized to trimethylamine (TMA) by specific gut bacteria[6]. These bacteria contain the cut*C/D* gene cluster that encodes choline TMA lyase[7]. Other important bacterial gene clusters include *CntA*/B and *YeaW*/X that may lead to TMAO formation from other substrates such as carnitine, betaine, and γ-butyrobetaine[8,9]. TMA is absorbed into circulating blood and further oxidized to TMAO by hepatic flavin-containing monooxygenase 3 (FMO3) in the liver[6]. Currently, there is no FDA-approved drug to control or lower TMA or TMAO levels, emphasizing the need for novel non-pharmaceutical strategies to manage TMAO production. There is a growing interest in the TMAO-lowering activities of dietary phenolics. Phenolics may reduce TMAO levels by various mechanisms, including reducing the abundance of bacterial genera that convert choline into TMA, or direct inhibition of TMA lyase. High concentrations of phenolics may accumulate in the gut, rendering them as promising TMAO-lowering compounds[7]. Chlorogenic acid (CGA) is a naturally occurring phenolic that our lab has identified as a potential TMA/TMAO production inhibitor[7]. Blueberries are rich in CGA and have been proven to have cardioprotective benefits; however, it is unknown whether variation in CGA level contributes to different TMAO-lowering activities between blueberry varieties. Additional information is needed to verify the effectiveness of CGA-rich blueberries as inhibitors of TMA/TMAO production and to determine if higher concentrations of CGA content correlates to greater activity.

1.2 Aims

1.2.1The overall aim of this thesis is to evaluate whether CGA is a key bioactive that enables blueberries to inhibit formation of the gut microbial metabolite TMA (a precursor to the pro-atherogenic compound TMAO). The **central hypothesis** is that CGA content is a primary determinant of the TMA-lowering benefits of blueberries.

1.2.2 The specific objectives of this thesis project are to:

1. Determine the relative impacts of phenolic (CGA) and non-phenolic components (sugar, fiber, skin, pulp, etc.) of blueberries on TMA production; and
2. Determine whether CGA content correlates with reduced TMA production in genetically diverse blueberry cultivars (germplasm diversity and biparental mapping populations) with a large spread of CGA using an *ex vivo-in vitro* human fecal fermentation model.

1.3 Rationale and Significance

1.3.1 Rationale: TMA and TMAO production are linked to specific gut microbiota expressing the *cutC/*D gene cluster. These metabolites are hypothesized to increase the risk of human cardiovascular disease. The inhibition of the gut microbiota-derived metabolite, TMA, and subsequent metabolite, TMAO, is a promising strategy to reduce incidences of CVD. Recent studies have noted the inhibition of TMA lyase through high concentrations of CGA and its metabolites in the gut[10–12]. Due to the limited bioavailability in the stomach and small intestine, CGA (or its microbial metabolite, caffeic acid) has the potential to accumulate in the lumen of the colon, where it can potentially reduce TMA production. CGA-rich foods offer a promising strategy to reduce TMA production without the need for pharmaceutical intervention. Identifying CGA-rich foods (like blueberries) as potential TMA/TMAO-lowering components of the diet provides a practical solution to implement in populations with elevated TMA/TMAO levels. CGA content varies significantly in blueberries; identifying blueberry bioactives, such as CGA, associated with greater TMA-lowering activity will drive the cultivation and consumption of berries with enhanced health benefits. By accomplishing the objectives outlined in this proposal, the data will facilitate further studies involving animal and human trials using CGA-rich blueberries to mitigate TMA levels in at-risk populations.

1.3.2. Significance: The prevalence of cardiovascular diseases necessitates the need for comprehensive research on diet and lifestyle interventions to mitigate its influence on global health. Atherosclerosis linked diseases are the leading causes of death in the United States[3]. Elevated TMAO levels in the blood is associated with atherosclerosis and other cardiovascular disease pathologies in specific models[13–17]. With no current FDA-approved medication for lowering TMA or TMAO levels, exploring complementary (i.e., non-drug) methods to reduce potential TMAO is crucial to aid in the prevention of atherosclerosis and other cardiovascular diseases. Utilizing dietary substrates to reduce pro-atherosclerotic compounds may offer an accessible, cost-effective strategy to help combat the burden of atherosclerosis.

1.4. Anticipated outcome: I anticipate that this study will generate evidence that chlorogenic content correlates to TMA-lowering activity in blueberries. The significance of this thesis is that this work will establish a fundamental understanding of whether consuming CGA-rich blueberries is a practical approach to regulate TMA (and potentially TMAO) levels. This is crucial due to the substantial burden of atherosclerosis and the current absence of effective TMA-lowering strategies.

CHAPTER 2 REVIEW OF THE LITERATURE

2.1 Introduction: In this literature review, I review production of the gut-microbiota-derived metabolite trimethylamine (TMA), its oxidation to trimethylamine N-oxide (TMAO), and their association with cardiovascular diseases (CVDs). I begin by first reviewing cardiovascular diseases (CVDs), specifically atherosclerosis, and the socioeconomic impact they have on populations worldwide. Next, I highlight the link between CVDs, dietary choline, TMA, and TMAO. Although dietary choline is a semi-essential nutrient, its metabolism by gut-microbiota produces TMA and subsequently TMAO, presenting a challenge in managing CVD risk. I then consider non-pharmaceutical interventions, focusing on phenolic compounds like chlorogenic acid (CGA) for inhibiting TMA production. Highbush blueberries are highlighted for their variable CGA levels across different cultivars, potentially leading to varying levels of TMA inhibition. I conclude the review by examining the connections between CVDs, blueberries, and their role in reducing TMA production. I also highlight existing unknowns and outline future research directions, which lead to the hypothesis of this project.

2.2 Cardiovascular Disease: Cardiovascular diseases (CVDs) is an encompassing name for a group of disorders that affects both the heart and blood vessels. Coronary artery disease is the most prevalent type of heart disease and is caused by plaque buildup in the artery walls, reducing blood flow to the heart and the remainder of the body[18]. Heart attack with symptoms of chest pain, weakness, shortness of breath, and pain in the arm is often the first sign of narrowed arteries[18]. People that smoke tobacco, are overweight, have low physical activity, have unhealthy eating habits, or have a family history of heart disease are at a higher risk of CVDs[18]. Although these CVD risks can be present in anyone, specific racial and ethnic groups are affected more by CVDs[19]. The social determinants of health may correlate to a person's likelihood of developing and treating cardiovascular disease. Due to a lack of access to effective health care, built environments, quality education, nutritious food, etc. people living in low-and middle-income countries make up at least three-quarters of the deaths caused by CVDs[18,19]. Emerging evidence indicates that CVDs can exacerbate poverty due to substantial health expenses and high out-of-pocket spending, placing a massive health and economic burden on populations worldwide[19]. Changes to lifestyle are often the first steps in treating CVDs and include eating a heart healthy diet, frequently participating in physical activity, controlling blood pressure and cholesterol levels, and managing stress[18,20,21]. Beyond lifestyle changes, medications to control blood pressure, cholesterol, blood sugar, and weight as well as procedures or heart surgery are becoming increasingly more common[21].

2.2.1 Atherosclerosis: Atherosclerosis is the buildup of atheromatous plaque formed from cholesterol, fat, blood cells, and other substances that cause the thickening or hardening of veins leading to reduced blood flow and the potential to develop CVDs[22,23]. Atherosclerosis linked diseases are the leading cause of death in the United States[3]. Risk factors include genetics, age, unhealthy diet, lack of exercise, high blood pressure, high blood cholesterol, and smoking[3,24]. Plaque develops in the intima and its underlying smooth muscle when small deposits of cholesterol crystals trigger the response of inflammatory cells to the damaged areas of the artery[3,22]. Inflammatory cell signals cause cholesterol, fat, and cellular waste to accumulate at the damaged site. The buildup attracts white blood cells, which work to eliminate cholesterol, forming plaque as they clump together[3]. As the plaques grow and bulge within the artery, blood flow is decreased. Deposits of calcium and production of connective tissue in the lesion causes hardening of the arteries, resulting in uneven artery surfaces that promote clot formation and thrombosis[22]. This disorder develops slowly overtime and may begin in childhood; about 50%

of individuals ages 45 to 84 in the United States have atherosclerosis and are unaware of it[3,24]. Many individuals experience symptoms and conditions of atherosclerosis such as heart attack, stroke, or organ damage suddenly and with no warning signs[3]. Prevention of atherosclerosis begins with lifestyle changes and include following a healthy diet, exercising, and avoiding tobacco products with more serious cases requiring medications, procedures, or surgeries[25,26]. Patients diagnosed with atherosclerosis face a significant economic burden, as the cost of health care can be difficult to manage for people that have a lack of insurance or are low-income. From the National Health Interview Survey (NHIS) reports from 2013-2017, it was noted that 45.1% of families that have at least one family member diagnosed with atherosclerotic cardiovascular disease (ASCVD) faced financial hardship. An additional 19% of families with at least one family member diagnosed with ASCVD were unable to pay their medical bills[27].

2.2.2 The Gut Microbiome and CVDs: The gut microbiome is part of the human gastrointestinal system, particularly the distal small intestine and large intestine. The intestines host trillions of microorganisms including bacteria, viruses and fungi, with bacteria being the most abundant, occupying distinct niches, producing metabolites and contributing to a complex ecosystem[28]. These bacteria are generally acknowledged as commensal, with their metabolic activity playing a critical role in processes such as the breakdown of plant polysaccharides and the support/ development of a well-functioning immune system[29]. In recent years, there has been growing evidence that certain gut-microbiota derived metabolites play a role in the development of inflammatory bowel disease, liver cirrhosis, rheumatoid arthritis, and CVDs[30]. In particular, the metabolite trimethylamine (TMA), produced by gut bacteria containing a TMA-lyase complex from choline, L-carnitine, and γ-butryobetaine, serves as a precursor to trimethylamine

N-oxide (TMAO). Elevated physiological levels of TMAO are linked to an increased risk of atherosclerosis[6,30].

2.2.3 Trimethylamine: Trimethylamine (TMA) is a tertiary amine produced by about 1% of total intestinal bacteria containing the *cutC/D* gene cluster using dietary quaternary amine substrates such as choline, phosphatidylcholine, and acetylcholine.[6,31,32]. This production primarily takes place in the digestive track where *cutC/D* gene-complex containing bacteria are commonly found in the human body[8]. The human gut microbiome is highly complex and dynamic, posing challenges in identify the bacteria containing the *cutC/D* gene cluster. The identification of these microbes has become increasingly accessible using meta-omics. Metagenomic analyses bridge the gap between functional and phylogenetic changes in gut microbes to their role in human pathological conditions, accurately identifying bacteria of interest[33]. The production of TMA relies on both the initial dietary substrate and the enzymatic system responsible for metabolizing the substrates into TMA (Figure 2.1). Choline trimethylamine-lyase (TMA-lyase), which metabolizes choline into TMA, is encoded by the *cutC/D* gene cluster and is found in the genetic code of specific anerobic gut microbiota[6,7]. However, Ramireddy *et al*. noted that TMA production is not solely dependent on bacterial gene expression of cut*C/D*. Rather, other factors, such as the glycyl radical-activating protein, the presence of S-adenosylmethionine (SAM), and transcriptional regulators, play a role in determining TMA production[9,34]. This is further supported by Ferrell *et al*., who documented that circulating TMAO levels may not be predictable based on microbiome composition, as gene copy number does not correlate with microbial metabolic activity[35]. Other TMA-lyase complexes include *cntA/B* and *yeaW/*X which metabolize L-carnitine and γ-butryobetaine, respectively[7,36] . The cutC enzyme cleaves the carbon-nitrogen bond in choline, subsequently the presence of the activating protein cutD and S-adenosylmethionine (SAM) produce TMA and acetaldehyde[7]. Choline and phosphatidylcholine are commonly found in animal products such as eggs, fish, red meats, and high-fat dairy products in sizable quantities[6]. A typical individual living in the United States today is estimated to eat ~730-1,040 mg/day of choline, this is almost double the daily dietary allowance recommended by The Food and Nutrition Board[37,38]. Although precursors to the formation of TMA are commonly found in the human diet, reduction of these dietary substrates as a primary solution to lowering TMA production is not recommended as it may result in adverse health effects[39]. Choline is a semi-essential nutrient that the body requires to produce acetylcholine, phospholipids, lipoproteins, etc. and plays a crucial role in membrane and brain development in fetuses[40]. The relationship between choline and CVDs shows inconsistent findings, suggesting that the issue may not stem directly from choline itself but rather from its conversion to TMAO, which is strongly associated with CVDs[39]. The conversion process varies significantly between individuals, highlighting the complexity of its impact[39].

2.2.4 FMO3: Flavin-containing monooxygenases (FMOs) is an encompassing name for FAD-dependent and NADPH-dependent enzymes responsible for catalyzing the oxidization of a wide variety of substrates that contain nitrogen, phosphorus, or sulfur. This process produces water and NADP+ across a diverse range of nucleophilic compounds[41,42]. This includes the oxidation of TMA into TMAO[7,42,43]. There are eleven different fmo genes identified in humans, however, FMO3 stands out as the predominant functional form located in human livers, and as the primary enzyme that oxidizes TMA to TMAO (Figure 2.1.)[41,43]. Factors such as sex, age, animal species, and external factors influence the expression of FMO3, with females exhibiting the highest FMO3 expression in the liver compared to males[7,42,44,45]. Although females express higher levels of the FMO3 enzyme, pre-menopausal woman that produce estrogen, a potential cardioprotective agent, have lower CVD risks compared to males[46,47]. FMO3 is genetically insufficient, meaning while its expression is influenced by genetics, mutations or variations in *fmo3* can impair the enzyme's function[48]. As FMO3 is responsible for the oxidation of TMA into TMAO, a dysfunctional form of FMO3 can lead to a buildup of TMA. TMA produces a distinct odor described as 'rotting fish'. This accumulation can lead to some individuals experiencing trimethylaminuria (TMAU), a condition where bodily excretions such as sweat, breath, or urine, smell of fish[48,49]. People with TMAU are often ridiculed and experience significant psychological and emotional distress. Variations in gut microbiota may provide a significant influence on the expression and activity of FMO3[44].

2.2.5 Trimethylamine N-Oxide: Trimethylamine N-Oxide (TMAO) is synthesized from trimethylamine (TMA) which is made when dietary choline or other substrates are metabolized by gut microbiota (Figure 1.). Once released, TMA is absorbed through the intestinal mucosa and travels through the bloodstream via the hepatic portal system which delivers blood directly to the liver rather than to the heart[50]. Once it reaches the liver, TMA is oxidized to TMAO by hepatic flavin monooxygenase 3 (FMO3)[51]. Preformed TMAO is present in fish and other seafood species, where it can bypass gut microbiota metabolism and be absorbed directly into the blood stream[52]. While the consumption of fish can lead to increased levels of circulating TMAO, studies have shown that TMAO levels return to baseline within a day after consumption[53]. Elevated TMAO levels in the blood is associated with atherosclerosis and other cardiovascular disease pathologies in specific models[13–17]. Although elevated TMAO levels is associated with CVDs, the link between the two is not consistent and varies depending on the studied population[54]. In human and rodent models, dietary choline is correlated to elevated TMAO and the promotion of atherosclerosis[17,55]. Though the mechanism by which TMAO promotes atherosclerosis remains theoretical, it is speculated that physiological levels of TMAO induces the expression of inflammatory cytokines that upregulate movement of immune cells to atherosclerotic sites[13,56]. The interruption of cholesterol transport enhances buildup of lipids such as very-low density lipoprotein-cholesterol (VLDL-c) and low-density lipoprotein-cholesterol (LDL-c) in aortic foam cells[57–59]. As immune cells work to remove these lipids and cholesterol from the atherosclerotic site, plaque forms as they clump together[3]. Blood flow is decreased as the plaques grow and bulge within the artery, which is described as atherosclerosis. TMAO has been shown to attenuate hepatic and serum levels of cholesterol, shedding light on the importance of the compound as a potential compensatory molecule in the reduction of cholesterol overload[60]. TMA production is dependent on metabolism by gut microbiota, hence dietary strategies to reduce TMA is of particular interest. Although reduction in dietary choline appears to be a plausible solution, choline is a semi-necessary nutrient in the diet and plays a crucial role in the synthesis of phospholipids and neurotransmitters[61]. Altering the composition of gut microbiota and suppressing TMA production could offer a more effective alternative to maintain original dietary choline requirements[39].

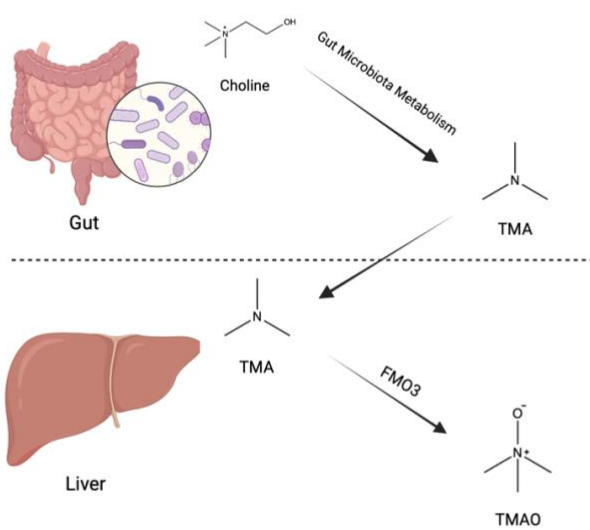


Figure 2.1 Pathway of trimethylamine (TMA) and trimethylamine N-oxide (TMAO) formation from choline. Visuals created in https://BioRender.com

2.2.6 Strategies to Reduce TMA/TMAO: With no current FDA-approved medication for lowering TMA or TMAO levels, exploring complementary (i.e. non-drug) methods to reduce TMAO may be beneficial in the prevention of atherosclerosis and other cardiovascular diseases. The gut microbiota plays a crucial role in TMA metabolism, and the addition of antibiotics decreases the presence of bacteria carrying the *cutC/*D gene, ultimately decreasing TMA production. This is evident by the decrease in TMA production during antibiotic treatment and its subsequent return after antibiotics are removed[62]. Although the use of antibiotics effectively reduces TMA production by bacteria with the cut*C/*D gene, the reduction of other probiotics and commensal bacteria could lead to health issues, such as the potential development of *C. difficile*[63], given their general positive impact on human health[64,65]. FMO3 is the enzyme responsible for converting TMA into TMAO. Lowering its expression or the direct inhibition may provide an effective strategy to decrease TMAO production[66]. TMA exudes an odor described as ‘rotting fish’, some individuals who have a dysfunctional metabolism of TMA may experience trimethylaminuria (TMAU) which causes sweat, breath, urine, or other bodily excretions to smell of fish[48,49,67]. The reduction or inhibition of FMO3 may result in a buildup of TMA, potentially causing unwanted side effects such as TMAU. Reducing dietary intake of choline and carnitine proves to be an effective strategy to reduce TMA and TMAO levels. This is supported by the correlation between higher choline intake and elevated TMAO levels, with vegetarians exhibiting lower fasting plasma TMAO levels compared to omnivores [62]. Although the reduction in dietary substrates effectively reduces levels of TMA and TMAO, individuals who produce low levels of TMAO gain no benefit, and choline, a semi-essential nutrient, plays an important role in the synthesis of phospholipids and neurotransmitters. Rather than reducing choline intake, the consumption of competing substrates, such as inhibitors like 3,3-Dimethyl-1-

butanol (DMB, a known TMA-lyase inhibitor[10,68,69]) or other potential inhibitors sourced from the diet or synthetic compounds can offer an alternative approach to reduce TMA levels without significant changes to diet. Direct inhibition of TMA production involves using an inhibitory compound in the presence of the enzyme and substrate. If the inhibitory compound is present in food, it can be consumed with choline-containing meals, ensuring that the inhibitor is present when the substrate is available. This method eliminates the need for dietary restrictions and offers the possibility of direct inhibition in the gut. However, it may not always be practical to consume the inhibitory compound simultaneously with a high-choline meal. Indirect inhibition of TMA allows the inhibitory compound to be absent when the substrate is present. This requires the chronic consumption of the inhibitor over an extended period, potentially reducing the number of bacteria containing the cut*C/D* gene complex or decreasing the expression of the TMA lyase enzyme. While this approach shows promise for TMA inhibition without the need to consume the inhibitor simultaneously, the effects would only be observed after prolonged consumption. Furthermore, since the gut microbiome can quickly adapt, the inhibitory effects may diminish if the compound is not continuously incorporated into the diet

Identifying potential TMA and TMAO-lowering agents is crucial. *In vitro* studies provide various strategies to discover TMA-inhibiting compounds and assess TMA production in their presence. Fecal culturing models, such as MiGut, SHIME, and TIM-2, as well as anaerobic fermentation chambers, simulate the gut environment, facilitating the measurement of labeled substrates over time to quantify the conversion of substrates to TMA[70–73]. *In vitro* studies also allow for the isolation and observation of individual bacterial strains or enzymes, helping to better identify the source and mechanism of TMA production inhibition[74,75]. While these *in vitro* models are effective for identifying inhibitory compounds, they do not fully replicate the complex physiological processes of an *in vivo* model, such as the absorption of digestive components like sugars, the absorption of TMA, or alternative metabolic pathways for TMA beyond its oxidation to TMAO[76,77]. Animal models allow for the study of both direct and indirect inhibition of TMA production after upper gastrointestinal digestion, which addresses the limitations of *in vitro* models[68], albeit with reduce control. Clinical studies in humans help account for individual variations in diet and lifestyle, which influence differences in the gut microbiome. Human studies overcome some limitations of *in vitro* research and offer a clearer understanding of how different people respond to TMA inhibitors, again with less fine control.

2.3 Phenolic compounds: Phenolic compounds are small, secondary metabolites known to contribute to the color, flavor, and antioxidant properties of plants (Figure 2.2)[78,79]. Secondary metabolites are compounds synthesized by plants in response to their environment, possessing diverse functions such as stimulating flowering, serving as antimicrobials, attractants, repellents, or antioxidants[80,81]. These compounds, ranging in size from simple to highly polymerized, are characterized by an aromatic ring that contains one or more hydroxyl substituents[78,82]. Phenolic compounds are classified based on chemical structure and placed into a subclass such as phenolic acids, stilbenes, coumarins, tannins, or flavonoids (Figure. 2.2). Phenolic acids, a group of phenolic compounds containing a carboxylic acid functional group, are one of the most abundant classes of plant phenolics[83]. Hydroxybenzoic and hydroxycinnamic acids are subclasses of phenolic acids and are derived from benzoic acid and cinnamic acid respectively[83,84]. Common derivatives of hydroxycinnamic acids include caffeic and ferulic acid[83]. Procyanidins, a derivative of proanthocyanidins, are synthesized through linking monomeric catechin or epicatechin (or other flavan-3ol monomer) and are classified as A-type or B-type depending on the number and types of connections between monomers[85]. Stilbenes are synthesized by plants as a response to environmental stressors with a monomeric or polymeric chemical structure that contains a 1,2-diphenylethylene nucleus[79,86]. Stilbenes, such as resveratrol, are commonly found in berries and are known to have chemoprotective and cardioprotective effects[86,87]. Flavonoids are divided into two subgroups, anthocyanins and anthoxanthins, representing the most abundant phenolic compound in the human diet[79,88]. The chemical structure of flavonoids features a benzopyrone ring with different phenolic group substituents linked at different positions[88,89]. Within the subgroup of flavonoids, flavanols and anthocyanins are abundantly common in berries. The chemical structure of flavanones is the same as flavanols, but with an added ketone group[90,91]. Anthocyanins are abundant in the vacuoles of plant cells and are responsible for the red, blue, and purple colors of many plants[92].

The bioavailability and bioaccessibility of phenolic compounds in food are limited, with most remaining unreleased and unabsorbed in the upper gastrointestinal tract (stomach and small intestine) following chemical, mechanical, and enzymatic digestion[93]. Due to the low bioavailability, released and unabsorbed free phenolics accumulate in the colon's lumen, where they can interact with anaerobic bacteria[93,94]. Microbial fermentation predominantly occurs in the colon, emphasizing that unabsorbed phenolics have the potential to inhibit formation of TMA[94,95]. Phenolic compounds are of interest due to their known cardiovascular benefits, yet the mechanisms through which they improve heart health remain poorly understood. *In vitro* studies have highlighted the potential of phenolic compounds to inhibit TMA production. Research by Iglesias *et al*. documented that chlorogenic acid, caffeic acid, epicatechin, 4-dihydroxybenzoic acid, and phenolic-rich beverages such as coffee, tea, and cocoa significantly inhibited TMA production compared to controls[12]. While these studies provide valuable preliminary data on potential TMA inhibitors, they do not clarify the mechanisms behind how phenolics cause this inhibition[12,96]. *In vivo* studies, such as the one by Jiang *et al*., examined the impact of polyphenols from hickory nuts on reducing the occurrence of atherosclerosis, obesity index, and inflammation in mice[97]. However, this study and many other animal studies lack a mechanistic component and primarily focus on exploring the potential effects of phenolic compounds without examining the underlying mechanisms. Clinical studies on phenolic compounds and their inhibitory effects are limited in the literature, as TMA and TMAO measurements are usually secondary analyses conducted after the primary focus of the study, which is not typically designed to target TMA and TMAO production. There is a clear need for in-depth, systematic research on phenolics and their potential to inhibit TMA and TMAO. While some studies suggest their role in inhibiting TMA and promoting overall health benefits, significant gaps remain in understanding the mechanisms by which they contribute to the reduction of heart disease.

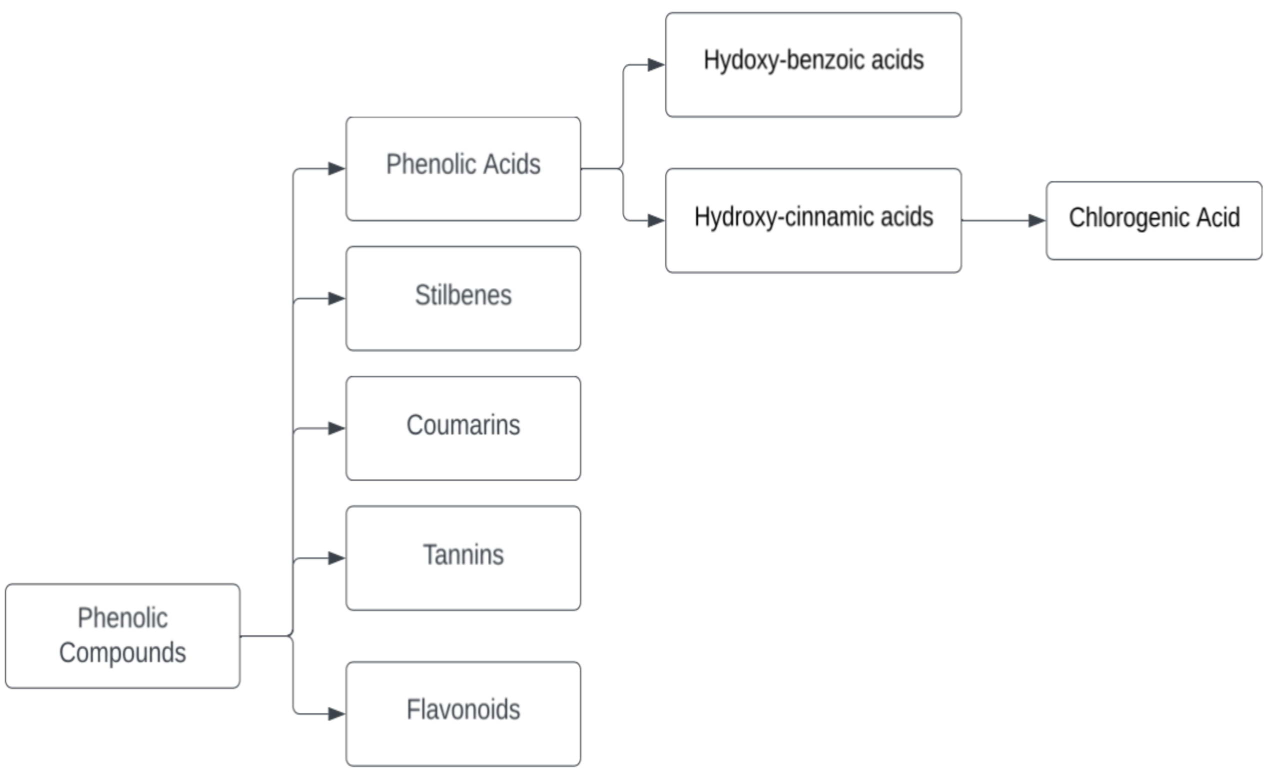


Figure 2.2. Phenolic compound classification chart.

2.3.1 Chlorogenic Acid: CGAs (CGAs) are polyphenolic compounds found in a large variety of fruits and vegetables that are common in the human diet[98,99]. CGA is predominantly found in the skin of fruit compared to the pulp[100]. CGAs is an inclusive term for a set of hydroxycinnamic esters that can be present in several isomeric forms and contain caffeoyl-, dicaffeoyl-, feruloyl-, ,and coumaroylquinic acids[101]. The most abundant CGA isomer found in food is chemically written as 5-caffeoyquinic acid (5-*O*-CQA)[102] and is derived from the esterification of caffeic acid and quinic acid (Figure. 2.3.)[99,103]. Plants exposed to environmental stress produce reactive oxygen species (ROS), which can cause damage to metabolic function within cells[104,105]. CGAs function as ROS scavenging compounds to help reduce oxidative stress and protect metabolic function in plants[106,107]. Major dietary sources of CGA include apples, artichoke, coffee beans, plums, tea, and blueberries (Table 1.1)[108–111]. Across a genetically diverse spectrum of blueberries, CGAs represent the primary cinnamic derived acids, constituting 10-16% of total acids and potentially contributing to the high antioxidant activity in the fruit[112,113]. CGAs are a well-studied group of phenols that exhibit anti-inflammatory, antihypertensive, and antioxidant properties that aid in reducing cardiovascular disease risk and improving overall human health[103,114,115]. Previous work by Iglesias *et al.* documented CGA as a significant inhibitor of gut microbial conversion of choline to TMA *in vitro* through a non-cytotoxic mechanism[10,11,96] It is speculated that CGA may not reduce TMA formation through direct inhibition of TMA lyase; rather, it is suggested that bacteria may prefer CGA as an energy source *in vitro* over choline substrates[10]. However, because there is a variety of substrates present in the colon *in vivo*, the preference of CGA over all other substrates by gut microbiota is unlikely[11]. The potential of CGA to inhibit formation of TMA, as well as the subsequent metabolism to TMAO, presents a promising approach to reduce the risk of atherosclerosis and other CVDs.

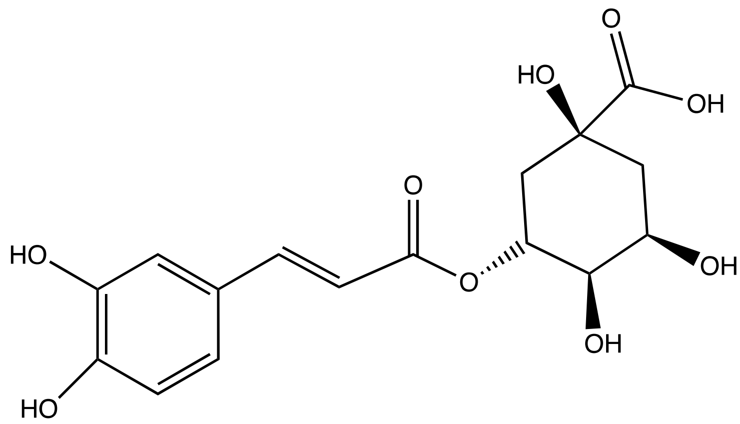


Figure 2.3. Structure of 5-caffeoyquinic acid (5-*O*-CQA), the most abundant form of chlorogenic acid (CGA) found in blueberries.

Table 2.1 Dietary Sources of Chlorogenic Acid	
Food	***Mean Content (mg/100 mL) or (mg/ 100 g)**
Sunflower Seeds	454
Artichoke Heads	202
Red chicory	168
Highbush Blueberry	131
Arabica Coffee	117
Green Chicory	101
Lowbush Blueberry	87
Robusta Coffee	76
Loquat Fruits	55
Rowanberry Juice	54
http://phenol-explorer.eu/contents/polyphenol/475 *Mean content of reported values, content varies greatly	

2.4 Blueberry Composition: Blueberry belongs to the Ericaceae Juss- Health family and the genus *Vaccinium* L.[116]. Blueberries, like most plant foods, exhibit varying levels of sugar, fiber, and phenolic compounds depending on cultivar, growth conditions, post-harvest conditions, and storage environment. Fresh blueberry composition is typically comprised of about 84% water, 9.7% carbohydrate, 0.7% protein, 0.3% fat, and 2.4% fiber[117,118]. A typical serving size of blueberries is about 1 cup (~150 g fresh weight) which contains 21.4 g carbohydrates by difference, 1.09 g of protein, 0.48 g fat, 14.7 g of sugar, and 3.55 g of fiber[118,119]. Of the total sugar content, roughly 0.163 g (1.11%) consist of sucrose, 7.22 g (49.11%) consist of glucose, and 7.36 g (50.06%) consist of fructose[118]. Of the total fiber content, roughly 0.40 g (11.26%) consists of soluble fiber (pectin) and 3.15 g (88.73%) consists of insoluble fiber (cellulose, hemicellulose, lignin)[120]. Procyanidins and flavonols, subclasses of flavonoids, and phenolic acids are the most common phenolic compounds present in blueberries and can make up to 0.3% of the total fresh fruit content[113,117,121,122]. Flavonoids are present in blueberries as flavanols and flavonols (subclasses of flavonoids) and include epi-catechin, catechin, kaempferol, and myricetin[123]. Stilbenes, another subclass of phenolic compounds, are present in blueberries in the form of pterostilbene and resveratrol[86,124,125]. It has been reported that blueberries contain a high concentration of anthocyanins, a subclass of flavonoid, primarily delphinidin, cyanidin, petunidin, peonidin, and malvidin, which can combine with sugar molecules to produce various anthocyanin compounds[123]. In addition to flavonoids, phenolic acids such as caffeic, ferulic, and CGAs represent a large number of phenolic compounds in blueberries. CGA stands out as the most abundant phenolic acid, representing more than 95% of total phenolic acid content in blueberries[113,123,126]. CGA, as well as other phenolic compounds, is predominately found in the skin of blueberries[100].

2.4.1 Variability of CGA in Blueberries: All blueberries, like any other plant food, contain varying levels of secondary metabolites depending on the cultivar (i.e. genotype), growth environment, harvest conditions, post-harvest environment, and other environmental stressors. Secondary metabolites are under tight genetic control and made in response to stressors such as drought, sunlight, foraging animals, insects, nutrient availability, soil, etc.[127,128]. Although all blueberries contain some level of CGA (Table 2.1.), the difference between cultivars can vary by >20-fold[123]. The variation of CGA and anthocyanins is associated with 35 different transcription factors and over 90 genes[129]. The large variety in CGA content is due to the strong genetic control of the identified major effect quantitative trait loci (QTL) in chromosome 2[130]. A QTL associated with high CGA in the same chromosome 2 region confirmed the association of this genome region with CGA[112]. Blueberry genotypes carrying the dominant allele (controlling for high CGA) have up to 11-fold more CGA than genotypes carrying the recessive allele (controlling for low CGA). A moderate to high broad sense heritability (>40%) of catechin, epi-catechin, and CGA is noted in blueberries, indicating the potential to improve these traits through selective breeding[123,130]. CGA stands out as the most predominant phenolic acid in blueberries, accounting for more than 95% of total phenolic acid content[113,123,126]. With a large mean content of CGA, blueberries may be an effective dietary approach for reducing TMAO. Since blueberries contain varying amounts of CGA, it is challenging to make a general claim that all blueberries offer the same health benefits, as their CGA levels (and other bioactive compounds) may influence these benefits. Clinical studies often focus on specific cultivars, which may not be readily available to everyone. Additionally, clinical studies are conducted with carefully controlled materials that have known or consistent CGA content and standardized serving sizes, which may not be applicable to people outside of the study. This issue could be addressed using genomic sequencing, specifically QTL analysis, to identify plant genes, determine phenolic compound levels, and assess their potential impact on the gut microbiome[131].

2.5 CGA, Blueberries, and CVD: CGA and CGA-rich foods exhibit anti-inflammatory, antihypertensive, and antioxidant properties that aid in reducing cardiovascular disease risk and improving overall health[103,114,115]. Current evidence from both animal models and humans supports the cardioprotective benefits of CGA and CGA-rich foods[132–135], including protection from atherosclerosis and other forms of cardiovascular diseases[98,132–136]. The mechanisms by which CGA achieves these benefits are not fully understood but speculated to include the improvement of vasodilation and hypertension (systolic/diastolic blood pressure over 140/90 mmHg), a major risk factor for stroke and congestive heart failure, through inhibition of NADPH oxidase, reduction of ROS, the direct scavenging of ROS, simulation of nitric oxide production, and inhibition of angiotensin-converting enzyme[132,134,137]. CGA and CGA-rich foods prevent the development of atherosclerotic lesions and improve associated biomarkers such as hypercholesterolemia, hyperlipidemia, reduced HDL cholesterols, and foam cell development[98,135,136]. Mechanisms by which CGA prevents the development of atherosclerotic lesions include the increase of cholesterol efflux and lipid metabolism, reducing macrophage activation, and increasing PPAR-α signaling[136,138–141]. Blueberries have gained attention, due to their high CGA content, for their potential to prevent atherosclerosis and other cardiovascular diseases[142–146]. Specifically, Xianli Wu *et al*. outlined various mechanisms through which blueberries help protect against atherosclerosis, including the reduction of inflammation, cholesterol, and oxidative stress, as well as improving endothelial function[142]. Xianli Wu *et al*. also reported that in mice fed with a blueberry diet designed to reflect human serving sizes, four key antioxidant enzymes in the aorta were upregulated, and the area of atherosclerotic lesions were 39% lower compared to mice fed an AIN-93G diet[143]. Researchers and nutritionists often label blueberries as a superfruit due to their well-known antioxidant and anti-hypertensive properties. The unknown question is "which characteristics must blueberries possess to optimize these benefits, and how can these blueberries become more accessible to consumers?". As indicated in the preliminary data section, CGA levels vary significantly depending on the cultivar and post-harvest conditions. The impact of CGA or CGA-rich blueberries on TMA and TMAO levels is unknown. It is unknown whether more CGA content in blueberries results in greater

TMAO-lowering and cardioprotective benefits. It remains unclear whether CGA is the primary factor driving the TMAO-lowering activities in blueberries or if other components like sugar and fiber play a larger role. It is unknown whether the CGA content directly inhibits bacteria containing *cut*C/D or if it is a preferred substrate over choline, resulting in a reduction of TMA production.

2.5.1 CGA, Bioavailability, Gut microbiome, and TMAO: The bioavailability of CGA, or the percentage of a dietary nutrient that is absorbed from the gut and enters the body's circulation, can be influenced by several variables[94]. These include the cell type studied, CGA dosage, the specific organs studied, and study duration. According to a study conducted by Wang *et al.*, only 0.8% of ingested intact CGA was recovered in urine with caffeic acid (a component of hydrolyzed CGA) accounting for less than 0.5% of the total ingested CGA. While 57.4% of the total intake of CGA was metabolized through microbial pathways and absorbed, with m-coumaric acid and its derivatives being the primary compounds found in urine and plasma. This suggests that the bioavailability of CGA is primarily due to its microbial metabolites rather than native CGA[147]. The gut microbial metabolites, such as caffeic and quinic acids as well as other small metabolites, are the primary circulating forms following CGA intake[148–151]. CGA can enhance the gut microbiome through increased bacterial diversity and lowered *Firmicutes/Bacteroidetes* ratios[152–154]. CGA reduces the prevalence of *Proteobacteria* and *Firmicutes* phyla, and genera such as *Desulfovibrio*, *Proteus*, and *Klebseilla*, which all include species carrying the *cutC/D* gene cluster responsible for TMA production[8,152,153,155–157]. The absorption of some compounds can be evaluated by analyzing fecal matter and calculating the amount presumed absorbed based on what is missing. However, CGA is susceptible to fermentation by gut microbiota, potentially leading to overestimation of CGA absorption by its absence in the native form in feces. Research conducted by Margreet *et al.* determined that among participants with an ileostomy bag, who lack a colon which contains most of the gut bacteria associated with that region capable of fermenting CGA (compared to the small intestine), approximately 33% of ingested CGA is absorbed in the small intestine[158]. However, this study did not consider the microbial metabolites of CGA, which likely makes up most of the absorbed CGA. Rather, it only focused on the loss of CGA measured by difference between oral dose and amount recovered in the ileostomy bag.

The systemic (i.e. blood) bioavailability of CGA and its intact Phase-II metabolites following oral consumption is low (≤1%), limiting the potential for CGA itself to act in peripheral tissues such as the vasculature[148–151]. With generally poor absorption, high concentrations of CGA reach the intestines with the potential to accumulate in the lumen, where it is available to inhibit TMA-lyase and subsequent TMAO formation through the modulation of gut microbiota[159].

2.6 Preliminary Data: The preliminary data are essential for understanding the rationale behind the project's approach and for establishing a foundation for the overall design. Most of the preliminary data presented in this section comes from recent studies conducted in our lab, particularly those involving CGA and its metabolites in fermentations, as well as blueberries in fermentation studies.

2.6.1 Chlorogenic Acid Content Varies Widely Amongst Blueberry Cultivars: Recent work from Iorizzo *et al*. documented the phenotypic variability of CGA across multiple blueberry accessions. From a genetic diversity panel of 128 blueberry accessions, where the plants were not directly related and do not share genetic material from the same immediate parental sources, CGA varied by >20-fold [8-173 mg/100 g fresh weight] (Figure 2.1A) [123]. Since all blueberries were harvested from Corvallis, OR, USA, the diversity of CGA among these accessions is predominately due to genetic influence, varying enzymatic activity, and protein expression. Further demonstration by Iorizzo *et al.* in a bi-parental mapping population of 196 F_1 blueberry individuals, that share genetic material from the same parental sources, exhibited a CGA variation of >99-fold [2.6-253 mg/100 g fresh weight] (Figure 2.1B)[130]. The large variety in CGA content is due to the strong genetic control of the identified major effect quantitative trait loci (QTL) in chromosome 2[130]. A QTL associated with high CGA in the same chromosome 2 region confirmed the association of this genome region with CGA[112]. Blueberry genotypes carrying the dominant allele (controlling for high CGA) can exhibit up to 11-fold more CGA than genotypes carrying the recessive allele (controlling for low CGA). In a separate bi-parental population, Iorizzo *et al.* identified a QTL linked to elevated CGA levels within the same Chromosome 2 region, validating the association of this genomic region with CGA[112]. Of note, these studies utilize tightly controlled blueberry material that remain consistent throughout the study and may not accurately reflect the blueberries in packages accessible to the average consumer. DNA markers associated with these QTLs can be utilized to select blueberry cultivars with high CGA content, increasing the availability to the average consumer. Generalizations that suggest all blueberries have cardiovascular benefits are likely inadequate due to the wide variety among cultivars. If CGA is the compound responsible for the TMA and TMAO-lowering effects, the blueberry cultivars rich in CGA could offer greater benefits compared to those with lower CGA content.

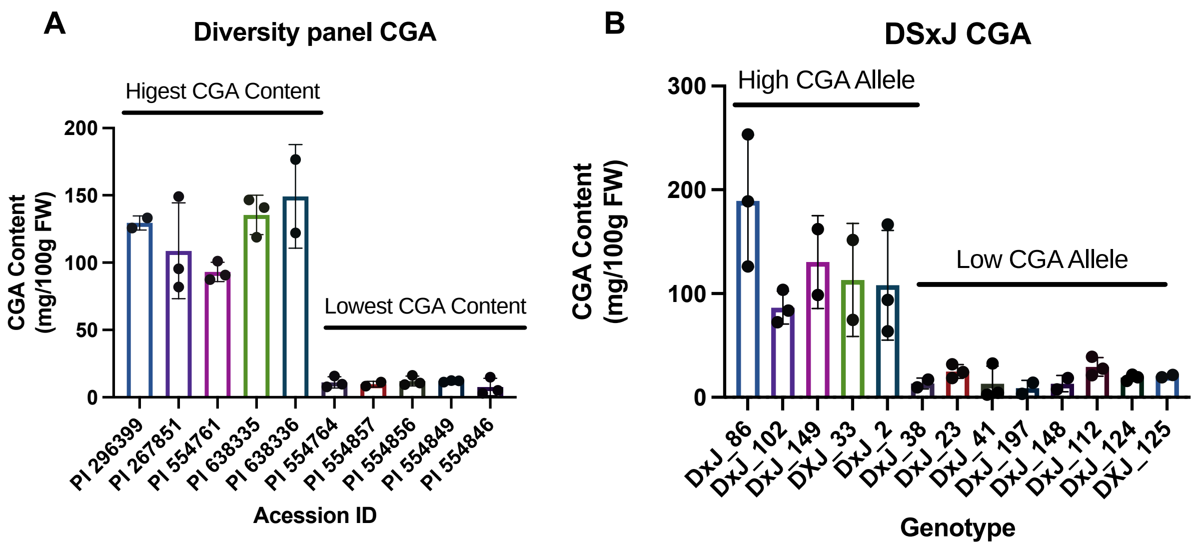


Figure 2.4. Chlorogenic acid (CGA) content in fresh weight (FW) blueberries from blueberry germplasm diversity (A) and the biparental mapping (B) populations. Black dots represent mean of individual years with error bars representing standard error of the mean (SEM). Data were adapted from Mengist *et al.* with permission (https://creativecommons.org/licenses/by/4.0/)[123,130].

2.6.2 Chlorogenic Acid Dose-dependently Inhibits TMA Formation and Choline Utilization *in vitro*: Recent work done by Iglesias *et al.* in our lab documented the impact of CGA on the retention of exogenous choline and the inhibition of microbial metabolism of TMA *in vitro*[10]. These data do not indicate acute or chronic mechanisms, rather an establishment of proof-of-concept and effective doses of CGA on TMA inhibition. To execute this, ideal fermentation conditions involved 20% fecal slurry (composed of 10% feces, so 2% final fecal concentration) mixed with 100 µM of exogenous choline, while varying concentrations of CGA ranging from 0.1-10 mM were added. Control groups included a choline-free and a 3,3-Dimethyl-1-butanol (DMB, a known TMA-lyase inhibitor[10,68,69]) treatment. To account for endogenous TMA and TMA-containing substrates, TMA and choline levels were quantified, and a calibration curve was constructed to detect the contribution of endogenous substrates on TMA production and choline retention. Samples of 50 µL from all CGA treatments were taken at 0, 8, and 12 hrs for choline and TMA analysis. CGA treatment AUCs for choline were significantly higher at ≥5 mM (Figure 2.5A) and AUCs for TMA were significantly lower at ≥ 2 mM (Figure 2.5B). Notably, at concentrations of 10 mM for CGA, TMA production rate was lower than those observed under choline-free conditions, emphasizing their significant ability to diminish TMA formation, including the reduction of background TMA levels. Notably, CGA dose-dependently affects TMA production (Figure 2.5 B). Given the CGA concentrations used in this experiment and the average colon volume of approximately 600 mL in a healthy adult, concentrations above 1mM are considered high and unlikely to occur in the colon after consuming a typical serving of blueberries[160]. For instance, even a cultivar with a high CGA content, such as one with 219 mg of CGA (value derived from figure 2.4) per150 g of whole blueberries (1 serving size), would only deliver approximately 1mM of CGA to the colon, as the concentration of CGA is diluted throughout the digestive process and is likely closer to 1mM after dilution in the 600 mL volume of the colon. This is further demonstrated by a cultivar with low CGA content, such as one containing 6.33 mg of CGA (value derived from figure 2.4) per 150 g of whole blueberries (1 serving), which would only deliver around 0.03 mM of CGA to the colon.

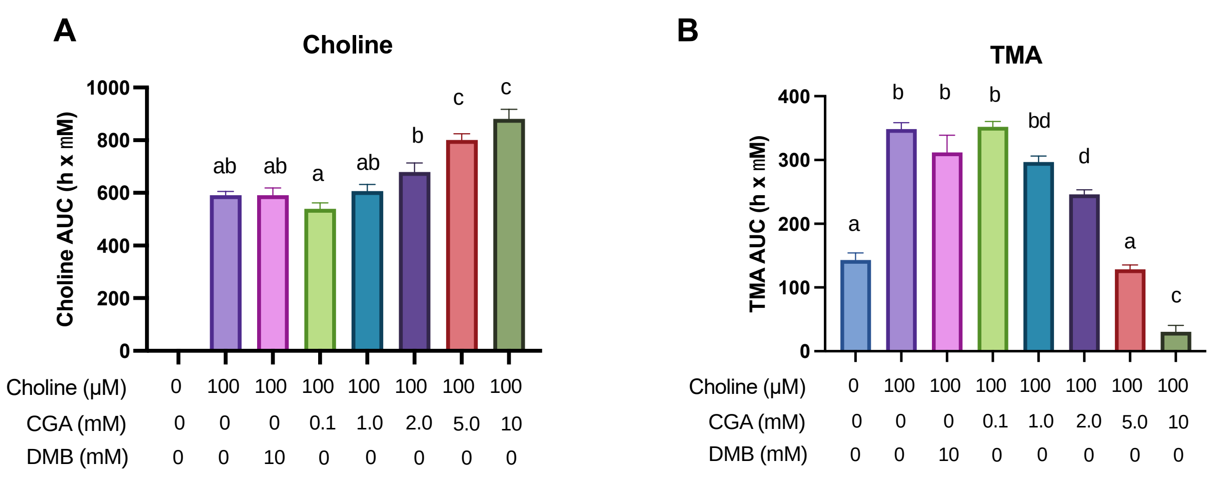


Figure 2.5. Area under the curve (AUC) of choline (A) and TMA (B) in fermentations with chlorogenic acid (CGA) and 3,3-dimethyl-1-butanol (DMB) over 12 hours. Different letters notate significantly different AUCs with error bars representing standard error of the mean (SEM). Data adapted from Iglesias *et al.* with permission (https://creativecommons.org/licenses/by/4.0/) [10].

2.6.3 Blueberries Inhibit TMA Production *in vitro*: Research performed by Neilson *et al.* (unpublished) tested the hypothesis that CGA drives TMA-lowering activity of blueberries. From the mapping populations described above, 8 blueberry genotypes with large differences in CGA contents were selected[123] (from among those in Figure. 2.4). Selection of 4 high CGA genotypes (170-250 mg/100 g FW) containing the dominant allele were compared to 4 low CGA genotypes (17-32 mg/100 g FW) containing the recessive allele. A simulated gastric and small intestinal digestion was performed using the blueberries at a dose equivalent to 133 g of fresh blueberry in a human with an upper digestive tract (stomach and small intestine) volume of 2 L. The blueberry digesta and control digesta were diluted 3X and fermented for 24 hrs with choline-d_9. Results indicated that blueberries are the most potent TMA-lowering food tested in this model compared to coffee, tea, coca[96], and artichokes[161]. The inhibition of TMA production across all blueberry cultivars appeared to be nearly zero (Figure 2.6 A). Therefore, a broken axis was used to better distinguish the TMA production levels (Figure 2.6 B.). High CGA content blueberries were generally more effective than low CGA content blueberries at inhibiting TMA formation. It is noted that all genotypes were effective in lowering TMA vs. control digesta; however, the kinetics suggest that differences between high and low CGA genotypes would increase if the assay were extended. Other components of blueberries such as sugar, fiber, or other non-phenolic compounds may have reduced TMA production *in vitro*[96,162]. Previous work by Bresciani *et al.* has shown that sugar, which would not be present in the colon after upper gastrointestinal digestion, can significantly inhibit TMA formation[162]. This is an assay interference, rather than physiologically relevant effect, and would not be observed *in vivo*. Of importance, there has not been a strong inhibition of TMA from other fiber-rich products such as cocoa[96], artichokes[161], or pure inulin[161]. It is also noted that in mice, blueberries reduced TMAO compared to a sugar and fiber matched control (Figure. 2.9 C).[11]

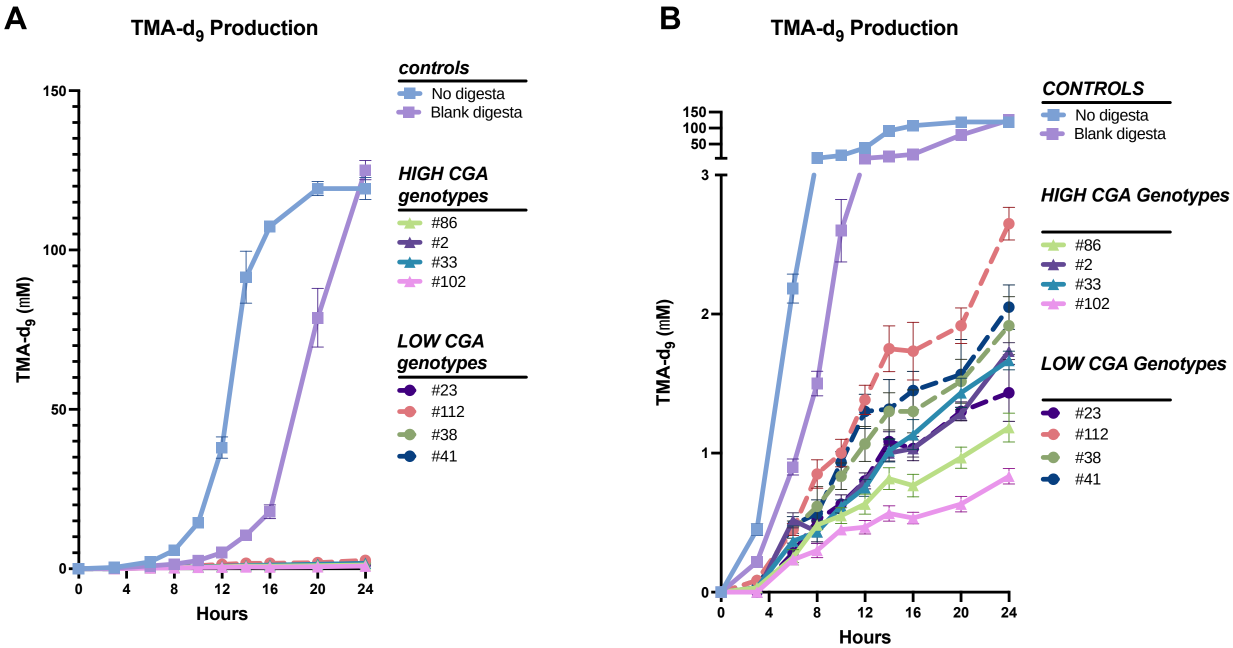


Figure 2.6. Kinetic curves of TMA-d_9 production in fecal fermentations with choline-d_9, No digesta, Blank digesta, or Digesta from blueberry genotypes were added. TMA production displayed without a broken axis (A) and with a broken axis (B). Unpublished data, used with permission from Dr. Neilson.

2.6.4 Microbial Metabolites of CGA Inhibit TMA Production *in vitro*: CGA is metabolized by gut microbiota into caffeic and quinic acids (Figure. 2.7), and then further metabolized to ferulic, hydroxyphenyl propionic, hydroxyphenyl acetic, and hydroxybenzoic acids[11,163,164]. Research performed by Iglesias-Carres *et al.* suggested that the microbial metabolites of CGA, rather than the native compound itself, is partly responsible for inhibiting TMA production *in vitro* and *in vivo*[12]. To test the inhibition properties of CGA and other phenolic microbial metabolites, an *ex vivo* anaerobic fecal fermentation assay was utilized to evaluate the suppression of TMA production from choline. At the start of the fermentation, most of the treatments did not significantly change the levels of choline- d_9. Most of the screened metabolites significantly reduced the utilization of choline- d_9 and the subsequent metabolism into TMA- d_9 between 6 and 10 hours. After 12 hours it was noted that caffeic acid and 3,4-dihydroxybenzoic acid maintained statistically significant levels of choline- d_9 compared to the control group. As noted in the TMA- d_9 AUCs (Figure. 2.8), most CGA metabolites reduced TMA- d_9 production vs control across metabolite classes[12]. These findings highlight the ability of CGA and its metabolites to interact with bacteria and maintain choline-d_9 levels. Thus, the bacterial metabolism of CGA in the colon does not imply that the activity diminishes once the native CGA is metabolized. This suggests the potential for further studies focusing on the inhibition potential of these metabolites. To account for inhibition due to toxicity, viability tests were used to monitor cell count and cell respiration rates. Inhibition was not due to cell toxicity from metabolites[12]. Caffeic and p-coumaric acids were the most effective in reducing the utilization of choline- d_9 and TMA- d_9 production.

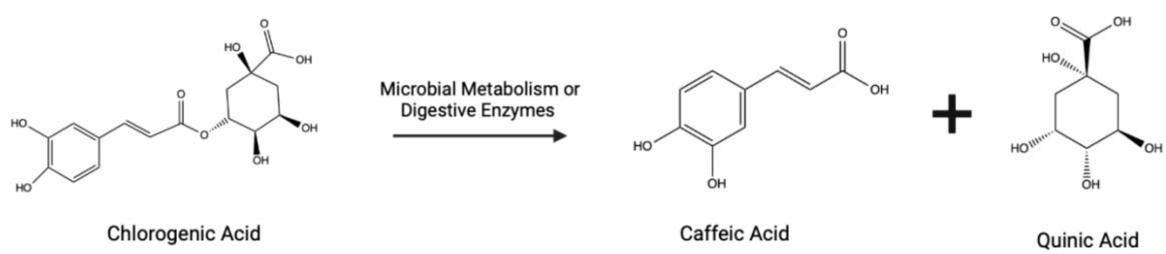


Figure 2.7. Gut hydrolysis of chlorogenic acid by microbial mechanisms or digestive enzymes into caffeic and quinic acid.

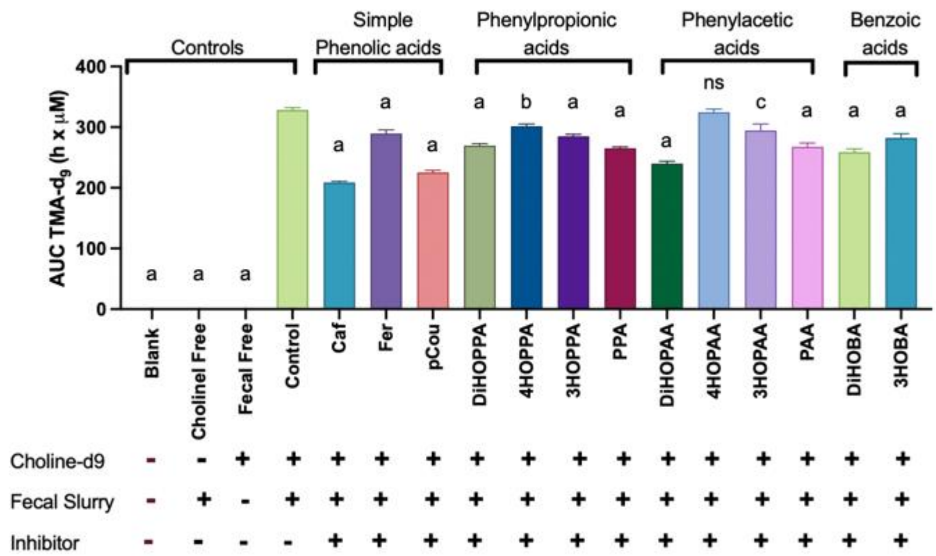


Figure 2.8. Areas under the curve (AUCs) of the kinetic curves of TMA-d_9 for the microbial metabolites of chlorogenic acid (CGA): blank, choline free, fecal free, control, Caffeic (Caf), Ferulic (Fer), *p*-coumaric (pCou), 3-(3,4-dihydroxyphenyl)propionic (DIHOPPA), 3-(4-hydroxyphenyl)propionic (4HOPPA), 3-(4-hydroxyphenyl) propionic acetic acid (3HOPPA), 3-phenylpropionic acid (PPA), 3-(3,4-dihydroxyphenyl)acetic acid (DIHOPAA), 3-(4-hydroxyphenyl) acetic acid (4HOPAA), 3-(3-hydroxyphenyl) acetic acid (3HOPAA), phenylacetic acid (PAA), 3,4-dihydroxybenzoic acid (DIHOBA), 3-hydroxybenzoic acid (3HOBS). Different letters notate statistical difference (P<0.05). Reprinted (adapted) with permission from {Iglesias-Carres, L.; Krueger, E. S.; Herring, J. A.; Tessem, J. S.; Neilson, A. P. Potential of Phenolic Compounds and Their Gut Microbiota-Derived Metabolites to Reduce TMA Formation: Application of an In Vitro Fermentation High-Throughput Screening Model. *J. Agric. Food Chem.* **2022**, *70* (10), 3207–3218. https://doi.org/10.1021/acs.jafc.2c00247.}. Copyright {2025} American Chemical Society[12].

2.6.5 Chlorogenic Acid-Rich Blueberries Lower TMAO in Mice: Recent work conducted by Satheesh Babu, Peterson, Iglesias-Carres, Paz, Wankhade, Neilson, and Anandh Babu documented the mitigation of trimethylamine N-oxide levels through alterations of gut microbes using blueberry supplementation in mice[11]. To accomplish this, 7-week-old mice were fed specific diets for 12 weeks. Diets included a control (C), a supplemented 3.8% freeze-dried blueberry (CB), a control with an antibiotic cocktail in drinking water (CA), and a supplemented 3.8% freeze-dried blueberry with an antibiotic cocktail in drinking water (CBA). Importantly, sugar and fiber content were matched for controls and blueberry supplemented diets. Antibiotic dosage, proven to deplete gut bacteria in mice, was gradually increased throughout the 12 weeks until a final dosage of 1 g/L ampicillin, 500 mg/L vancomycin, 1 g/L neomycin sulfate, and 1 g/L metronidazole was reached. Blueberry powder content of 3.8% (14.94± 0.86 mg CGA/g dry weight), measured as w/w, was determined to be equivalent to a human consumption of ~240 g, or 1.5 servings, per day. After the 12 weeks, choline, TMA, and TMAO were extracted from blood plasma and analyzed through UPLC-ES-MS/MS. There was not a statistically significant reduction in plasma choline levels in mice treated with blueberry and/or antibiotics (Figure 9A). There was no statistical difference in TMA plasma levels between C and CB mice, but it was noted that antibiotics reduced TMA plasma levels below the limit of quantification (Figure 9B). There was a significant reduction of TMAO plasma levels in CB compared to C mice (Figure 9C) and it is noted that blueberry supplementation reduced TMAO levels by ~48%. Mice body weight, blood glucose, and body composition were measured for all treatments, noting that the significant changes were not due to metabolic alterations. Classification of gut microbiota was executed using DNA extracted from fecal contents and analyzed using a DNeasy PowerSoil kit. Blueberry supplementation is noted to increase bacteria that are negatively associated with plasma TMA and TMAO (not shown). These findings raise interesting questions about which components of blueberries contribute to reducing TMAO levels in plasma.

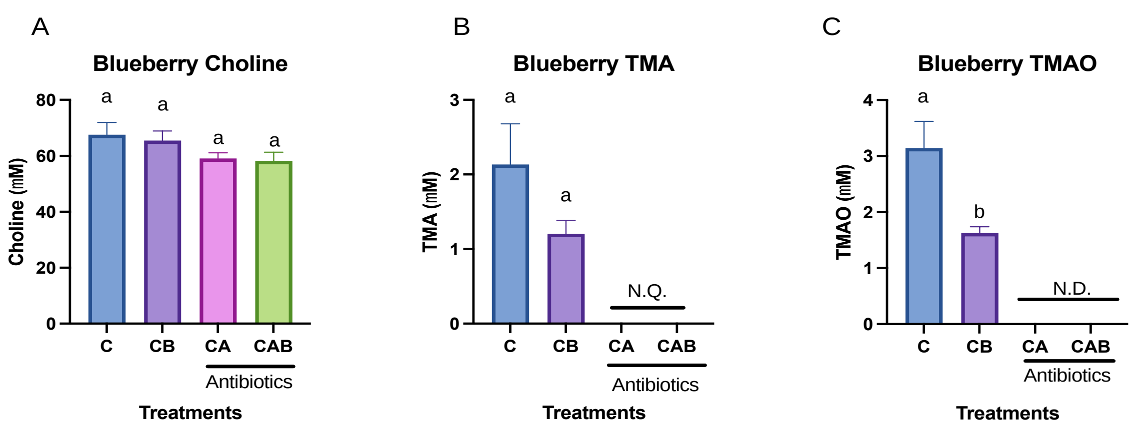


Figure 2.9. Choline concentration, trimethylamine (TMA) concentration, and trimethylamine N-oxide (TMAO) concentration in standard diet fed mice (C); blueberry-fed mice (CB); standard diet-fed mice treated with antibiotics (CA); blueberry fed mice treated with antibiotics (CAB).;). Different letters notate significantly different (P<0.05). Adapted with permission from (Satheesh Babu, A. K.; Petersen, C.; Iglesias-Carres, L.; Paz, H. A.; Wankhade, U. D.; Neilson, A. P.; Anandh Babu, P. V. Blueberry Intervention Mitigates Detrimental Microbial Metabolite Trimethylamine N-Oxide by Modulating Gut Microbes.) Copyright (2025), Figure 3, IUBMB Journals,Wiley[11].

The preliminary data presented in this section guided the hypothesis that CGA content is a primary determinant of the TMA-lowering benefits of blueberries, while also validating the feasibility of the proposed work. The data in Figure 2.4 revealed significant variability in CGA content across different blueberry cultivars. However, the inhibition observed in all the whole blueberry cultivars shown in Figure 2.6 suggested that both high and low CGA content cultivars exhibit inhibitory activity. Additionally, the dose-dependent response of CGA and corresponding TMA inhibition presented in Figure 2.5 led to the hypothesis that cultivars with higher CGA content may have stronger TMA-inhibiting effects than those with a lower CGA content. These findings helped shape the framework for further exploration into the specific component of blueberries responsible for the observed inhibitory activity.

CHAPTER 3 METHODS AND MATERIALS

3.1 Introduction: This chapter outlines the experiments conducted for this thesis, including optimization of ATP assays for fecal bacteria and fermentation matrices, optimization of fermentation parameters to identify and minimize assay interferences, the identification of sugar-related interferences, and the inhibitory effects of various blueberry components. Finally, I examine the inhibition effects of peels from a genetically diverse group of blueberry cultivars that have different levels of CGA.

The BacTiter-Glo ATP assay was optimized by testing different plate colors to determine which maximizes luminescence of treatments. The BacTiter-Glo protocol does not provide specific guidelines for bacteria in complex matrices, so modifications were necessary to minimize background interferences in the fermentation model. To clarify the luminescence signal, fecal cells in fermenta were “washed” to remove matrix components. Lastly, fecal slurry concentration on luminescence signals of a BacTiter-Glo assay were determined.

Once the BacTiter-Glo assay was optimized, anaerobic fermentation parameters were optimized to account for fiber/sugar-rich fruits and determine the potential influence of non-phenolic blueberry components on the inhibition of TMA in our assay. Fecal slurry was pre-fed unlabeled choline 12 hrs prior to the fermentation to shift bacterial metabolism towards choline. Various blueberry fractions (whole blueberry, sugar, fiber, and phenolic) were tested for TMA inhibition. Building on these results, further analysis was conducted to identify which specific blueberry components most effectively reduce TMA production (Whole blueberry, peel, pulp, sugar, and fiber).

Finally, to determine whether CGA content correlates with reduced TMA production in multiple blueberry accessions, peels from a genetic diversity population and bi-parental mapping population from the National Clonal Germplasm Repository (NCGR), Corvallis OR, USA was compared to controls in the fermentation model.

3.2 General Methods

3.2.1 Chemicals and Reagents: OpenBiome fecal samples (Cambridge, MA, USA), urea (Fisher, CAS# 57-13-6), uric acid (Fisher, CAS# 69-93-2), α-amylase (porcine pancreas, Sigma, CAS# 0009000902), mucin (porcine stomach, Sigma, CAS# 84082-62-4), KCl, Na_3PO_4, Na_2SO_4, NaCl, $NaHCO_3$, N_2 gas, saline (0.9% NaCl in water), HCl, pepsin powder (porcine gastric mucosa, Sigma, CAS# 9001-75-6), NaOH, pancreatin (porcine pancreas, Sigma, CAS# 8049-47-6) , lipase (porcine pancreas, Sigma, CAS# 9001-62-1), bile extract (porcine, Sigma, CAS# 8008-63-7), peptone water, yeast extract, Na_2HPO_4, $MgSO_4 \times 7H_2O$, $CaCl_2 \times 6H_2O$, $NaHCO_3$, hemin, bile salts, Tween 80, vitamin K1, resazurin, L-cysteine, PBS, formic acid, methanol, choline- d_9, TMA- d_9, $ZnSO_4$, choline-1-^{13}C-1,1,2,2, -d_4, choline-1,2-$^{13}C_2$ (Cambridge Isotopes), TMA-$^{13}C_3$-^{15}N, TMA-$^{13}C_3$-d_9 (Cambridge Isotopes), acetonitrile, concentrated ammonia, ethyl bromoacetate, ammonium formate, Diaion HP-20 resin, Milli-Q water, acetone, methanol, acetic acid, formic acid, sodium acetate, sodium carbonate, Folin-Ciocalteu reagent, 4-Dimethylainocinnamaldehyde (DMACA).

3.2.2 Blueberry Sources: Commercially available frozen (distributed by Food Lion, LLC Salisbury, NC 28147) and fresh (SBROCCO International INC. Mount Laurel, NJ 08054) blueberries for assay development were obtained from Food Lion, Kannapolis, NC, USA on March 4, 2024. Various blueberry cultivars were obtained with help from Dr. Iorizzo and Dr.

Bassil in the fall (2024) from a 2024 harvest crop through the National Clonal Germplasm Repository (NCGR), Corvallis OR, USA.

3.2.3 General Gastrointestinal Digestion Simulated *in vitro* upper gastrointestinal digestions were performed to mimic oral, gastric, and small intestinal digestion and absorption[96,161]. Digestions were scaled from ~1 serving in human volume to an equivalent dose for the *in* vitro digestion final volume. The volume of the upper gastrointestinal track, the stomach and small intestine, of a human is approximated at 2 L.

For a 50 mL oral digestion a ratio of 1 g of solid sample was mixed with 6 mL of an oral phase solution (0.4 g/L urea, 30 mg/L uric acid, 10.6 g/L α-amylase, 50 mg/L mucin, 1.792 g/L KCl, 1.776 g/L Na_3PO_4, 1.140 g/L Na_2SO_4, 0.596 g/L NaCl, 3.388 g/L $NaHCO_3$) homogenized in a 50 mL falcon tube and blanketed with N_2 gas, capped, and placed in an incubator at 37°C, and shaken at 120 opm (oscillations per minute) for 10 minutes. The gastric digestion occurred after the 10 minutes of shaking. The tubes were removed from the incubator and immediately placed on ice, brought to a volume of 30 mL using saline (0.9% NaCl in water), 2 mL of pepsin solution (20 mg/mL in HCL 0.1M) was added. Adjustment of pH to 2.5 ± 0.1 was achieved using 1M HCl and a pH probe. The volume was brought to 40 mL using saline. The tube was blanketed with N_2 gas, capped, placed in an incubator at 37°C, and shaken at 120 opm for 1 hour. The intestinal digestion occurred after shaking for 1 hour. The tubes were removed from the incubator and immediately placed on ice. Adjustment of pH to 6.5 ± 0.1 was achieved using 1M $NaHCO_3$. Then 2 mL of pancreatin (20 mg/mL in 0.1 NaHCO3)/ lipase (10 mg/mL in 0.1 NaHCO3) mixture was added. 3 mL of bile extract (30 mg/ mL in 0.1 $NaHCO_3$) was added. The final volume was brought to 50 mL with saline, blanketed with N_2 gas, capped, and placed in an incubator at 37°C, and shaken at 120 opm for 2 hours. The crude digesta was utilized. 5 mL of the same treatments were pooled in a new tube and stored in a –80°C freezer. The frozen digesta was lyophilized to remove water with solubilized oxygen and stored in a – 80°C freezer.

For a 15 mL oral digestion, a ratio of 1 g of solid sample per 1.8 mL in oral phase solution (0.4 mg/mL urea, 0.03 mg/mL uric acid, 10.g mg/mL α-amylase per g food digested, 0.05 mg/mL mucin, 1.792 g/L KCl, 1.776 g/L Na_3PO_4, 1.140 g/L Na_2SO_4, 0.596 g/L NaCl, 3.388 g/L $NaHCO_3$), homogenized in a 15 mL falcon tube and blanketed with N_2 gas, capped, placed in an incubator at 37°C, and shaken at 120 rpm for 10 minutes. The gastric digestion occurred after the 10 minutes of shaking. The tubes were removed from the incubator and immediately placed on ice, brought to a volume of 9.6 mL using saline (0.9% NaCl in water), 0.6 mL of pepsin solution (20 mg/mL in HCL 0.1M) was added. Adjustment of pH to 2.5 ± 0.1 was achieved using 1M HCl and a pH probe. The volume was brought to 12 mL using saline. The tube was blanked with N_2 gas, capped, placed in an incubator at 37°C, and shaken at 120 rpm for 1 hour. The intestinal digestion occurred after shaking for 1 hour. The tubes were removed from the incubator and immediately placed on ice. Adjustment of pH to 6.5 ± 0.1 was achieved using 1M $NaHCO_3$. Then 0.6 mL of pancreatin (20 mg/mL in 0.1 NaHCO3)/ lipase (10 mg/mL in 0.1 NaHCO3) mixture was added. 0.9 mL of bile extract (30 mg/ mL in 0.1 $NaHCO_3$) was added. The final volume was brought to 15 mL with saline, blanked with N_2 gas, capped, and placed in an incubator at 37°C, and shaken at 120 rpm for 2 hours. The crude digesta was utilized. 5 mL the same treatments was pooled in a new tube and stored at –80°C. The frozen digesta was lyophilized to remove water with solubilized oxygen and stored in a –80°C freezer

A "blank" or "control" digesta was made with saline in place of food material and treated the same as the other treatments.

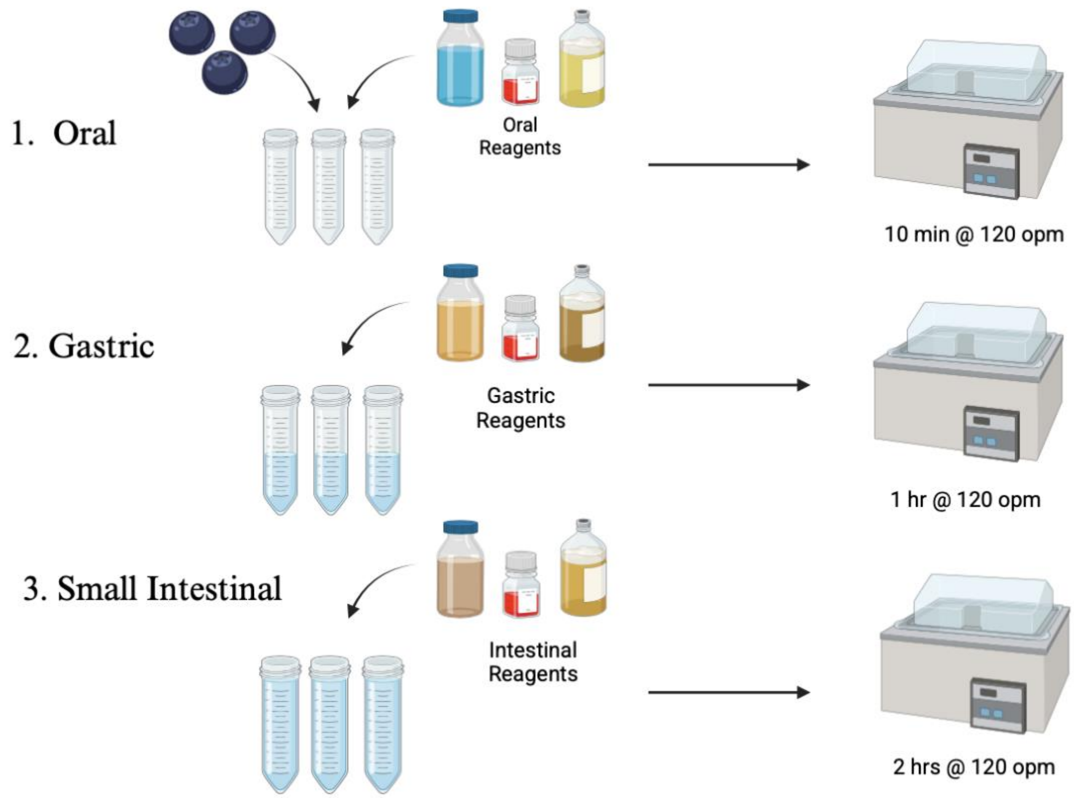


Figure 3.1 General gastrointestinal digestion schematic overview**.** Visuals created in https://BioRender.com

3.2.4 Anaerobic Fecal Fermentation

3.2.4.1 Growth Media Preparation: A final volume of 500 mL growth media was composed of 1 g peptone water, 1 g yeast extract, 50 mg NaCl, 20 mg Na_2HPO_4, 20 mg KH_2PO_4, 5 mL $MgSO_4 \times 7H_2O$ + $CaCl_2 \times 6H_2O$, 1 g $NaHCO_3$, 25 mg hemin, 250 mg bile salts, 1 mL Tween 80, 5 µL vitamin K1, 2 mL resazurin, and 250 mg L-cysteine. To prepare the 500 mL solution, two separate solutions at 250 mL each were prepared at 2X final concentration. Solution one included all components of growth media except for resazurin and L-cysteine. Solution two only contained resazurin and L-cysteine. The pH of both solutions was adjusted to 6.8 then separately filtered through a 0.22 µm sterile filter. Solution two was boiled for 10 minutes until clear. Both solutions were sparged to remove oxygen. The bottles containing the media were sealed with a septa disk, which allowed needles to pass N_2 (g) through without exposing the contents to ambient air. For proper gas exchange, the number of inlet needles (introducing N_2) is equal to the number of outlet needles. The bottles were continuously stirred at 250 rpm while N_2 is passed through. This process was maintained for a minimum of 8 hrs to effectively remove oxygen. After sparging, solutions were combined 1:1 in the anaerobic chamber (O_2 ≤10 ppm) to a final volume of 500 mL at 1X concentration.

3.2.4.2 Fecal Slurry Preparation: Fecal samples collected from healthy donors and that are screened for infectious diseases were obtained through OpenBiome (Cambridge, MA, USA) and stored immediately upon arrival in a –80°C freezer. OpenBiome provides information for all health histories including clinical data, pathogen screen results, and 16S rDNA sequences. Samples are prepared by OpenBiome in sterile 12.5% glycerol and o.9% saline buffer (2.5 mL of buffer per one gram of stool) then filtered through a 330 μm filter and frozen at –80°C. The fecal slurries were prepped 12 hrs before the start of the fermentation. Fecal slurry volume varied based on individual experimental parameters. At least two different fecal samples were combined with at least 16 mL of growth media in a 50 mL tube and vortexed until homogenized. The fecal slurry was left uncapped in the anaerobic chamber for at least 12 hrs. The slurry was filtered immediately before the start of the fermentation. The remaining 2 mL of media was used to rinse residual fecal slurry through the filter to bring to final volume.

3.2.4.3 General Fermentation Conditions: Fermentations took place inside a 4-glove 855-ACB anaerobic chamber (Plas-Labs, Lansing, MI, USA). The chamber was shifted to anaerobic conditions 24 hrs before the start of the fermentation. This was done by sanitizing the chamber, and all equipment inside the chamber, using 70% ethanol and left to dry. The chamber was then be purged of oxygen using a mixed gas and N_2. A day before the fermentation was performed, $\geq$10 gas exchanges first with N_2 (Airgas, Charlotte, NC, USA) then mixed gas containing 5% H_2, 5% CO_2 and 90% N_2 (ARC3, Raleigh, NC, USA) until chamber conditions reached H_2 (2-3%) and O_2 levels below 20 ppm monitored with a CAM-12 anaerobic monitor (Coy Lab Products, Grass Lake, MI, USA). Once anaerobic conditions were achieved, the heater inside the chamber was turned on to 37°C, and a palladium catalyst was placed on top before leaving it to stabilize overnight. Conditions were monitored throughout the fermentation experiment.

3.2.4.4 Optimization of Choline and Fecal Slurry Conditions: Optimization of choline and fecal slurry conditions were determined through previous procedures described by Iglesias-Carres *et al.*[10]. Optimized conditions were determined to be 100 μM concentrations of choline-d_9 and 20% fecal slurry (which itself is 10% fecal sample from OpenBiome, resulting in a final concentration of 2% fecal sample).

3.2.4.5 General Fermentation Procedures: Fermentations were carried out as per methodology developed by Iglesias-Carres *et al.*[10]. The anaerobic chamber was prepped as described above. To control for endogenous choline and other TMA-lyase substrates leading to TMA metabolism in the absence of exogenous choline, choline-d_9 was added as a substrate at a final concentration of 100 μM to all treatments. Subsequent TMA-d_9 was measured to evaluate the effectiveness of the treatments. Lyophilized, digested samples (if any) were prepared as described in general gastrointestinal digestions and were reconstituted to 1X concentration of the initial volume before lyophilization using filter-sterilized, overnight-sparged PBS 1X inside anaerobic chamber. In 2 mL 96-well plates, 750 μL of growth media was mixed with 90 μL of choline- d_9 stock solution (2mM) in PBS 1X, 600 μL of reconstituted digesta samples, and 360 μL of fecal slurry (aliquots from at least two different donors pooled). The dilution of 600 μL of digesta into a final volume of 1800 µL is a conservative method, as it reflects a more diluted concentration, avoiding the potential overestimation of the inhibitory effect that might occur with less dilution of digesta. Additionally, one of the functions of the colon is to absorb water, which results in a more concentrated mixture of digestive enzymes and food in this region. As a result, higher concentrations of digesta may be present *in vivo*, potentially leading to greater inhibition. This method aims to mimic the physiological environment of the colon by accounting for the relatively dilute matrix containing intestinal fluids. Solutions were pre-heated at 37°C, and the start of fermentation (time 0 h) began when fecal slurry was inoculated into reaction mixture with the substrate. From 0 to 24 or 30 hours, 100 µL of sample was collected at various time points, combined with 100 µL of acetonitrile, and frozen at −80°C.

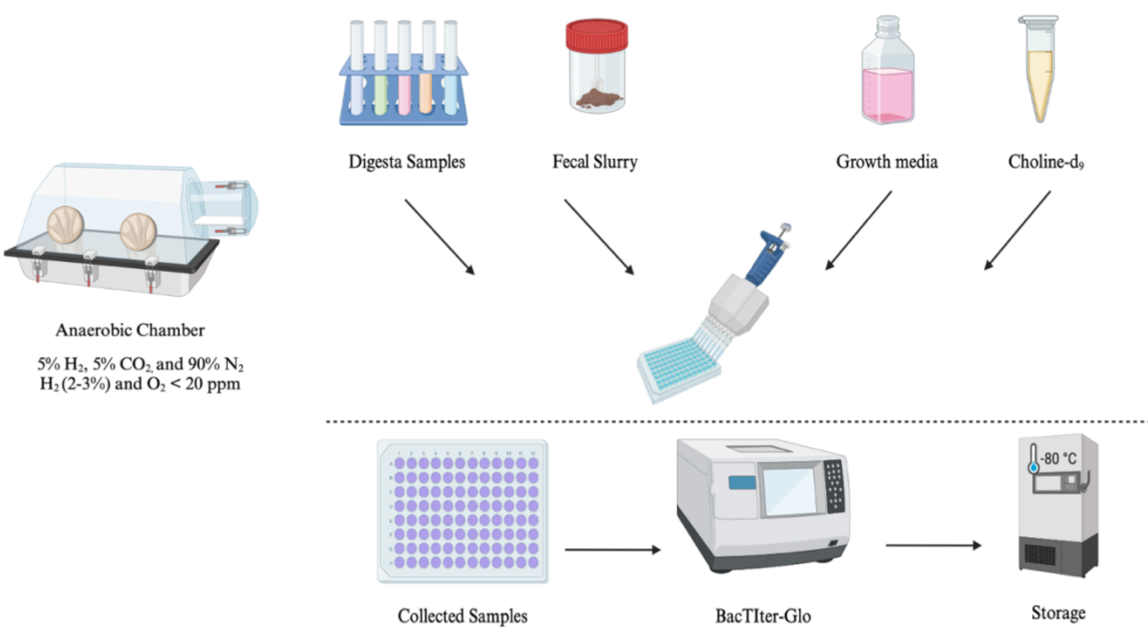


Figure 3.2 General Fermentation procedure schematic overview**.** Visuals created in https://BioRender.com

3.2.5 ***Phenolic Fraction Preparation:*** A phenolic extraction was performed to generate a blueberry fraction that accurately represents phenolic compounds in an average serving of blueberries (~150 g) for use in a fermentation. To achieve this, an equal mixture of frozen (distributed by Food Lion, LLC Salisbury, NC 28147) and fresh (SBROCCO International INC. Mount Laurel, NJ 08054) whole blueberries were homogenized using a ninja bullet blender for 5 min. After homogenization, the mixture was weighed (246.83 g), aliquoted, spread thinly on three separate pans, and lyophilized until all water was removed. The resulting lyophilized material was weighed (43.40657 g) and frozen at −80°C.

3.2.5.1 Phenolic Fraction Extraction: For extraction, 26.007 g of lyophilized blueberry mixture representing a serving size of fresh weight blueberry (~150 g) was combined with 234 mL of extraction mixture containing acetone, water, and acetic acid (70:29.5:0.5). A total of 2 L of extraction mixture was made for a total of 8 extractions. The mixture of solvent and freeze-dried material was vortexed, blended for 1 min using a polytron (VWR 200), sonicated for 5 min in a water bath at 50 °C, and then centrifuged at 3248 x g for 10 min. The steps were repeated seven times until the supernatant was clear. Due to the soft, fibrous nature of the blueberry pellet, completely removing the supernatant proved challenging. As a result, approximately 90% of the supernatant was carefully removed without disturbing the pellet after each extraction. A total of 8 extractions were performed, and the remaining supernatant did not affect the extract material, as multiple extraction repeats included the leftover supernatant. About 1600 mL of the supernatant was collected from the extractions, pooled, and concentrated using a rotary evaporator at 45 °C under partial vacuum. After removing the material, the flask was rinsed twice with 25 mL of Milli-Q water to remove leftover extract material. The rinses were pooled with the extract (~600 mL total). The extract material was frozen at −80°C then lyophilized until all water was removed.

The resulting lyophilized extract material was weighed (23.05 g) and resuspended in ~ 200 mL acidified mill-Q water (0.1% formic acid). 607.43 g of dry, Diaion HP-20 resin was transferred to a beaker and covered with methanol by approximately 2”, stirred, and left to sit for 15 minutes. After the 15 minutes, the methanol was decanted and the resin was covered with milli-Q water by approximately 2”, stirred, and left to sit for 10 minutes. Enough milli-Q water was added to an empty 24/40 column to reach a 1” height. The resin was slowly poured into the column, as the excess water slowly drained out. Once all the resin was added, five 9 cm filter papers were added followed by a 1” layer of sand. The resin was equilibrated by adding 10 column volumes of milli-Q water, and the water was discarded. The resuspended extract material was added slowly to form a uniform layer on top of the sand layer. Another 1” layer of sand was added on top of the extract material. Four liters of acidified milli-Q water (0.1% formic acid) was added to the column, and the resulting eluent was collected in large bottles. Once all acidified water was collected; three liters of acidified methanol (0.1% formic acid) was added to the column, and the resulting eluent was collected in large bottles. Lastly, three liters of acidified acetone (0.1% formic acid) was added to the column, and the resulting eluent was pooled with the methanol fractions. The pooled methanol and acetone fractions were transferred into a round bottom flask and concentrated using a rotary evaporator under partial vacuum starting at 80°C for the methanol fractions and then lowering the temperature to 45°C for the acetone fractions. After removing the concentrated material, the flask was rinsed and sonicated twice with 50 mL milli-Q water and once with 25 mL of milli-Q water. The rinsed fractions were pooled with the extract material (~170 mL total), frozen at –80°C, and lyophilized to remove water. The final lyophilized extract was weighed (1.01251 g) and stored at –80°.

3.2.6 Plant Phenolic Extraction: Plant-derived phenolic extract can be used in phenolic assays to measure relative amounts of phenolics in blueberry fractions. 250 mg of blueberry material was mixed with 2.5 mL of solvent A (80% methanol 2% formic acid in water). The samples were vortexed for 30 seconds, sonicated for 20 min, and centrifuged for 4 minutes at 3428 x g. The supernatant was removed and dried under nitrogen. 2.5 mL of solvent B (98%methanol 2% formic acid) was then added to all samples. The samples were vortexed for 30 seconds, sonicated for 20 min, and centrifuged for 4 min at 3428 x g. The supernatant was removed and combined with the previous supernatant. Steps for solvent B were repeated. Once supernatant was dried, the samples were stored at –80°. To resolubilize the dried supernatant, 3 mL of 0.1% formic acid in water was mixed into each sample. The samples were vortexed until homogenized and then filtered. Resolubilized extracts were stored at –80°.

3.2.7 Phenolic Analysis: Folin- Ciocalteu Assay: To quantify the total phenolics in the plant extracts, prepared as described in "*Plant Phenolic Extraction*", a Folin assay was carried out in a 96 well plate. Each extract underwent a series of dilutions. Starting with the pure extract then diluting 10x, then diluting 100x, etc. A standard curve was made with a 2mg/mL gallic acid mixture with a series of 23- 2/3rds dilutions with the last dilution as pure water to represent a "blank". 20 µL of both the standard and extract samples were plated in triplicate. 140 µL of Folin reagent (2mL of Folin-Ciocalteu reagent mixed in 18 mL water) was mixed with all samples. The plate was covered with foil and protected from light for 30 min. After the 30 min, 100 uL of stop solution (25% sodium carbonate in water) was added, and the absorbance was taken at 765 nm. Total phenolics are expressed as mg of gallic acid equivalent (GAE)/ g of whole blueberry.

3.2.8 Phenolic Analysis

3.2.8.1 SPE: To quantify the relative amounts of CGA in plant material, a solid phase elution (SPE) followed by analysis using LC-MS was utilized following methodology described by Mengist *et al.*[165]. Plant extracts described in "*Plant Phenolic Extraction"* underwent a series of dilutions. Starting with the pure extract then diluting 10x, then diluting 100x, etc. A standard of 0.2 mM ethyl gallate was used to determine loss during the SPE process. An internal standard of 0.2 mM Taxifolin was used for the LC/MS. A standard curve was made with a 1mg/mL CGA mixture with a series of dilutions. Samples were plated in triplicate. 500 µL of sample and standard curve were mixed with 500 µL of 1% formic acid in water. A waste collection plate was placed on top of the StrataX filter plate (Phenomenex, Strata-X 96well plate 10mg/ well, Cat # 8E-S100-AGB) and was conditioned with 1 mL 1% formic acid in methanol, placed on a positive pressure manifold, and methanol was pulled through. The waste plate was emptied before 1 mL of 1% formic acid in water was pulled through using the positive pressure manifold. The waste plate was emptied before 1 mL of the sample mixed with 1% formic acid and 20 µL of Ethyl Gallate were plated and pulled through. Once the sample was pulled through, the filter was washed with 1 mL 1% formic acid in water twice and then washed with pure water. The waste plate was emptied, and samples were dried. The dried samples were resolubilized using 300 µL of 1% formic acid in water and left to sit for 10 min. After the 10 min, the samples were slowly pulled through the filter. 20 µL of Taxifolin was plated on a new 96-well plate with 160 µL of the final sample.

3.2.8.2 LC-MS Analysis: Samples were then characterized on LC-MS. Separation was accomplished using a Waters Acquity UPLC system (Milford, MA, USA) with an ACQUITY UPLC BEH C18 column (1.7 µm, 2.1 mm x150 nm). Mobile phases include 0.1% formic acid in acetonitrile (A) and 2% formic acid in water (B) and phenolics were separated using a gradient elution method which involved a 6-minute run time, started with 100% solvent B for the first 30 seconds. Over the next 5 minutes, the solvent composition gradually shifted to 35% solvent A and 65% solvent B. In the final minute of the elution, the solvent composition shifted back to 100% solvent B. Flow rate was 0.5 mL/min, column temperature was set to 30°C, and autosampler was set to 4°C. The above system was coupled with a Waters Acquity triple quadrupole mass spectrometer. Source temperatures was 150°C. Capillary voltage was 2 kV, desolvation (N_2) and gas flow (N_2) were set to 600°C and 650 L/h, respectively. Electrospray ionization (ESI) was operated in negative mode. Data were collected using multiple reaction monitoring (MRM) in MS/MS mode (Table 3.1). CGA (Figure 3.2) was the focus of this experiment; however, the method used to analyze CGA also detected other phenolic compounds, which will be further analyzed in future experiments. Total CGA content was expressed as mg CGA/ g whole blueberry.

3.2.9 BacTiter-Glo ATP Assay: To confirm treatments did not have a cytotoxic or cytostatic effect on fecal bacteria cells, a luminescent assay is used to measure the presence of ATP and monitor the number of viable microbial cells during fermentation. A BacTiter-Glo Microbial Cell Viability Assay (Promega, USA) reagent was prepped by mixing equal amounts of the provided BacTiter-Glo substrate and BacTiter-Glo buffer and leaving to rest for at least 15 min at room temperature before use. 100 µL of fermenta sample was plated on a white 96-well opaque round-bottom plate and centrifuged at 500 x g for 5 min. After centrifugation, the supernatant was removed, and the pellet was resuspended in 100 μL of PBS 1X. After the pellet was resuspended, 100 μL of the reconstituted BacTiter-Glo reagent was mixed with the fermenta samples and covered for 5 min before reading luminescence. This test is used to determine whether the treatment's inhibitory effects on choline-d_9 utilization and TMA-d_9 production are not a result of low cell viability or general cytotoxicity.

3.2.10 Extraction and Quantification of Choline-d_9 and TMA-d_9: Sample extraction and quantification of choline-d_9 and TMA-d_9 from the stable isotope dilutions were carried out as described by Iglesias-Carres et al.[110]. Two internal standards are available for use, with only one utilized per experiment. After sample collection, choline- d_9 is extracted in a 96-well plate. 25 μL of fermenta sample were mixed with 10 μL of $ZnSO_4$ solution, 100 μL acetonitrile, and 20 μL of either choline-1-^{13}C-1,1,2,2, -d_4 (Figure 3.1A.) or choline-1,2-$^{13}C_2$ (Figure 3.1 B.) solution (internal standard; 20μM). The plate was sonicated for 5 minutes in a water bath, and samples were then filtered through AcropreAdv 0.2 μm WWPTFE 96-well filtering plates (Pall Corporation, Port Washington, NY, USA) using centrifugation for 10 minutes at 3,400 x g. Samples are collected in a fresh 700 μL 96-well plate and frozen at –80°C. TMA- d_9 requires a derivatization process to the quaternary amine compound, ethyl betaine- d_9 (Figure 3.1C.). To analyze TMA- d_9, 25 μL of fermenta sample is mixed with 120 μL ethyl bromo acetate (20 mg/mL) solution, 9 μL of 28-30% ammonia, and 20 μL TMA-$^{13}C_3$-^{15}N (Figure 3.1D.) or TMA-$^{13}C_3$-d_9 (Figure 3.1E.) solution (internal standard; 20 μM, for derivatization to ethyl betaine-$^{13}C_3$-^{15}N or ethyl betaine-d_9, respectively). The samples are left to rest for 30 minutes at room temperature. After the 30 minutes, 120 μL 50% acetonitrile/0.025% formic acid in HPLC grade water is added to stop the reaction. TMA-d_9 samples are filtered as described for choline- d_9. After extraction, TMA- d_9 (Figure 3.3), TMA-$^{13}C_3$-^{15}N (Figure 3.4) and TMA-$^{13}C_3$-d_9 (Figure

3.5) derivatized samples are analyzed using UPLC-ES-MS/MS. Choline-d_9 (Figure 3.6), choline-1,2-$_{13}C_2$ (Figure 3.7), and choline-1-^{13}C-1,1,2,2, -d_4 (Figure 3.8) were analyzed separately, but with the same method, from TMA-d_9, TMA-$^{13}C_3$-d_9,and TMA-$^{13}C_3$-^{15}N. Separation was accomplished using a Waters Acquity UPLC system (Milford, MA, USA) with an ACQUITY BEH HILIC column (1.7 µm, 2.1x100 nm) and an AQUITY BEH HILIC pre-column (1.7 µm, 2.1x4 nm). Mobile phases include 15 mM ammonium formate in water (pH 3.5) (A) and acetonitrile (B). Gradient is isocratic at 80% B for 3 minutes, flow rate 0.65 mL/min, column temperature set to 30°C, and autosampler set to 4°C. The above system is coupled with a Waters Acquity triple quadrupole mass spectrometer. Source and capillary temperatures are 150°C and 400°C, respectively. Capillary voltage is 0.60 kV, desolvation (N_2) and gas flow (N_2) set to 800 and 20 L/h, respectively. Electrospray ionization (ESI) operated in positive mode; data were collected using multiple reaction monitoring (MRM) in MS/MS mode (Table 3.1). Standard curves are created by dividing choline-d_9 or TMA-d_9 values by their respective internal standard, then plotting those ratios against the known concentration of the standard analyte, to generate a slope equation. For fermentation samples, the choline-d_9 or TMA-d_9 values are divided by their respective internal standard and input into the slope equation to get calculate the actual concentration. These values are then multiplied by a factor of two to correct for dilution with acetonitrile. Finally, the values are plotted over time to produce kinetic curves for choline-d_9 utilization and TMA-d_9 production.

Table 3.1 Analyte Multiple Reaction Monitoring (MRM) Values			
Analyte	**MRM**	**Collision Energy (V)**	**Cone Voltage (V)**
TMA-d_9	155.33→127.21	34	20
choline-d_9	113.32→69.08	40	16
TMA-$^{13}C_3$-^{15}N	150.27→122.19	34	18
TMA-$^{13}C_3$-d_9	158.20→130.13	34	16
choline-1-^{13}C-1,1,2,2, -d_4	109.28→60.36	36	18
choline-1,2-$^{13}C_2$	106.10→60.04	36	14
chlorogenic acid	353.0228→84.9980	40	26

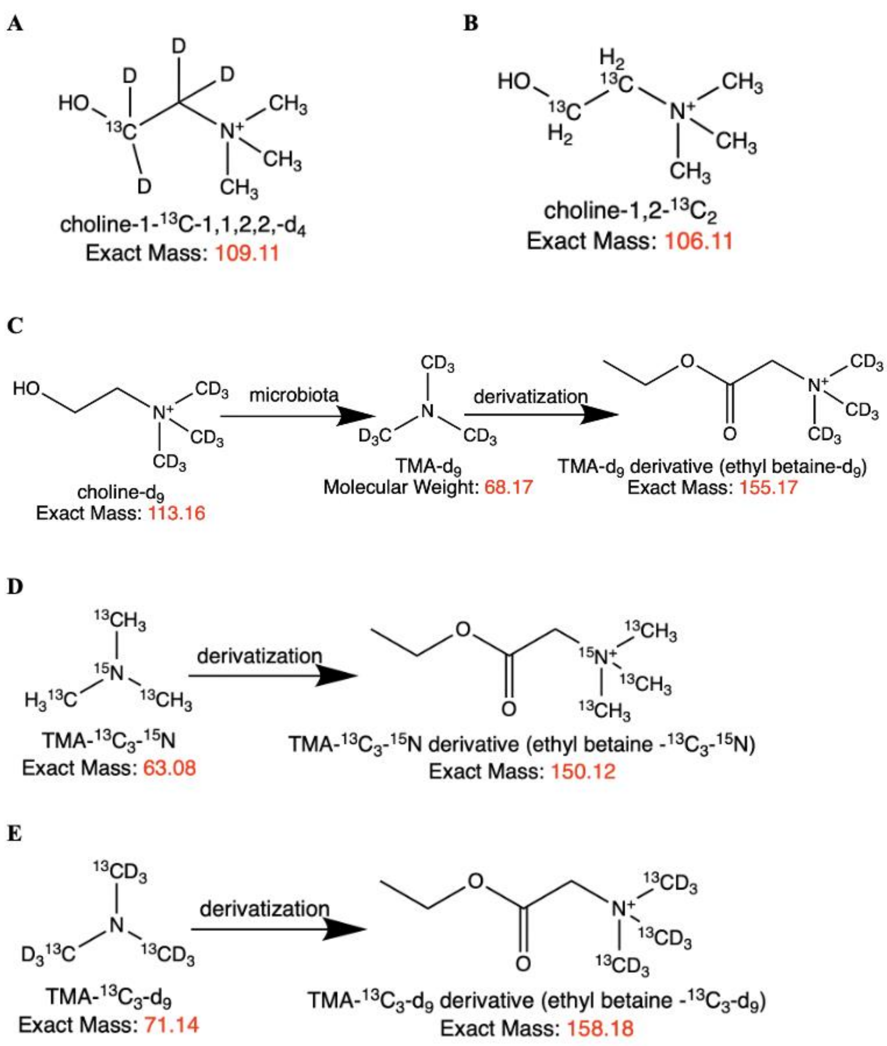


Figure 3.3. Internal standard choline-1-^{13}C-1,1,2,2, -d_4 (A), internal standard choline-1,2-$^{13}C_2$ (B), choline-d_9 metabolism to TMA-d_9 derivatization (C). TMA-13C_3-15N derivatization (D), TMA-13C_3-d_9 derivatization.

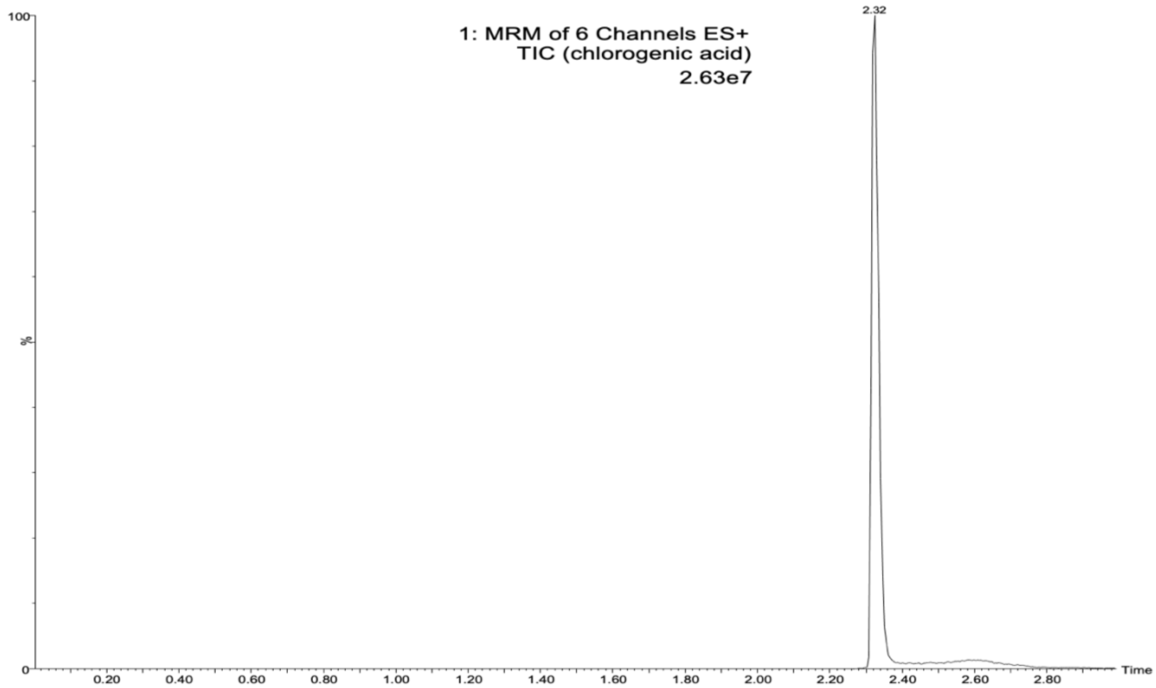


Figure 3.4. Chromatogram of chlorogenic acid

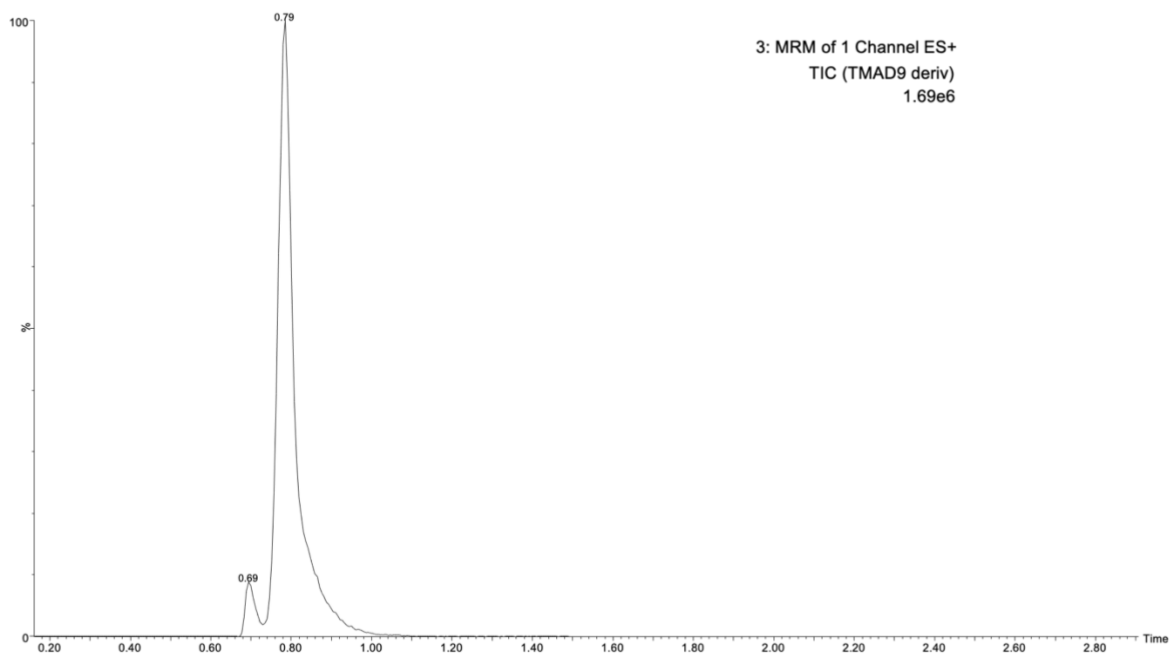


Figure 3.5. Chromatogram of derivatized TMA-d_9

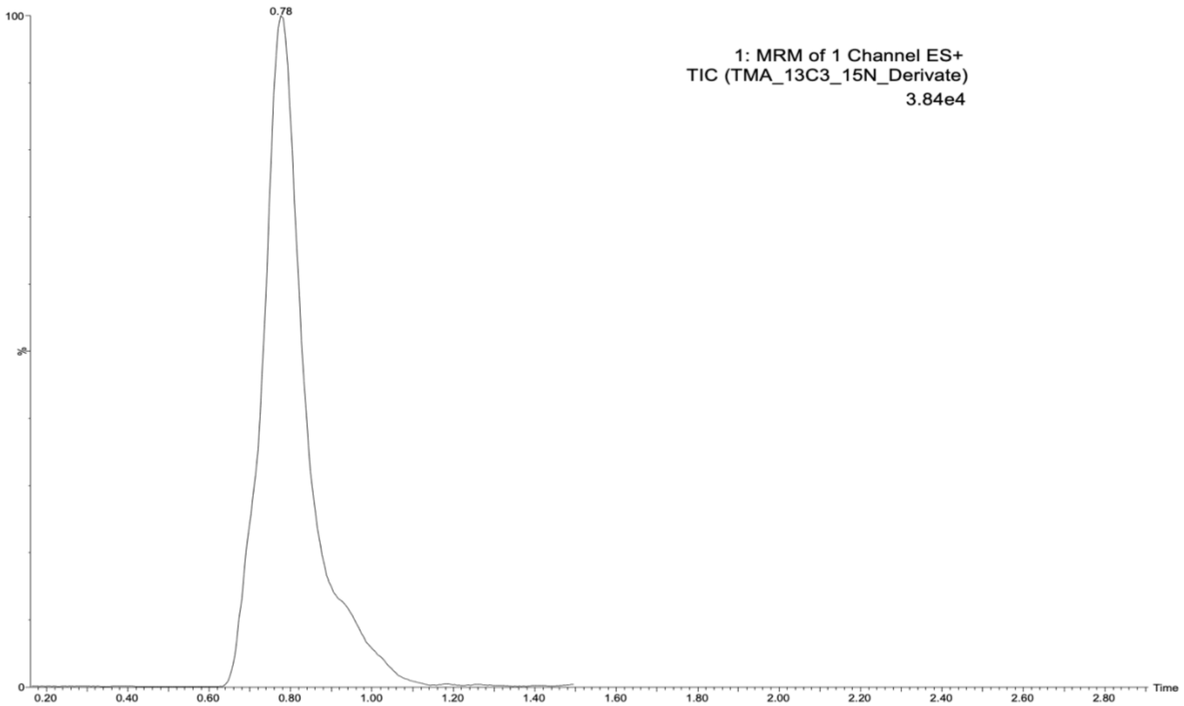


Figure 3.6 Chromatogram of derivatized internal standard TMA-$^{13}C_3$-^{15}N (ethyl betaine $^{13}C_3$-^{15}N)

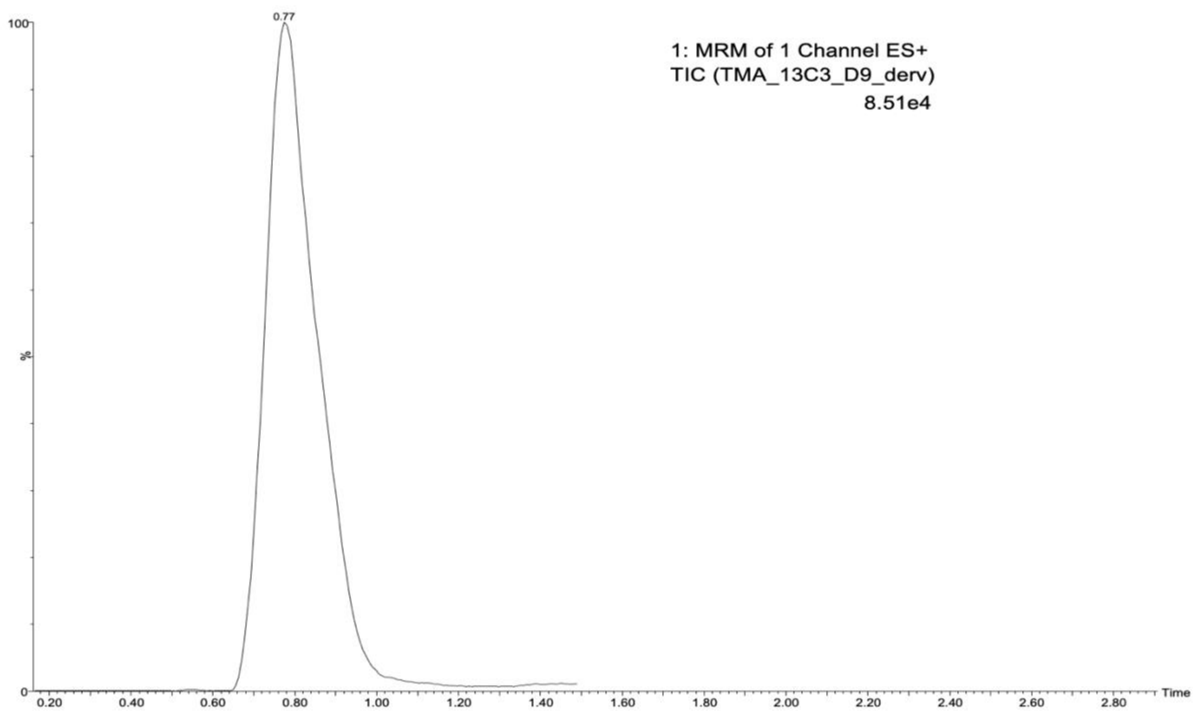


Figure 3.7. Chromatogram of derivatized internal standard TMA-$^{13}C_3$-d_9 (ethyl betaine $^{13}C_3$- d_9)

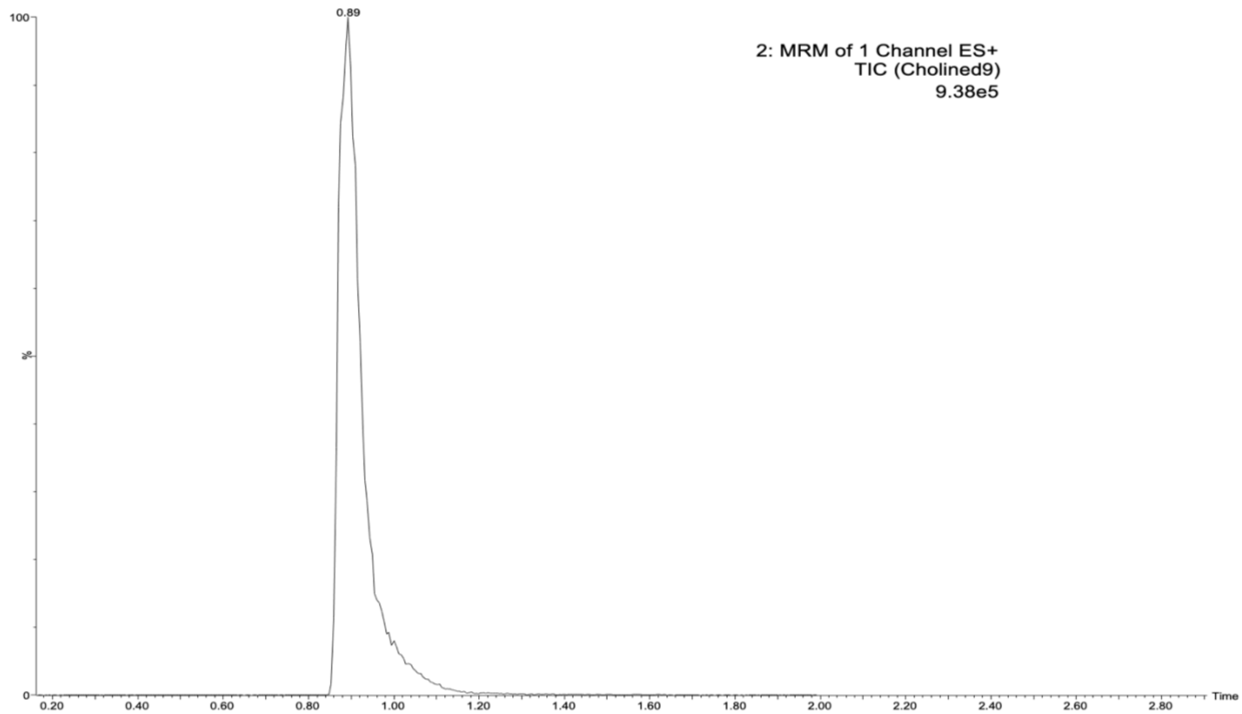


Figure 3.8. Chromatogram of choline-d_9

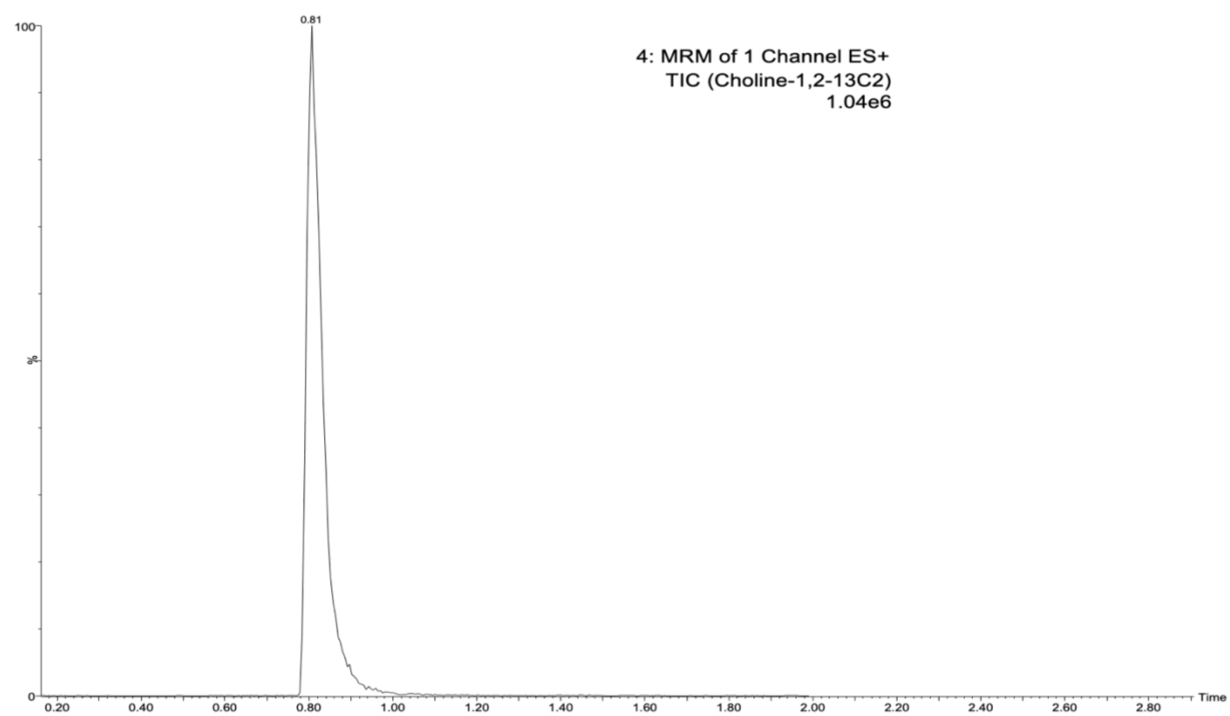


Figure 3.9. Chromatogram of internal standard choline-1-^{13}C-1,1,2,2, -d_4

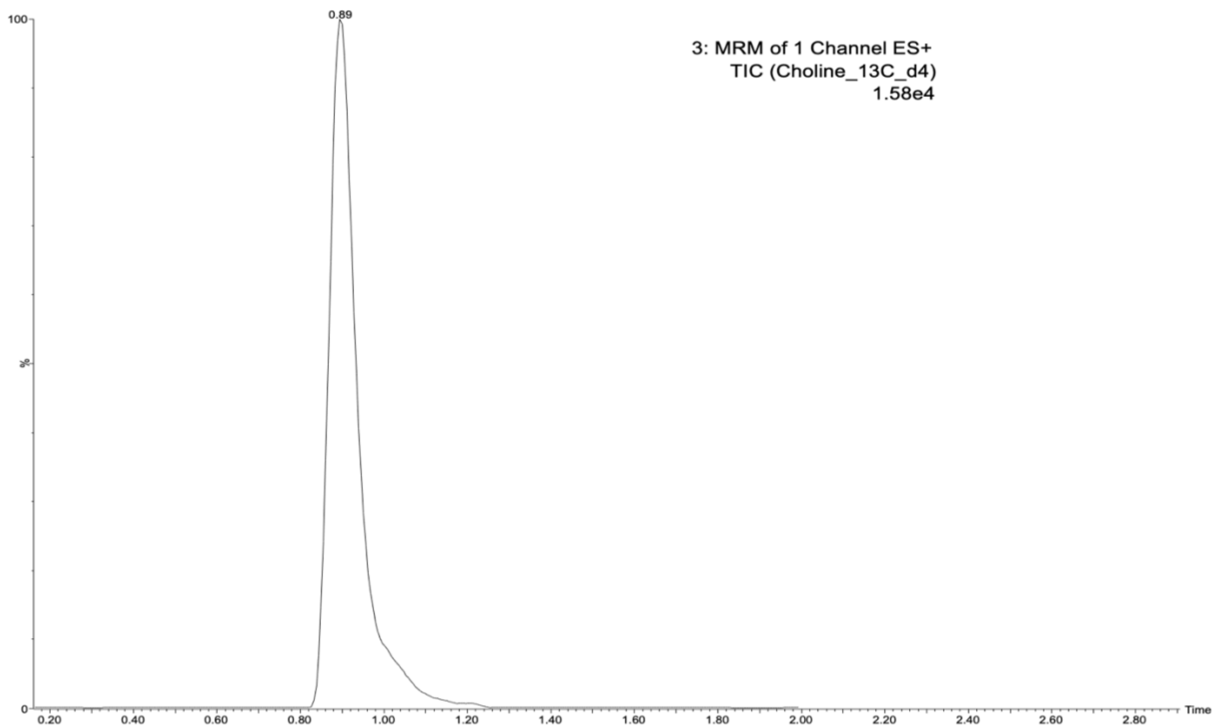


Figure 3.10 Chromatogram of internal standard choline-^{13}C-d_4

3.3 Specific Experiments and Methods

3.3.1 Objective 1: Determine the relative impacts of phenolic (CGA) and non-phenolic components (sugar, fiber, skin, pulp, etc.) of blueberries on TMA production.

3.3.1.1. Experiment 1: BacTiter-Glo Optimization: The BacTiter-Glo ATP assay (Promega, USA) protocol does not provide specific guidelines for adapting the assay for more complex matrices. Given the complexity of the fermentation solution in our model, modifications are necessary to minimize background interferences.

Plate Color Determination

Goal: The goal of this experiment was to determine whether a black or white plate is the most suitable for luminescence readings and to identify potential background interferences in the fermentation mixture.

Specific methods: To identify the color of 96-well assay plate that would produce the strongest luminescence signal and to identify potential background interferences of the fermentation mixture, 1mM ATP in PBS 1X was prepared and ATP standard curves were made in varying mediums that represented a component of the final fermentation mixture: PBS, growth media, PBS+ choline-d_9, PBS +CGA (10mM, 1mM, 10 μM and 1 μM), PBS + blueberry digesta, PBS + blank digesta. 100 μL of the serial dilutions of ATP in the various mediums were plated *n*=3 on white 96-well opaque round-bottom plates (Figure 4.1.) and *n*=3 on black 96-well clear bottom plates (Figure 4.2.). Each well was mixed with 100 uL of reconstituted BacTiter-Glo reagent at room temperature and placed in the plate reader (SpectraMax iD3, Molecular Devices, USA) for 5 min to rest. Luminesce was read (Shake: 10s, integration: 2000, read height: 0.50mm) and results recorded. 10 mM and 1 mM standards were not plated on black plates.

Evaluating the Effects of Washing Cells to Remove Supernatant Interferences:

Goal: Based on previous results from the plate color determination experiment, which showed inhibited luminescence in treatments with digesta, particularly blueberry digesta, an experiment was conducted to evaluate whether "washing" fecal bacteria reduces fermentation background interferences.

Methods: A fermentation was carried out in a 2 mL, 96-well plate following methodology described above in "*general fermentation procedures*". Treatments included growth media, growth media with fecal, blueberry digesta, blueberry digesta with fecal, blank digesta, and blank digesta with fecal. 100 uL of fermenta samples were plated in *n*=2 on a white 96-well opaque round-bottom plate at 12 h (Figure 4.3.) and 24 h (Figure 4.4.). The plate was centrifuged for 5 min at 4°C at 500 x g. The supernatant was removed from the first set of samples, and the pellet was resuspended in 100 μL of PBS 1X, this set of samples is referred to as “washed”. The second set of samples were resuspended back into the original fermenta sample. All samples were mixed with 100 μL of reconstituted BacTiter-Glo reagent and placed in the plate reader for 5 min before reading the luminescence. Results were recorded and it was determined that samples are to be centrifuged and resuspended in 100 μL PBS 1X before adding 100 μL BacTiter-Glo reagent to reduce matrix interference and obtain a clear signal.

Validating the Relationship Between Cells and BacTiter-Glo Response

Goal: The goal of this experiment was to identify the impact of fecal slurry concentration on luminescence signals of a BacTiter-Glo assay and to determine whether the signal is influenced by cell concentration rather than the mere presence of cells.

Methods: To evaluate the impact of fecal slurry concentration on luminescence signal, a 5-fold diluted fecal slurry (1 mL of fecal + 4 mL of media) was prepped under anaerobic conditions 12 hrs prior to the start of the experiment. Six serial dilutions were then created by diluting the 5-fold fecal slurry to achieve 10-fold, 20-fold, 40-fold, 80-fold, and 160-fold concentrations by diluting 1:1 using the fecal suspension and media at each step. 100 μL from each dilution were plated *n*=3 on a white 96-well opaque round-bottom plate. The plate was centrifuged at 500 x g for 5 min, the supernatant was removed, and the pellet resuspended in PBS 1X. Following resuspension of the pellet, 100 μL of reconstituted BacTiter-Glo was mixed into the samples and left to sit in the dark for 5 min. Luminescence was read (Figure 4.5.).

3.3.1.2 Experiment 2: Accounting for Assay Interferences: To account for possible sugar, fiber, or phenolic substrate preferences by the bacteria, a longer fermentation experiment (30 hrs as opposed to 24 hrs) provided insight on whether other components that are metabolized first are inhibiting the metabolism of choline. As shown in Figure 2.6, documented by Mike Sweet in our lab group (unpublished data), whole blueberries almost completely inhibit TMA production. However, this inhibition may be due to the sugar content, which could lead to physiologically irrelevant effects as seen in a study published by Bresciani *et al.*, as sugar would not remain in the colon following upper gastrointestinal digestion[162]. If bacteria exhibit a preference for sugar/fiber or other non-chlorogenic substrates over choline, prioritizing the consumption of these substrates may occur initially, which may result in misleading inhibition by whole blueberries. Prolonged fermentation durations may clarify whether choline is subsequently metabolized by bacteria, potentially explaining the TMA- lowering effects of blueberries. In addition to a prolonged fermentation, increasing the abundance of bacteria that express the choline-utilizing genes such as cut*C/*D and/or shifting metabolic preferences in specific bacteria towards dietary choline over other substrates such as sugar/fiber/phenolic compounds is advantageous for assessing subsequent TMA formation. The data is used to determine whether increasing choline metabolism will eliminate or minimize the influence of interfering compounds and to help determine what component of blueberries has the greatest TMA inhibitory activity.

Goal: To establish desired anaerobic fermentation parameters to account for fiber/sugar-rich fruits and determine the potential influence of non-phenolic blueberry components on the inhibition of TMA in our assay.

Methods: To identify what portion of blueberry tissue exhibits the greatest effect on TMA-lowering activities, whole blueberry, sugar, fiber, and phenolic acid fractions were used in the extended fermentation model (30 hrs as opposed to 24 hrs). Blueberries from two sources (fresh + frozen) were pooled to make a "representative" blueberry sample. Whole blueberries were used to extract phenolic compounds as per methodology described in "*polyphenol extraction*" adopted from Mohamedshah *et al*.[166]. Digestions were performed as described in the "*general gastrointestinal procedures*" adopted from Iglesias-Carres *et al*.[10]. Digestions are scaled from ~1 serving in human volume (1 cup or ~150 g fresh blueberries, ~14.7 g sugar content, ~3.55 g fiber content, and ~1.0125 g phenolic extract content) to an equivalent does for the *in vitro* digestion final volumes (table 3.2). A "blank" digesta was made with saline in place of food material but contained all other components of the digestion

Fermentations were carried out as per methodology described in "*general fermentation procedures*". Three different fecal samples were combined with 27 mL of growth media and filtered to remove solids. 10 mL of the filtered fecal slurry was aliquoted into a new 50 mL tube and 10 µL of unlabeled choline (100 µM final conc.) was added in attempt to "shift" metabolic preferences in specific bacteria towards dietary choline over other substrates. Both 50 mL tubes containing the fecal the slurry (one containing unlabeled choline and the other without) was left uncapped in the anaerobic chamber for 12 hrs. Treatments inoculated with the fecal slurry containing the unlabeled choline are indicated as "pre-fed". From 0 to 30 hours, 100 µL of sample was collected at 0, 2, 4, 6, 10, 14, 18, 22, 26, and 30 hr time points, combined with 100 µL of acetonitrile, and frozen at −80°C. To confirm the treatments did not have a cytotoxic or cytostatic effect on fecal bacteria cells, an ATP assay was used at 14 hr (Figure. 4.4 A.) and 30 hr (Figure. 4.4 B.) time points to monitor cell growth and cell respiration following methodology described in "*BacTiter-Glo Assay*" and "*BacTiter-Glo Analysis".*

Sample extraction and quantification was carried out as described in "*extraction and quantification*". Internal standards for this experiment included choline-1-^{13}C-1,1,2,2, -d_4 and TMA-$^{13}C_3$-^{15}N. Data analysis was carried out as described in "choline and TMA analysis" utilizing a two-way ANOVA test to evaluate the statistical differences between choline-d_9 (Figure. 4.5.) and TMA- d_9 (Figure. 4.7.) kinetic curves, and AUCs for Choline (Figure. 4.6.) and TMA (Figure. 4.8.) "pre-fed" vs "non pre-fed" fermentations.

Table 3.2 Weights of Food Material for a 50 mL Digestion.

Food (*n*=2)	**Amount in 1 serving (g) per 2 L upper GI volume**	**Target weight (mg) in 50 mL digestion**	**Actual weight (mg) in 50 mL digestion**
Whole Blueberry	150	3750	A: 3.7275 B: 3.7275
Sugar	14.7	367.5	A: 367.6 B: 367.5
Fiber	3.55	88.75	A: 88.8 B: 88.7
Phenolic extract	1.0125	25.3125	A: 25.4 B: 25.9

3.3.1.3 Experiment 3: Identifying Blueberry Components with the Greatest TMA Inhibition Potential: Building on the results from Experiment 2, which showed that whole blueberry and sugar fractions exhibited the greatest TMA inhibition, further analysis was conducted to identify blueberry components with the most significant TMA-lowering effect in the TMA assay. Store-bought blueberries were separated into skin and pulp, and their respective ratios were used to compare inhibition potential against whole blueberry, sugar fraction, and fiber fraction.

Goal: To further identify blueberry components with the most significant TMA-lowering effect in the TMA assay.

Methods: Total phenolic levels were determined for whole blueberry, skin, and pulp fractions using methodology described in "*plant phenolic extraction*" and "*Phenolic analysis: Folin- Ciocalteu assay*". CGA content was determined using a solid phase elution (SPE) and LC/MS quantification as described in "*Solid Phase Elution (SPE)*". Sugar and fiber fractions were included in the digestion and fermentation to compare inhibition levels.

To separate the skin from the pulp, a revised blanching method was used. The whole blueberries were submerged in liquid nitrogen for 10 seconds, removed from the liquid nitrogen and submerged in boiling water for 5 seconds, and then removed from the water and submerged in ice water for 15 seconds. After this process, the peels separated from the pulp with ease. The blueberries were weighed before separation, and both the peel and pulp fractions were weighed. 16.58 g of peel and 107.44 g of pulp were recovered. These values were used to determine weights for the digestion (~13.37% peel and ~86.67% pulp in a serving size of blueberries). Whole blueberry, skin fraction, and pulp fraction were blended separately using a polytron. The blended fractions were flash frozen and stored at –80°. It is important to note that this method may influence the enzymatic oxidation of polyphenols in the blueberries. When the berries are consumed and chewed, the polyphenols are exposed to polyphenol oxidase (PPO), the enzyme responsible for the oxidation of phenolic compounds. The blanching method used can deactivate PPO, preventing the phenolics from oxidation and leading to effects in the *in vitro* experiment that may not be observed *in vivo*[167].

For the Folin extract, 249.72 mg of whole blueberry, 250.94 mg of peel, and 250.90 mg of pulp were used. To quantify the total phenolics in the extracts, a Folin assay was carried out in a 96 well plate. Each extract underwent a series of dilutions. Starting with the pure extract then diluting 10x, then diluting 100x, etc. A standard curve was made with a 2mg/mL gallic acid mixture with a series of 23- 2/3rds dilutions with a "blank" as pure water. Total phenolics were expressed as mg of gallic acid equivalent (GAE)/ g or whole blueberry (Table 4.3).

To further quantify the relative CGA amounts in the blueberry fractions, the extracts were analyzed using the LC-MS following the SPE (solid phase elution). For the SPE extract, 249.7 mg of whole blueberry, 250.5 mg peel, and 499.6 mg pulp were used. A standard curve was made with a 1mg/mL CGA mixture with a series of dilutions. Samples were plated in triplicate. Total CGA content was expressed as mg CGA/ g whole blueberry (Table 4.4).

Digestions were performed as described in the " *general gastrointestinal procedures*" adopted from Iglesias-Carres *et al.*[10]. Blueberries from two sources (fresh + frozen) were pooled to make a "representative" blueberry sample. Digestions are scaled from ~1 serving in human volume (1 cup or ~150 g fresh blueberries, ~20.05 g peel content, 129.95 g pulp content, ~14.7 g sugar content, and~3.55 g fiber content.) to an equivalent does for the *in* vitro digestion final volumes (Table 3.3). A "blank" digesta was made with saline in place of food material but contained all other components of the digestion.

Fermentations were carried out as per methodology described in "*general fermentation procedures*". Two different fecal samples were combined with 16 mL of growth media and left uncapped in the anaerobic chamber for 12 hrs. The slurry was filtered immediately before the start of the fermentation. The remaining 2 mL of media was used to rinse residual fecal slurry through the filter. The 50 mL tube containing the fecal the slurry was left uncapped in the anaerobic chamber for 12 hrs. From 0 to 24 hours, 100 μL of sample was collected at 0, 2, 4, 6, 8, 10, 12, 16, 20, and 24 hr time points, combined with 100 μL of acetonitrile, and frozen at

−80°C. To confirm the treatments did not have a cytotoxic or cytostatic effect on fecal bacteria cells, an ATP assay was used at 12hr (Figure. 4.9 A.) and 24 hr (Figure. 4.9 B.) time points to monitor cell growth and cell respiration following methodology described in "*BacTiter-Glo Assay*" and "*BacTiter-Glo Analysis".*

Sample extraction and quantification was carried out as described in "*extraction and quantification*". Internal standards for this experiment included choline-1,2-$^{13}C_2$ and TMA-$^{13}C_3$-d_9. Data analysis was carried out as described in "*choline and TMA analysis*" displaying kinetic curves for choline- d_9 (Figure. 4.10 A.) and TMA- d_9 (Figure 4.10 B.), utilizing a one-way ANOVA test to evaluate the statistical differences between choline-d_9 (Figure 4.11 A.) and TMA- d_9 (Figure 4.11 B.) AUCs.

Table 3.3. Weights of Food Material for a 50 mL Digestion

Food (*n*=2)	**Amount in 1 serving (g) per 2 L upper GI volume**	**Target weight (mg) in 50 mL digestion**	**Actual weight (mg) in 50 mL digestion**
Whole Blueberry	150	3750	A: 3770 B: 3750
Peel	20.05	501.25	A: 501.73 B:501.89
Pulp	129.95	3248	A: 3260 B:3270
Sugar	14.7	367.5	A: 366.25 B: 367.24
Fiber	3.55	88.75	A: 87.44 B: 87.82

3.3.2 Objective 2: Determine whether CGA content correlates with reduced TMA production in multiple blueberry accessions from a genetic diversity population with a large spread of CGA in our *ex vivo-in vitro* human fecal fermentation model.

3.3.2.1 Experiment 4 Determination of TMA Inhibition Potential of Varying Levels of CGA Content in Genetically Different Blueberries: To account for the varying levels of CGA among genetically diverse blueberry populations, 20 different blueberry cultivars from the National Clonal Germplasm Repository (NCGR), Corvallis OR, USA were used to determine the impact of CGA on the inhibition of TMA in our assay. In the fermentation, a total of eight blueberry cultivars were used, including four high-CGA and four-low CGA varieties from the biparental mapping and diversity panel populations. The CGA content of these blueberries aligned with values reported in previous years, as shown in Figure 2.4, confirming their relevance and importance in the study. The data was used to help identify blueberry cultivars with greater TMA-lowering activity, which may drive the cultivation and consumption of berries with enhanced health benefits.

Goal: To compare the relative amounts of CGA across the 20 different blueberry cultivars and use the peel of the four highest and four lowest CGA content blueberries in a fermentation to determine the impact on TMA production.

Specific Methods: Extracts were prepared for each of the 20 different blueberry cultivars as described in "*plant phenolic extraction*". A SPE (solid phase elution) and LC-MS analysis was completed using the extracts as described in "*Solid Phase Elution (SPE) and Analysis*". For the extract, ~250 mg of blueberries from each cultivar (n=4) were used in four separate replicates. This allowed each replicate to consist of a unique set of blueberries from the same cultivar, ensuring a more varied representation of each cultivar. (Table 3.4). A standard curve was made with a 4mg/mL CGA mixture with a series of 35- 2/3rds dilutions with the 35^{th} well as pure water. Samples were plated in n=4. Total CGA content was expressed as mg CGA/ 100g whole blueberry (Table 4.5.). CGA content is used to determine four of the highest CGA content and four of the lowest CGA content blueberry cultivars to use in the fermentation model (Figure. 4.12.).

The highest four CGA content cultivars (DxJ 033, PI 267851, DxJ 002, PI 296399) and the lowest four CGA content cultivars (PI 554856, PI 554857, DxJ 197, DxJ 148) were peeled by hand, blended with a polytron (VWR 200), and weighed for a 15 mL digestion. Digestions were performed as described in the " general gastrointestinal procedures" adopted from Iglesias-Carres et al.[10]. Digestions are scaled from ~1 serving in human volume (150 g FW providing ~20.05 g peel content) to an equivalent does for the in vitro digestion final volumes (Table 3.5.). A "blank" digesta was made with saline in place of food material but contained all other components of the digestion.

Fermentations were carried out as per methodology described in "general fermentation procedures". Three different fecal samples were combined with 27 mL of growth media and left uncapped in the anaerobic chamber for 12 hrs. The slurry was filtered immediately before the start of the fermentation. The remaining 2 mL of media was used to rinse residual fecal slurry through the filter. The 50 mL tube containing the fecal the slurry was left uncapped in the anaerobic chamber for 12 hrs. From 0 to 24 hours, 100 µL of sample was collected at 0, 2, 4, 6, 8, 10, 12, 16, 20, 24 hr time points, combined with 100 µL of acetonitrile, and frozen at −80°C. To confirm the treatments did not have a cytotoxic or cytostatic effect on fecal bacteria cells, an ATP assay was used at 12hr (Figure 4.13 A.) and 24 hr (Figure 4.13 B.) time points to monitor cell growth and cell respiration following methodology described in "BacTiter-Glo Assay" and "BacTiter-Glo Analysis".

Sample extraction and quantification was carried out as described in "*extraction and quantification*". Internal standards for this experiment included choline-1,2-$^{13}C_2$ and TMA-$^{13}C_3$-d_9. Data analysis was carried out as described in "choline and TMA analysis" displaying kinetic curves for choline- d_9 (Figure 4.14 A.) and TMA- d_9 (Figure 4.15 B.). Utilizing a one-way ANOVA test to evaluate the statistical differences between choline-d_9 (Figure 4.15 A.) and TMA- d_9 (Figure 4.15 B.) AUCs.

Table 3.4. Weights for Cultivar Extracts

Cultivar	Replicate Weight (mg)
DxJ 197	A: 252 B: 252 C: 251.3 D: 251.8
DxJ 148	A: 251 B: 251.5 C: 253.1 D: 253.1
DxJ 112	A: 247.3 B: 253.7 C: 248.9 D: 251.6
DxJ 041	A: 248.8 B: 250.7 C: 249.5 D: 251.8
DxJ 125	A: 249.1 B: 251.6 C: 253.2 D: 249
DxJ 124	A: 251.1 B: 249.9 C: 252 D: 248.6
DxJ 023	A: 252.9 B: 251.7 C: 251.6 D: 249.4
DxJ 102	A: 251.1 B: 251.7 C: 250 D: 251
DxJ 038	A: 250.6 B: 252.5 C: 250.6 D: 252.8

Table 3.4. Weights for Cultivar Extracts Cont.	
DxJ 149	A: 253 B: 249.7 C: 252.7 D: 252.1
DxJ 002	A: 252 B: 250.2 C: 251.3 D: 249.5
DxJ 033	A: 250.5 B: 249.9 C: 251.6 D: 251.1
PI 554857	A: 251.3 B: 253 C: 250.9 D: 250.5
PI 554856	A: 251.5 B: 250.6 C: 250.5 D: 251.1
PI 554764	A: 253.5 B: 250.8 C: 251.5 D: 250.7
PI 554846	A: 250.6 B: 252.9 C: 250.1 D: 251
PI 554849	A: 253 B: 251.3 C: 251.1 D: 252.9
PI 638335	A: 249.9 B: 250.3 C: 253.5 D: 253.6
PI 267851	A: 249.9 B: 248.8 C: 252.7 D: 249.8
PI 296399	A: 252.3 B: 252.3 C: 251.4 D: 251.6

Table 3.5. Weights of Peel Material Used in 15 mL Digestion				
Food (*n*=2)	**CGA level**	**Amount in 1 serving (g) per 2 L upper GI volume**	**Target weight (mg) in 50 mL digestion**	**Actual weight (mg) in 50 mL digestion**
DxJ 197	Low	20.05	150.375	A: 152.4 B: 149.1
DxJ 148	Low	20.05	150.375	A: 150.5 B: 149.8
DxJ 002	High	20.05	150.375	A: 150.6 B: 150.0
DxJ 033	High	20.05	150.375	A: 149.6 B: 151
PI 554857	Low	20.05	150.375	A: 154.3 B: 151.8
PI 554856	Low	20.05	150.375	A:151.7 B: 149.7
PI 267851	High	20.05	150.375	A: 151.9 B: 151.6
PI 296399	High	20.05	150.375	A: 151.8 B: 150.6

Average cultivar size was measured to determine whether CGA content correlates with size of the blueberry. If smaller blueberries contain a higher amount of CGA, a smaller quantity will need to be consumed to achieve the health benefits associated with blueberries. 15 blueberries from each cultivar were selected at random and the size (diameter) was measured in mm with an electric Mitutoyo digimatic caliber and recorded. The average size and standard error of the mean was calculated and used to plot against average CGA content of the same cultivar (Figure 4.17).

3.3.3 Digesta Sugar Removal: Sucrose, glucose, and fructose in foods are readily absorbed during upper gastrointestinal digestion. In attempt to remove sugar from digesta material, enzymes were introduced at varying concentrations and allowed to react with sugar material for different durations, to determine the optimal amount of time for reducing the amount of sugar molecules.

Goal: The objective of the initial reaction is to eliminate all sucrose molecules.

Specific Methods: Invertase is an enzyme that breaks down sucrose into glucose and fructose molecules. The sucrose assay reagent (Sigma Product No. S 1299) was reconstituted with 2 mL of Milli-Q water. Initially, 100 µL of the reconstituted reagent was combined with 100 µL of the digesta sample (either whole blueberry or sugar digesta, n=2) and incubated for 30 or 50 minutes at room temperature. Subsequently, 250 µL of sucrose reagent was mixed with 100 µL of the digesta sample (either whole blueberry or sugar digesta, n=2) and left to sit for 30 or 50 minutes at room temperature. The LC-MS analysis for sucrose indicated there was not enough degradation and did not yield the expected results, and therefore the data is not presented.

3.3.4 GraphPad Prism Analysis:

3.3.4.1 **Choline and TMA Analysis:** Prism 10.4.1(GraphPad, La Jolla, CA) was used for statistical analyses and graph creation. One-way ANOVA test was used to evaluate statistical differences in experiments with one independent variable between control conditions and test solutions (treatment effects). Two-way ANOVA test was used to evaluate statistical differences in experiments with multiple independent variables between control conditions and test solutions as well as choline-d_9 and TMA- d_9 kinetic curves (significant main effect/ interaction). If a significant difference ($P < 0.05$) is noted, Tukey post hoc test or Sidak's multiple comparison test will be used to assess the significance of the difference between means. If kinetic curve calculated values are negative, a zero is imputed for the AUCs calculations. One-way ANOVA test was used to estimate differences in choline- d_9 and TMA- d_9 AUCs between treatments and controls.

3.3.4.2 BacTiter-Glo Analysis: Prism 10.4.1 (GraphPad, La Jolla, CA) was used for statistical analysis and graph creation. One-way ANOVA test was used to evaluate statistical differences, if a significant difference was noted ($P<0.05$) then a Tukey's post hoc multiple comparison test was used to evaluate statistical differences between treatments means.

CHAPTER 4 RESULTS AND DISCUSSION

4.1 Introduction This chapter presents the results of the experiments conducted for this thesis, including a series of graphs and tables that visually summarize the data collected. Each figure and table are accompanied by a detailed description, highlighting the key trends, patterns, and significant observations.

4.2. Objective 1: Determine the relative impacts of phenolic (CGA) and non-phenolic components (sugar, fiber, skin, pulp) of blueberries on TMA production.

4.2.1. Experiment 1: BacTiter-Glo Assay Optimization: This experiment aimed to determine whether a white or black plate is the most suitable for luminescence readings, identify potential background interferences in the fermentation mixture, evaluate whether "washing" fecal bacteria reduces fermentation background interferences, and to identify the impact of fecal concentration on luminescence signal.

Plate Color Determination: The goal of this experiment was to identify the color plate that will produce the strongest luminescence signal and to identify potential background interferences of the fermentation mixture.

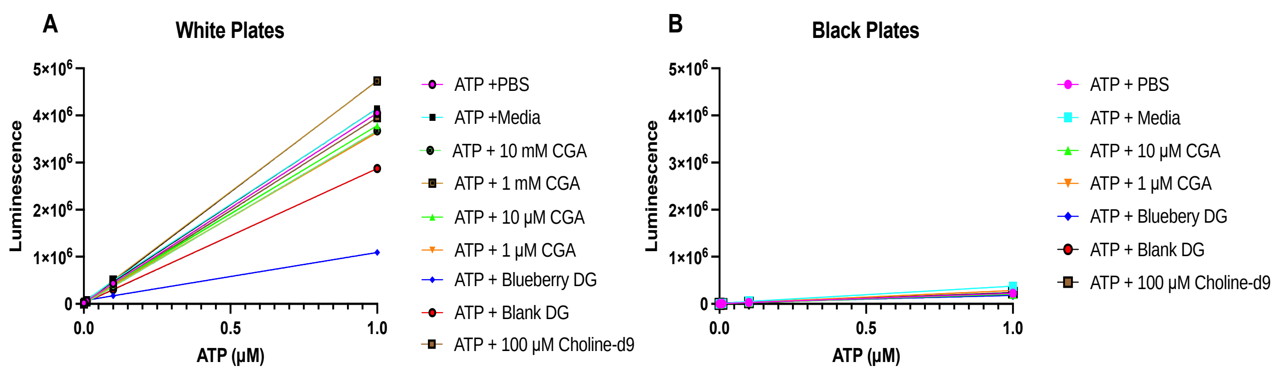


Figure 4.1. ATP standard curves in varying mediums representing a component of the final fermentation mixture plated on a black 96-well white round-bottom plate (A) and black 96-well clear-bottom plate (B). chlorogenic acid (CGA), digesta (DG): PBS, growth media, 10 mM CGA, 1 mM CGA, 10 µM CGA, 1 µM CGA, blueberry DG, blank DG, and choline-d_9 (100 µM). Data represents mean SEM from *n*=3.

The results of the plate color determination experiment are presented in Figure 4.1 where ATP standard curves prepared in varying mediums exhibited different luminescence signals. On the white plate, standard curves prepared in media representing blueberry digesta and blank digesta yielded the lowest luminescence signals compared to other treatments, with blueberry digesta showing the greatest suppression, likely due to the blue color of the blueberry. This suggests that digestive enzymes, along with the presence of food and associated color, interfere with the luminescence signals. In contrast, ATP standard curves made with choline-d_9 displayed the highest luminescence among all treatments. PBS and media treatments, which constitute the largest proportion of the final fermentation mixture, exhibited high luminescence readings. This suggests that any potential reduction in luminescence signals in fermenta containing fecal bacteria is more likely due to interference from the digesta background (particularly blueberry digesta), rather than from PBS or the media background. In comparison to the white plates, the ATP standard curves on the black plates showed a significantly greater reduction in signal magnitude. This is likely due to the black plates absorbing the luminescence, combined with the clear bottom of the plate, which allows the luminescence to escape through the bottom. Since the plate reader detects the signal from the top, this plate setup resulted in a reduced signal being measured. Additionally, the treatments plated on the black plates exhibited less variation between treatments, making it more challenging to detect differences in background interferences. Overall, white plates were determined to be the most suitable for the BacTiter-Glo ATP Assay, as they provided higher luminescence signals and better differentiation between treatments.

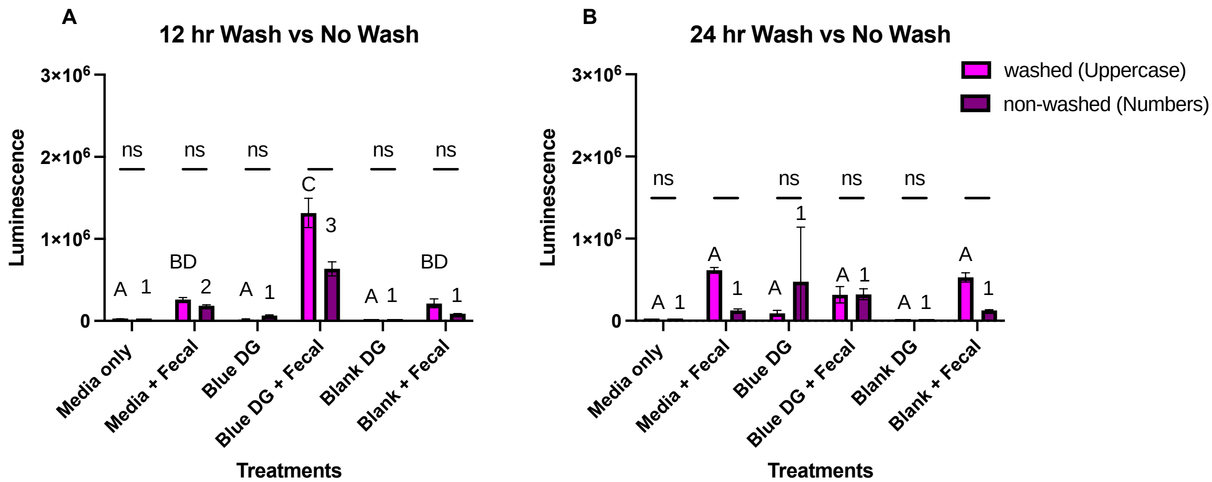
Table 4.1. Simple Linear Regression Tabular Results for ATP Standard Curves in Varying Mediums of PBS, Growth Media, Chlorogenic acid (CGA), Digesta (DG) and Choline-d9 Plated on White 96-well Opaque Plates (*n*=3)

	PBS	**Growth Media**	**10 mM CGA**	**1 mM CGA**	**10 µM CGA**	**1 µM CGA**	**Blueberry DG**	**Blank DG**	**Choline-d9 100 µM**
R-Squared	1.000	0.9999	1.000	1.000	1.000	1.000	1.000	1.000	1000
P value of the slope	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Table 4.2. Simple Linear Regression Tabular Results for ATP Standard Curves in Varying Mediums of PBS, Growth Media, Chlorogenic acid (CGA), Digesta (DG) and Choline-d9 Plated on Black 96-well Clear Round-Bottom Plates (*n*=3)

	PBS	**Media**	**10 µM CGA**	**1 µM CGA**	**Blueberry DG**	**Blank DG**	**Choline-d9 100 µM**
R-squared	0.9991	0.9991	0.9984	0.9994	0.9992	09997	0.9994
P value of the slope	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Testing the Luminescence Signal of "Washed" Fecal Cells: Building on the results from the plate color determination experiment, a follow-up experiment was conducted to assess whether "washing" the fecal bacteria could reduce the fermentation background interferences identified previously, particularly those associated with the blueberry and blank digesta. For this experiment, all fermentation samples were plated onto white 96-well plates, as these plates were found to provide the strongest luminescence signal. It was hypothesized that removing the fermentation background would isolate the fecal bacteria in the sample, eliminating the interference from the complex fermentation matrix. This would result in a clearer luminescence signal, better reflecting ATP levels from the fecal bacteria, with less background interference.



Source of Variation	% of total variation	P value	P value summary
treatments x wash	11.32	<0.0001	****
treatments	83.38	<0.0001	****
wash	3.564	<0.0001	****

Fixed effects (type III)	P value	P value summary
treatments	0.2732	ns
wash non wash	<0.0001	****
treatments x wash non wash	<0.0001	****

Figure 4.2. Luminescence signals of all treatments at the 12 hr (A) and 24 hr (B) mark. Washed (W), digesta (DG), and blue (whole blueberry digesta): media only, W media only, W media + fecal, media + fecal, W blue DG, Blue DG, W blue DG + fecal, blue DG + fecal, W blank DG, blank DG, W blank DG + fecal, blank DG + fecal. Different uppercase letters notate statistical differences between washed treatments (P<0.05) using a 2-way ANOVA and Sidak's multiple comparison test. Different numbers notate statistical differences between non-washed treatments (P<0.05) using Mixed-effect analysis and Sidak's multiple comparison test. Asterisks notate statistical differences between washed and non-washed within treatments (P<0.05) using a 2-way ANOVA and Tukey's multiple comparison test. Data represents mean SEM from n=2.

Results of the washing experiment are presented in Figure 4.2. A (12 h) and B (24 h). Treatments inoculated with fecal slurry exhibited a clear luminescence signal, providing evidence that the signal is originating from the fecal bacteria, rather than from other components of the fermentation matrix. Notably, the washed blueberry digesta and fecal treatment produced a luminescence signal that was twice as high as the same unwashed treatment, providing strong evidence that washing effectively reduces the background interferences from the digesta. At the 12-hour mark, a statistically significant difference was observed between the washed and unwashed samples that contained fecal bacteria, supporting the rationale for washing the fermenta samples to minimize background interference. At the 12 hr mark, treatments containing blueberry digesta exhibited significantly higher luminescence signals compared to all other treatments, suggesting blueberry digesta may promote bacterial growth. By the 24 hr mark, all treatments exhibited a luminescence signal, with W media + fecal and W blank + fecal exhibiting the highest signal. Interestingly, whole blueberry treatments at 24 hr displayed a signal even in the absence of fecal inoculation, which may be attributed to a potential bacterial contamination in those treatments. Overall, washing the fecal bacteria in the fermenta samples significantly reduced background interference, enhancing the detection of luminescence. This allowed the assay to specifically measure luminescence from the bacteria, rather without quenching interferences from the fermentation matrix. As a result, all fermentation treatments were "washed" in subsequent experiments

Relationship between cell concentration and luminescence: With the plate color established and the sources of interference identified in the previous experiments, the aim of this experiment was to assess the impact of fecal slurry concentration on the luminescence signals in a BacTiter-Glo assay. Specifically, the experiment sought to determine whether the luminescence signal correlates with cell concentration, rather than just the presence of cells. This aimed to reveal whether there is a linear relationship between cell concentration and luminescence.

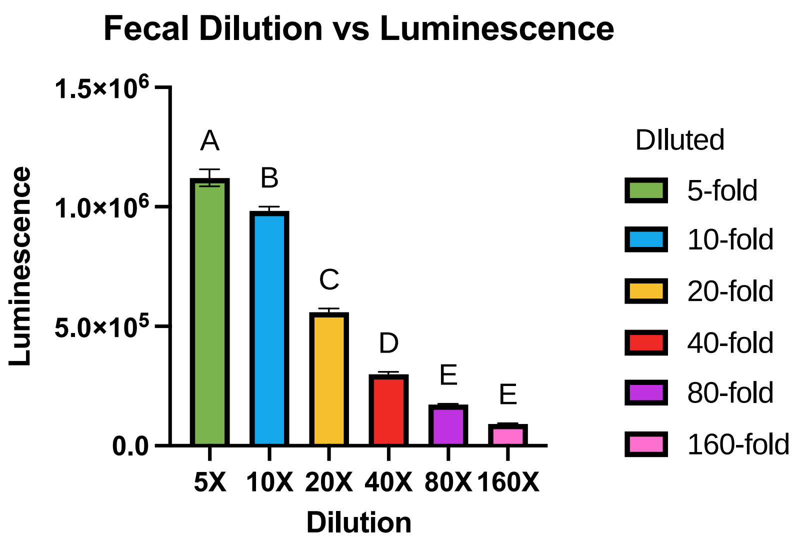
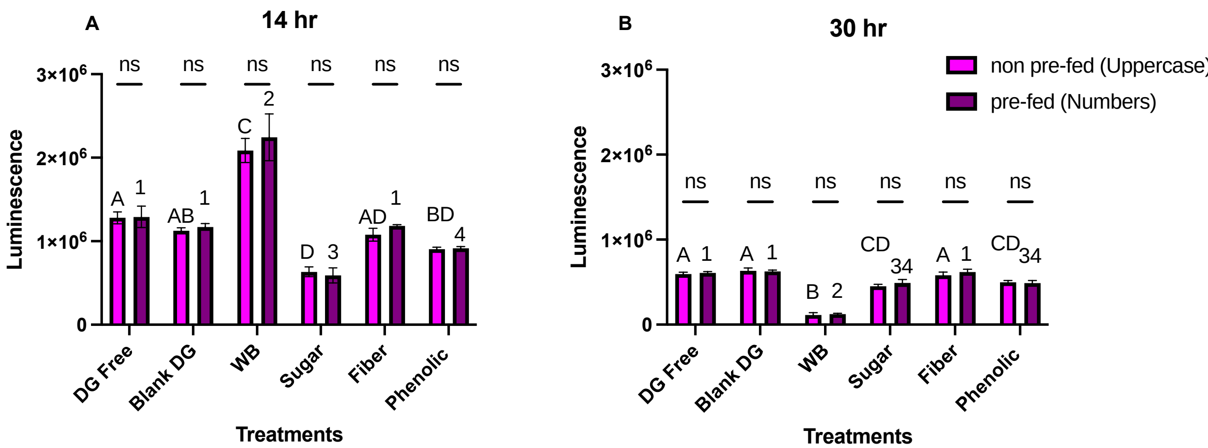


Figure 4.3. BacTiter-Glo luminescence of varying concentrations of diluted fecal slurry: 5-fold, 10-fold, 20-fold, 40-fold, 80-fold, 160-fold. Different letters notate a statistical difference ($P<0.05$) using an ordinary one-way ANOVA with Tukey's multiple comparison test. Data represent mean ± SEM from n=3

Results from the relationship between cell concentration and luminesce are presented in Figure 4.3, where luminescence signals for all dilutions are statistically different from one another. A linear trend is observed for the 10-fold, 20-fold, 40-fold, 80-fold, and 160-fold dilutions, with luminescence signals decreasing as fecal concentration becomes more diluted. However, the difference in luminescence signals between the 5-fold and 10-fold dilutions is less pronounced, suggesting that at very high and very low concentrations, differentiation becomes less linear. At higher concentrations, the luminescence signal levels off, rather than continuing to follow a linear pattern.

4.2.2 Experiment 2: identifying fermentation interferences: The goal of this experiment was to establish desired anaerobic fermentation parameters and to account for fiber/sugar-rich fruits and determine the potential influence of non-phenolic blueberry components on the inhibition of TMA in our assay. Unpublished preliminary data shown in Figure 2.6 on the inhibition effects of different blueberry cultivars revealed significant (almost total) inhibition compared to other foods previously studied in our lab. Additionally, previous work by Bresciani *et al.* has shown that sugar, which would not be present in the colon after upper gastrointestinal digestion, can significantly inhibit TMA formation[162]. Since the majority of the fruit's sugar content is found in the whole blueberry pulp, it was necessary to investigate whether components of blueberries, such as sugar, were interfering with the assay. Given the nature of the digestion model, removing sugar is not possible without also eliminating other water-soluble compounds, such as chlorogenic acid, that are not well-absorbed. To account for possible sugar, fiber, or phenolic substrate preferences by the bacteria, a longer fermentation experiment (30 hrs as opposed to 24 hrs) provided insight on whether other components, such as sugar, that are metabolized first are inhibiting the metabolism of choline. The data is used to determine whether increasing choline metabolism will reduce or eliminate the impact of interfering compounds, and to identify which component of blueberries has the greatest TMA inhibition potential. This includes evaluating potentially physiologically irrelevant effects, such as inhibition by sugar, which would not occur *in vivo* due to absorption in the upper GI tract that prevents interaction with bacteria that produce TMA.



Source of Variation	% of total variation	P value	P value summary
treatments x pre-feed	0.4537	0.1947	ns
treatments	95.58	<0.0001	****
pre-feed	0.2367	0.0519	ns

Source of Variation	% of total variation	P value	P value summary
treatments x pre feed	0.3177	0.2899	ns
treatments	97.84	<0.0001	****
pre feed	0.1539	0.0876	ns

Figure 4.4. BacTiter-Glo luminescence reading at 14 hr (A) and 30 hr (B) of "pre-fed" (PF) vs "non pre-fed" treatments using one-way ANOVA to compare between treatments, and a multiple comparison Tukey post hoc test to compare between PF and non-PF. Different uppercase letters note a statistical difference (P<0.05) between non-pre fed treatments and different numbers notate statistical difference (P<0.05) between pre-fed treatments. Digesta (DG), Pre-fed (PF), whole blueberry (WB): DG free, PF DG free, Blank DG, PF blank DG, WB DG, PF WB DG, Sugar DG, PF Sugar DG, Fiber DG, PF fiber DG, Phenolic DG, and PF phenolic DG. Data represent mean ± SEM from *n*=6

BacTiter-Glo luminescence results are presented in Figure 4.4. Luminescence readings relative to controls (blank digesta and digesta free) for blueberry fractions in the fermentation at 14 hr (A) and 30 hr (B). A reduction in bacterial growth would be indicated by a very low or absent luminescence signal. Compared to the controls there is no evidence suggesting that any treatment caused a cytotoxic effect that would lead to the perceived effect of TMA inhibition. Luminescence readings for pre-fed vs non pre-fed, within the same treatment, did not show a statistical difference in cell viability at 14 hr. Whole blueberry (WB) luminescent signal is higher compared to all other treatments at the 14 hr time point, likely due to the blueberry promoting cell growth. By the 30 hr time point, the WB signal is much smaller compared to the other treatments. This may be due to the fast consumption of sugar and other readily available substrates, as the bacteria quickly metabolize the blueberry digesta, leaving fewer substrates available for growth by the 30-hour mark. Since no statistical differences in cell growth were observed between pre-fed vs non-pre-fed within the same treatments, it was determined that pre-feeding had no impact on growth and was therefore not continued for subsequent experiments.

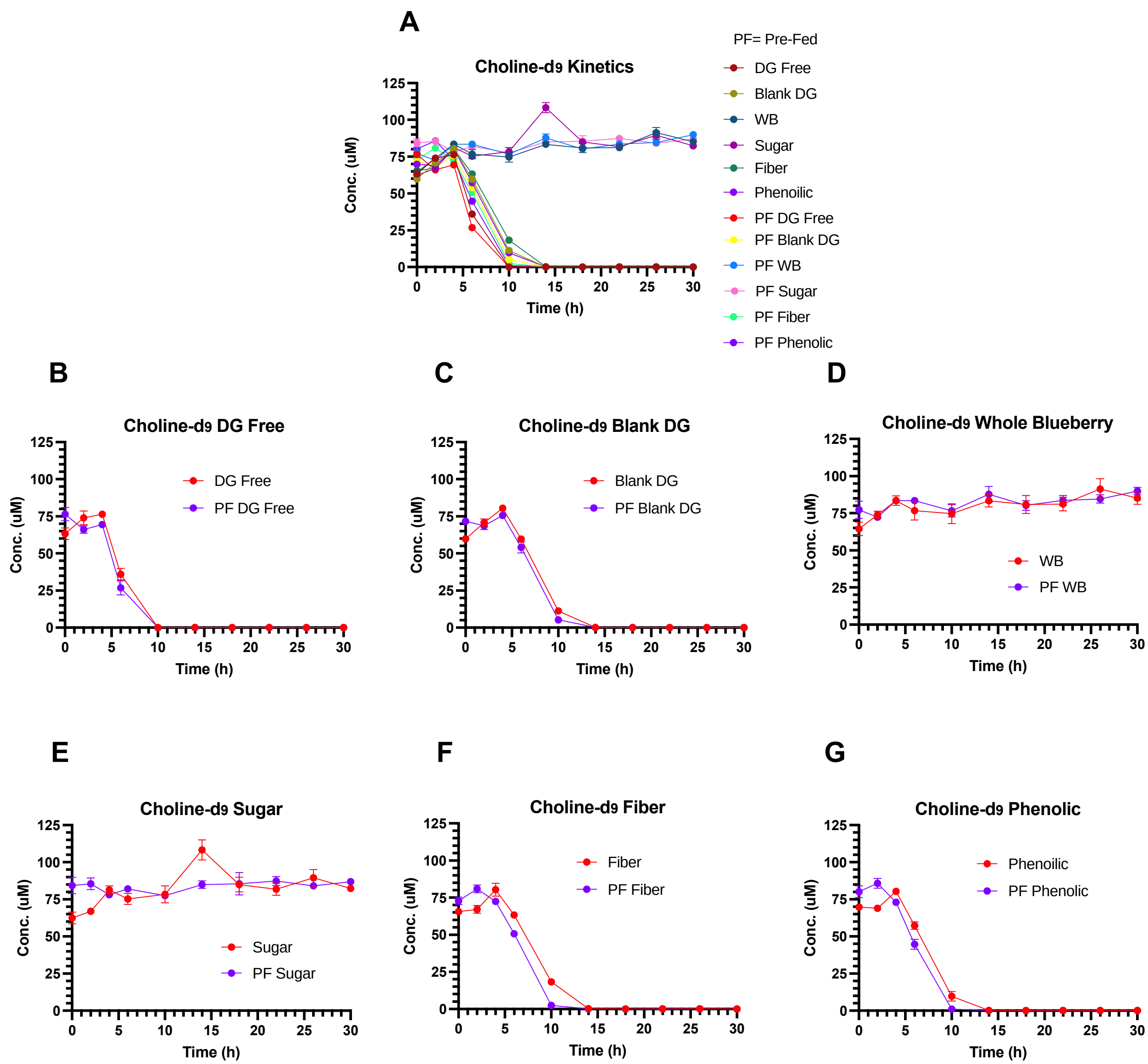
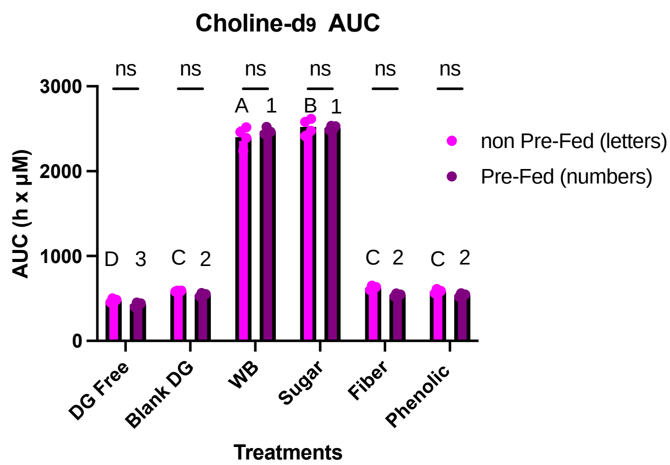


Figure 4.5. Kinetic curve of choline-d_9 concentration in a fermentation (0-30 hr) of all treatments: digesta (DG), Pre-fed (PF), whole blueberry (WB): DG Free vs PF DG Free (B), Blank DG vs PF Blank DG (C), WB vs PF WB (D), Sugar vs PF Sugar (E), Fiber vs PF Fiber (F), and Phenolic vs PF Phenolic (G). Data represent mean ± SEM from *n*=6.

Kinetic curves of choline-d_9 concentration are presented in Figure 4.5 for fermentations with digesta free (B), blank digesta (C), whole blueberry (D), sugar fraction (E), fiber fraction

(F), and phenolic fraction (G). Whole blueberry and sugar fraction treatments exhibited higher concentrations of choline-d_9 throughout the 30 hr fermentation period (essentially no choline-d_9 breakdown) compared to all other treatments. This is reflected in the statistically different AUCs, which quantify the analyte's concentration over the fermentation period, providing clear values for the total choline-d_9 present. A greater AUC indicates higher choline-d_9 levels and less TMA-d_9 production, highlighting the significant impact whole blueberry and sugar have on the inhibition of TMA-d_9. Small differences in choline-d_9 concentrations were observed in the fiber and phenolic fractions, with pre-fed treatments metabolizing the substrates slightly faster than the non-pre-fed counterparts. In contrast, digesta free, blank digesta, fiber, and phenolic treatments exhibited similar curves to one another, with choline-d_9 concentration significantly decreasing by the 10 hr time point, indicating comparable levels of choline-d_9 utilization.



Source of Variation	% of total variation	P value	P value summary
Treatments x Pre-feed	0.06600	0.0934	ns
Treatments	99.70	<0.0001	****
Pre-feed	0.02034	0.0789	ns

Figure 4.6. Areas under the curve (AUCs) for choline-d_9 concentration in fermentation (0-30 hr) for all treatments using 2-way ANOVA and Tukey's multiple comparison to test for treatment effects and 2-way ANOVA and Sidak's multiple comparison to test pre-fed effects. Different letters notate statistical differences between non-pre-fed treatments and different ($P<0.05$), numbers notate statistical differences between pre-fed treatments ($P<0.05$), and asterisks notate statistical differences between pre-fed effects ($P<0.05$): digesta (DG), Pre-fed (PF), whole blueberry (WB): DG free vs pre-fed DG free, blank DG vs PF blank DG, WB vs PF WB, sugar vs PF sugar, fiber vs PF fiber, and phenolic vs PF phenolic. Data represent mean ± SEM from *n*=6.

Area under the curve (AUC) of choline-d_9 kinetic curves are presented in Figure 4.6 obtained from fermentations with choline-d_9 100 µM, fecal slurry 1:10 in PBS 1X, and digested treatments. There were no statistically significant differences in AUCs between pre-fed and non-pre-fed for all treatments, suggesting pre-feeding does not have an effect on bacterial metabolism of choline-d_9. The choline-d_9 AUCs for Whole blueberry (WB) and sugar treatments, both pre-fed and non-pre-fed treatments, showed a significant difference in choline-d_9 utilization compared to other treatments, highlighting their significant inhibition (which is likely a non-physiologically relevant assay interreference). In contrast, the choline-d_9 AUCs for DG free, blank DG, fiber, and phenolic treatments are not statistically different from each other, but are significantly smaller from WB and sugar treatments. This suggests that sugar in the whole blueberry may be the main inhibitory contributor.

Since there were no significant differences in choline-d_9 AUCs between pre-fed and non-pre-fed treatments, pre-feeding was not included in subsequent experiments.

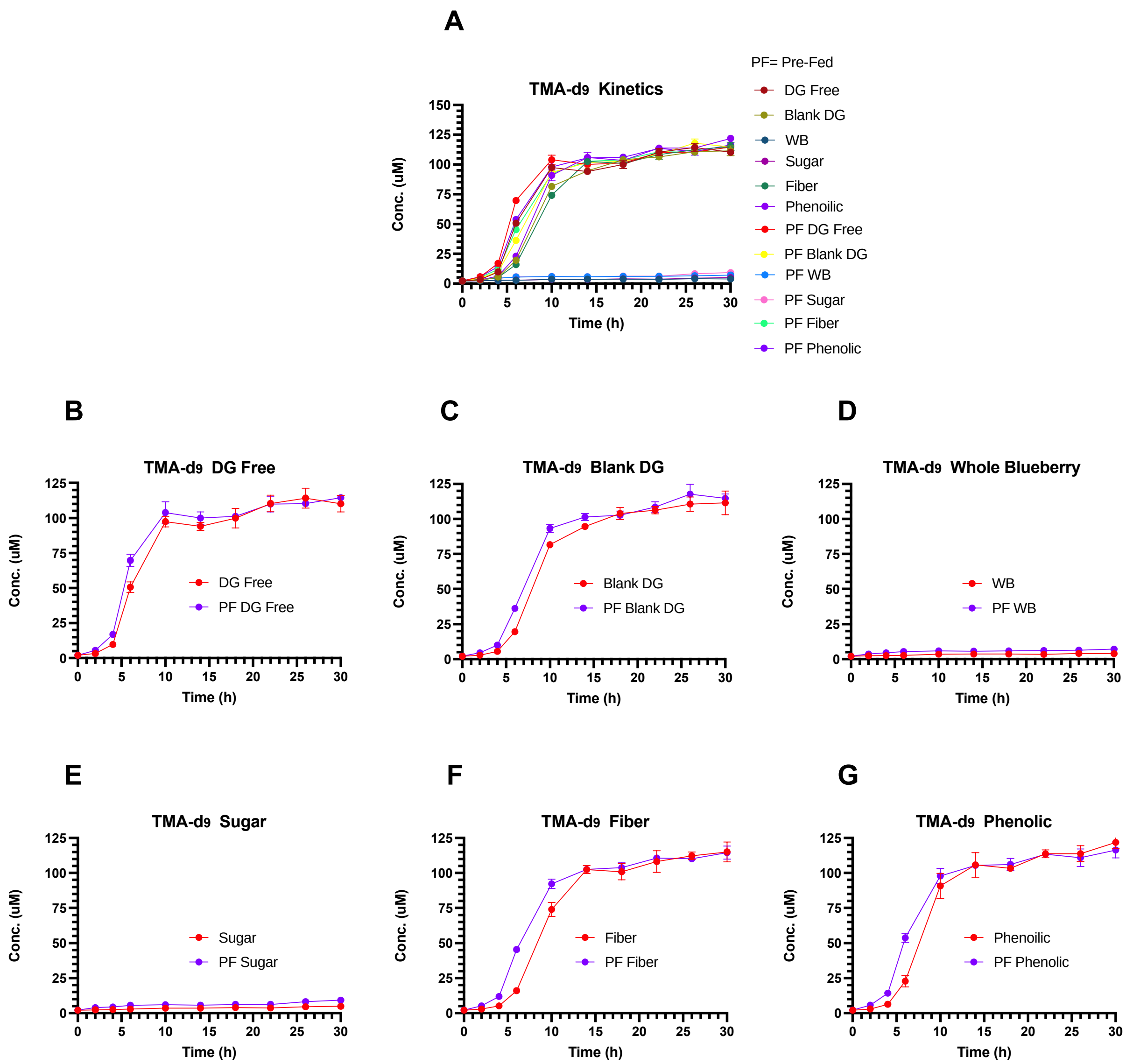
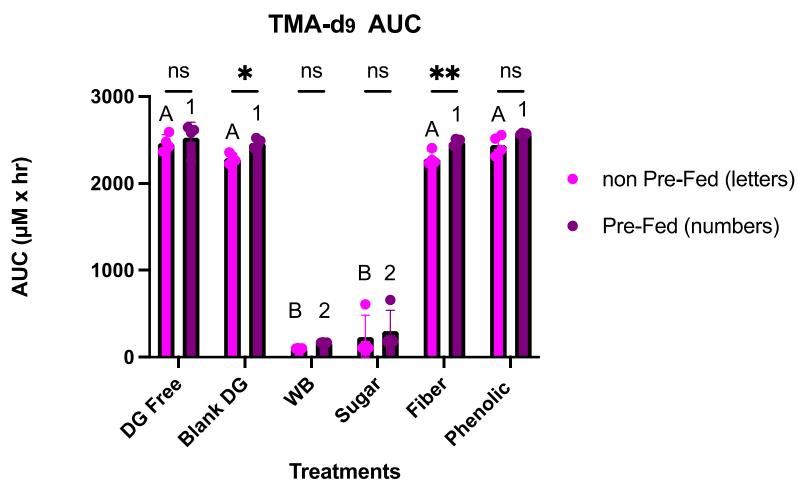


Figure 4.7. Kinetic curve of TMA-d_9 concentration in fermentation (0-30 hr) of all treatments digesta (DG), Pre-fed (PF), whole blueberry (WB): DG Free vs PF DG Free (B), Blank DG vs PF Blank DG (C), WB vs PF WB (D), Sugar vs PF Sugar (E), Fiber vs PF Fiber (F), and Phenolic vs PF Phenolic (G). Data represent mean ± SEM from *n*=6.

Kinetic curves of TMA-d_9 concentrations are presented in Figure 4.7 for fermentations with digesta free (B), blank digesta (C), whole blueberry (D), sugar fraction (E), fiber fraction

(F), and phenolic fraction (G). Whole blueberry and sugar fractions demonstrated significant (and almost complete) inhibition of TMA-d_9 production throughout the 30 hr fermentation period, with statistically smaller AUCs compared to all other treatments. In contrast, digesta free, blank digesta, fiber, and phenolic treatments exhibited similar curves to one another, with TMA-d_9 concentration significantly increasing by the 10 hr time point, indicating comparable levels of TMA-d_9 inhibition.



Source of Variation	% of total variation	P value	P value summary
Treatments x pre-feed	0.06217	0.2511	ns
Treatments	98.61	<0.0001	****
pre-feed	0.2940	<0.0001	****

Figure 4.8. Area under the curve (AUC) for TMA-d_9 concentration in fermentation (0-30 hr) for all treatments using 2-way ANOVA and Tukey's multiple comparison to test for treatment effects and 2-way ANOVA and Sidak's multiple comparison to test pre-fed effects. Different letters notate statistical differences between non-pre-fed treatments and different ($P<0.05$), numbers notate statistical differences between pre-fed treatments ($P<0.05$), and asterisks notate statistical differences between pre-fed effects ($P<0.05$): digesta (DG), Pre-fed (PF), whole blueberry (WB): DG free vs pre-fed DG free, blank DG vs PF blank DG, WB vs PF WB, sugar vs PF sugar, fiber vs PF fiber, and phenolic vs PF phenolic. Data represent mean ± SEM from *n*=6.

Area under the curve (AUC) of TMA-d_9 kinetic curves are presented in Figure 4.8 obtained from fermentations with choline-d_9 100 µM, fecal slurry 1:10 in PBS 1X, and digested treatments. There are statistically different AUCs for TMA-d_9 production between pre-fed and non-pre-fed treatments for blank DG and fiber, suggesting that pre-feeding bacteria with unlabeled choline may slightly increase the rate of choline-d_9 metabolism in these treatments. There were no statistically significant differences in AUCs between pre-fed and non-pre-fed for the other treatments, emphasizing that pre-feeding had little to no effect on shifting bacterial preferences toward metabolizing choline-d_9, compared to non-pre-fed treatments. The TMA-d_9 AUCs for DG free, blank DG, fiber, and phenolic treatments are not statistically different from each other, but are statistically different from WB and sugar treatments. This is further supported by the low choline-d_9 utilization observed in the choline-d_9 AUCs and the high TMA-d_9 production seen in the TMA-d_9 AUCs.

This experiment provided valuable insight into the source of inhibition in whole blueberries. Given the significant similarity in inhibition between whole blueberry and sugar treatments, it is reasonable to conclude that the sugar in the pulp may be responsible for the observed effect. However, since sugar would not remain in the colon after upper gastrointestinal digestion, it is physiologically irrelevant in the fermentation process. Additionally, the extended 30-hour fermentation period proved unnecessary, as choline-d_9 concentrations were nearly gone in most treatments by the 10-hour mark. Pre-feeding was also determined unnecessary, as there were no significant differences in cell growth, choline-d_9 AUCs, or TMA-d_9 AUCs, and any minor differences did not significantly impact the assay. Based on these findings, the subsequent experiments focused on reducing the presence of sugar in the assay, fermentation periods were set to 24 hrs, and pre-feeding the bacteria was excluded.

4.2.3 Experiment 3. Identifying Blueberry Components with the Greatest TMA Inhibition Potential: Building on the results from Experiment 2, which showed that whole blueberry and sugar fractions exhibited the greatest TMA-d_9 inhibition, further analysis was conducted to identify blueberry components with the most significant TMA-lowering effect in the TMA assay. Breaking the blueberry into individual components, such as the peel and pulp, and comparing their inhibitory effects to those of sugar and whole blueberry was a useful next step. If the pulp exhibits the same level of inhibition as sugar and whole blueberry, it would further support the conclusion that sugar is likely the key factor contributing to TMA-d_9 inhibition

Phenolic Composition of Blueberry Extracts: Total phenolic levels were determined for whole blueberry, skin, and pulp fractions using methodology described in "*plant phenolic extraction*" and "*Phenolic analysis: Folin- Ciocalteu assay*". CGA content was determined using a solid phase elution (SPE) and LC/MS quantification as described in "*Solid Phase Elution (SPE)*".

Table. 4.3 Folin Assay Mean and Standard Error of the Mean (SEM) of Phenolic Content of Commercially Available Blueberry Fractions (*n*=3)

Extract	mg GAE/ mg blueberry fraction	mg GAE/ g blueberry fraction	mg GAE/ 100 g whole blueberry*	SEM for mg GAE/ g whole blueberry means
Peel	9.2×10^{-3}	9.18	122.76	2.60
Pulp	4.7×10^{-4}	0.47	40.77	0.17
Whole blueberry	1.8×10^{-3}	1.81	180.05	1.29

*Whole blueberry value was measured for the bottom row and were calculated for the top two rows based on ratios of peel and pulp in a whole blueberry

The results from the Folin-Ciocalteu assay are presented in table 4.3which shows the relative phenolic content of commercial whole blueberry, peel fraction, and pulp fraction extracts. The majority of total phenolic content are contained in the peel fraction of the blueberry

Table 4.4 Mean and Standard Error of the Mean (SEM) of Chlorogenic Acid (CGA) Content for Commercially Available Blueberry Fractions After Solid Phase Elution (SPE) and LC-MS Analysis (*n*=2)

Extract	mg CGA/ mg blueberry fraction	mg CGA/ g blueberry fraction	mg CGA/ 100 g whole blueberry*	SEM for mg CGA/ g whole blueberry means
Peel	8.89×10^{-4}	8.89×10^{-1}	11.86	0.04
Pulp	7.29×10^{-5}	7.29×10^{-2}	6.32	0.50
Whole blueberry	8.06×10^{-5}	8.07×10^{-2}	8.07	1.23

*Whole blueberry value was measured for the bottom row and were calculated for the top two rows based on ratios of peel and pulp in a whole blueberry

The chlorogenic acid (CGA) content of commercial whole blueberry, peel fraction, and pulp fraction extracts after an SPE and LC-MS analysis is presented in Table 4. 4. The majority of total CGA content is coming from the peel. Commercial blueberries used in this assay are classified as having "low" CGA content, as their CGA level is well below 50 mg/100 g of fresh weight (FW). This contrasts with the higher CGA content reported by Iorizzo *et al.* in Figure 2.4, where the CGA content in high CGA cultivars were closer to 100 mg/100 g of FW berries[123,130].

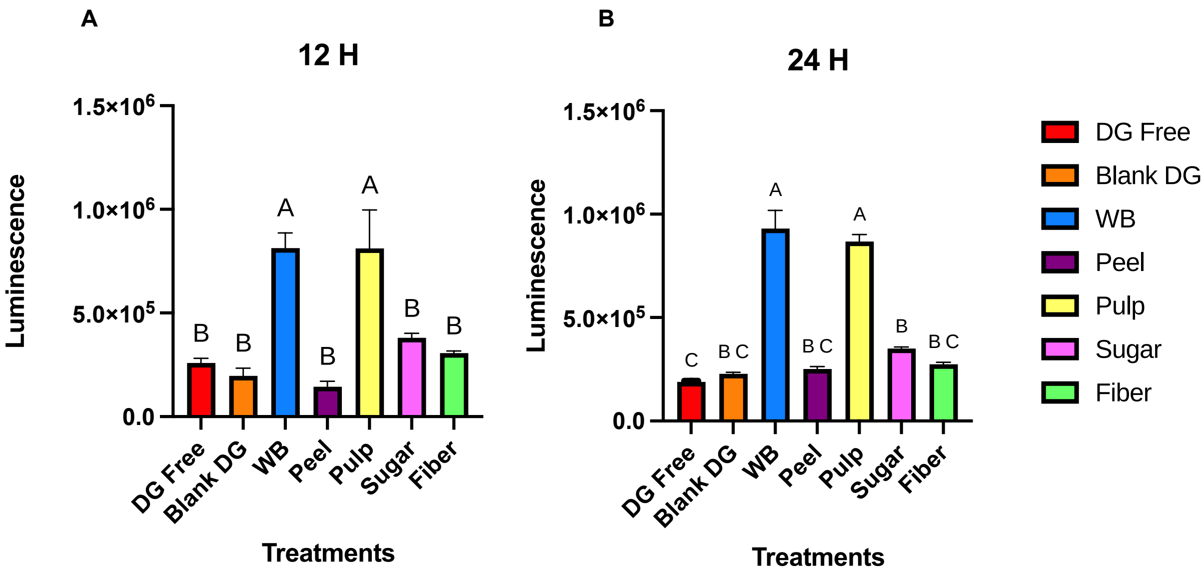


Figure 4.9. BacTiter-Glo luminescence reading at 12 hr (A) and 24 hr (B) time points of treatments Digesta (DG), whole blueberry (WB): DG free, blank DG, WB, Peel, Pulp, Sugar, and Fiber. Using one-way ANOVA and Tukey's multiple comparison to determine statistical differences between treatments. Different letters notate a statistical difference ($P<0.05$). Data represent mean ± SEM from n=6.

BacTiter-Glo luminescence reading for treatments in blueberry fraction fermentations at 12 hr (A) and 24 hr (B) are presented in Figure 4. 9. A reduction in bacterial growth would be indicated by a very low or absent luminescence signal. Compared to the controls there is no evidence suggesting that any treatment caused a cytotoxic effect that would lead to the perceived effect of TMA-d_9 inhibition. Luminescence readings for whole blueberry and pulp are significantly higher than all other treatments at both the 12 hr and 24 hr time points, likely due to the presence of easily metabolized substrates, such as sugar, which may promote rapid bacterial growth. The strong inhibition observed with whole blueberry and pulp, along with the high luminescence signal indicating substantial fecal cell growth, is likely due to the presence of sugar. However, since sugar would not remain in the colon after upper gastrointestinal digestion, it is physiologically irrelevant to the inhibition of TMA-d_9 in this assay. DG free, blank DG, and peel have the lowest luminescence readings at both 12 hr with no statistical differences between them and some small statistical differences at 24 hr time point. Luminescence for the peel is the lowest at the 12 hr time point but increases slightly at the 24 hr time point, likely due to the dark color of the peel digesta and subsequent metabolism of the peel and associated color by the bacteria at later time points. The luminescence for blank DG, WB, and peel increases after the 12 hr time point. In contrast, DG free, pulp, sugar, and fiber luminescence decreases after the 12 hr point.

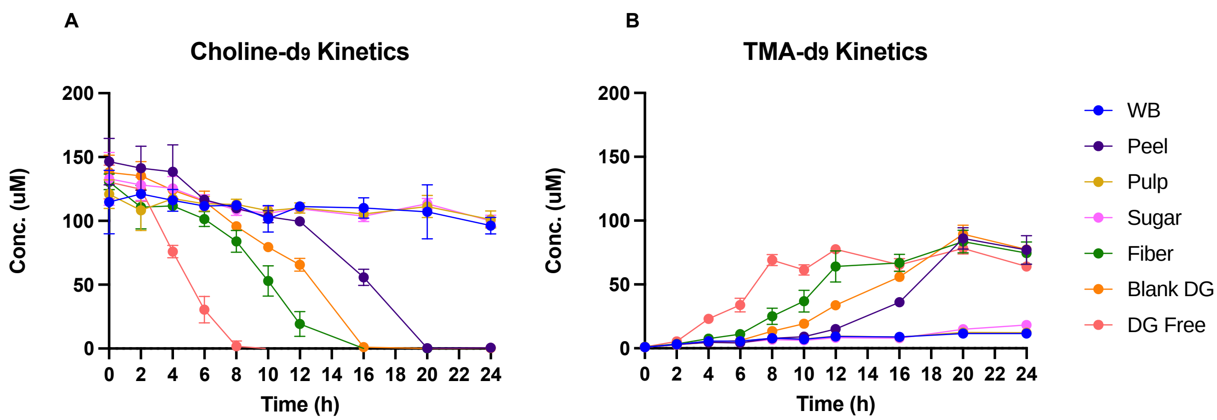


Figure 4.10. Kinetic curves for choline-d_9 use (A) and TMA-d_9 production (B) in fermentation (0-24 hr) for all treatments. digesta (DG) and whole blueberry (WB): Blank DG, DG free, WB, Peel, Pulp, Sugar, and Fiber. Data represent mean ± SEM *n*=6.

Kinetic curves of choline-d_9 concentration (A) and TMA-d_9 production (B) is presented in Figure 4.10 for fermentations with digesta free, blank digesta, whole blueberry, peel fraction, pulp fraction, sugar fraction, and fiber fraction. Whole blueberry, pulp, and sugar demonstrated significant inhibition of TMA production throughout the 24 hr fermentation period compared to all other treatments. This further supports the idea that sugar is primarily responsible for inhibiting TMA-d_9 production, as the pulp displayed strong, almost total inhibitory effects like those seen with whole blueberry and sugar treatments. Digesta free, blank digesta, fiber, and peel treatments were different from the WB, pulp, and sugar treatments, as well as from each other. DG free treatments exhibited the quickest choline-d_9 utilization, with choline-d_9 concentrations decreasing and TMA-d_9 rising sharply by the 8 hr mark. Fiber treatments demonstrated the second quickest choline-d_9 utilization, with a significant decrease in choline-d_9 and corresponding increase in TMA-d_9 occurring between the 12 and 16 hrs. Blank DG displayed similar results to the fiber treatments, with choline-d_9 concentrations declining and TMA-d_9 rising by the 16 hr mark. Peel treatments exhibited low choline-d_9 utilization until the 18-20 hr time frame, though not to the same extent as the WB, pulp, and sugar treatments. Since the peel contains most of the fiber, the stronger inhibition observed with the peel compared to the fiber fraction suggests that another component of the peel is contributing to the inhibition of TMA-d9 production. This is likely a more accurate reflection of the inhibition process, as the peel components are more likely to reach the colon, where the bacteria reside, compared to the sugar-containing treatments.

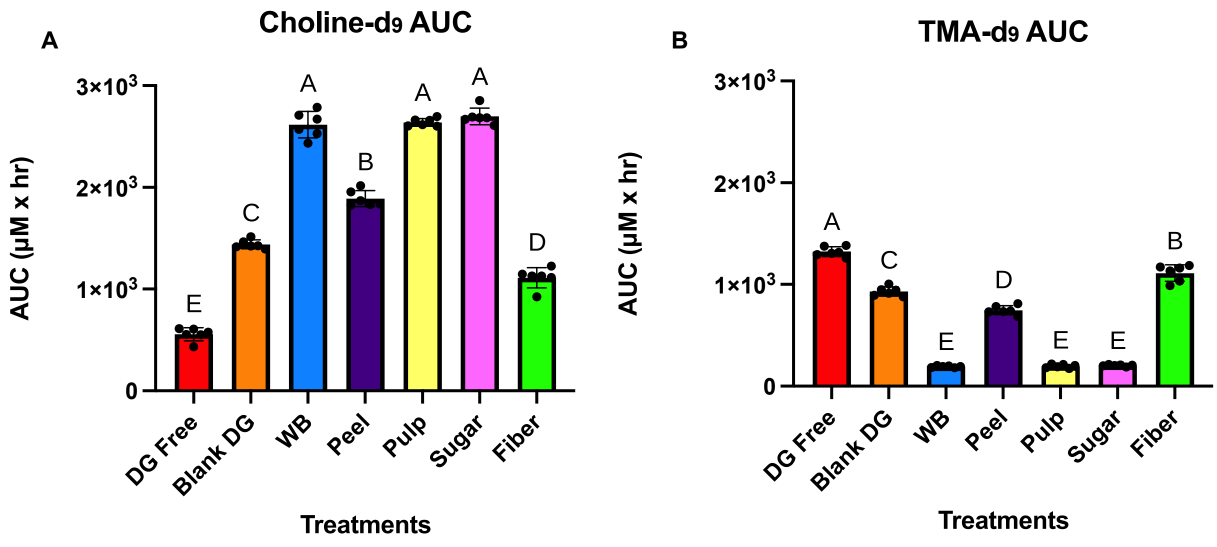


Figure 4.11. Areas under the curve (AUCs) for choline-d_9 utilization (A) and TMA-d_9 (B) production in fermentation (0-24 hr) for all treatments using one-way ANOVA and Tukey's multiple comparison to test for treatment effects. Digesta (DG) and whole blueberry (WV): DG free, Blank DG, WB, Peel, Pulp, Sugar, and Fiber. Data represent mean ± SEM *n*=6.

Area under the curve (AUC) of choline-d_9 (A) and TMA-d_9 (B) kinetic curves obtained from fermentations are presented in Figure 4.11 with choline-d_9 100 µM, fecal slurry 1:10 in PBS 1X, and digested treatments. Whole blueberry (WB), pulp, and sugar treatments showed no statistically significant differences in choline-d_9 or TMA-d_9 AUCs. As shown in experiments 2 and 3, whole blueberry inhibits TMA-d_9 production, primarily due to the pulp, which is rich in sugar. However, the sugar in these treatments wouldn't reach the colon, making its inhibitory effect physiologically irrelevant. In contrast, DG free, blank DG, peel, and fiber treatments displayed statistically significant differences in choline-d_9 and TMA-d_9 AUCs both among themselves and compared to WB, pulp, and sugar AUCs. The fiber appears to stimulate TMA-d_9 production, likely due to bacteria's quick metabolism of fiber and subsequent growth of all bacteria, including those that produce TMA. Although the peel contains most of the fiber, its distinct AUCs for both choline-d_9 and TMA-d_9 suggest another component in the peel contributes to the observed inhibition.

4.3 Objective 2: Determine whether CGA content correlates with reduced TMA production in multiple blueberry accessions from a genetic diversity population with a large spread of CGA in our *ex vivo-in vitro* human fecal fermentation model. For this experiment, the peels of eight cultivars were used, as previous experiments showed that removing the pulp from whole blueberries reduced interference from the sugar. This allowed for a clearer focus on the peel components, which are more likely to accumulate in the colon and interact with TMA-producing bacteria.

***4.3.1 Experiment* 4** Determination of TMA inhibition potential of varying levels of CGA content in genetically different blueberries

Determining CGA Content: To account for the varying levels of CGA among genetically diverse blueberry populations, 20 different blueberry cultivars from the National Clonal Germplasm Repository (NCGR), Corvallis OR, USA were used to determine the impact of blueberry CGA on the inhibition of TMA in our assay. CGA content was measured for the whole blueberry to identify cultivars with the highest and lowest levels for use in the fermentation to determine the impact CGA has on TMA inhibition. The data will help to identify blueberry cultivars with the greatest TMA-lowering activity, which may drive the cultivation and consumption of berries with enhanced health benefits.

Table 4.5 Average Cultivar Chlorogenic Acid (CGA) Content (*n*=4)		
Cultivar	**mg CGA/ 100 g whole blueberry**	**SEM**
DxJ 197	4.22	0.36
DxJ 148	5.00	0.72
DxJ 112	7.28	1.17
DxJ 041	8.39	0.90
DxJ 125	9.50	3.01
DxJ 124	11.95	0.68
DxJ 023	13.57	2.69
DxJ 102	17.88	2.88
DxJ 038	20.17	1.22
DxJ 149	43.35	19.16
DxJ 002	105.56	5.79
DxJ 033	133.52	7.92
PI 554857	2.63	0.36
PI 554856	4.47	0.82
PI 554764	5.07	0.89
PI 554846	6.56	1.69
PI 554849	9.67	2.37
PI 638335	58.16	21.31
PI 267851	115.83	6.79
PI 296399	146.29	14.82
DxJ: Biparental Mapping Population PI: Diversity Panel Population		

The relative chlorogenic acid (CGA) content of the whole blueberries from 20 different cultivar extracts after an SPE and LC-MS analysis are presented in table 4.5. The CGA content of these cultivars were generally consistent with that of the same cultivars from previous years, as documented by Mengist *et al.*[123,130]. This is significant because the CGA content is unlikely to vary significantly from year to year, supporting the idea that if one cultivar exhibits greater TMA inhibition, its inhibitory potential will likely remain consistent over time.

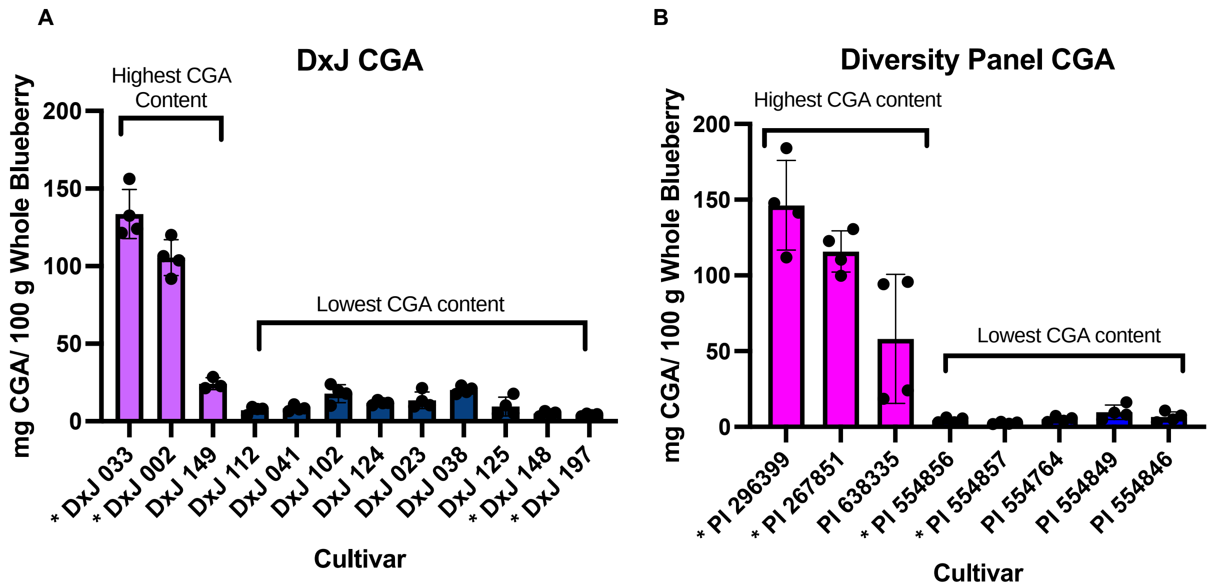


Figure 4.12. Chlorogenic Acid (CGA) content of multiple genetically diverse blueberry cultivars from the summer of 2024. Two of the lowest CGA and two of the highest CGA content blueberries from the biparental mapping population (A) and two of the lowest CGA and two of the highest CGA content blueberries from the diversity panel (B) were selected and noted with an asterisk. Data represent mean ± SEM from *n*=4.

The chlorogenic acid content of the whole blueberries from 20 different cultivars is presented in Figure 4. 12. Extracts from each cultivar were prepared and analyzed as four separate replicates, with each replicate representing a unique set of blueberries from the same cultivar to ensure a more diverse sample. The CGA levels obtained from the 2024 harvest generally align with the CGA levels observed in the same cultivars from previous years, as shown in Figure 2.4. Cultivars such as PI 296399, PI 267851, PI 638335, DxJ 033, DxJ 002, and DxJ 149 consistently exhibit some of the highest CGA content in both the 2024 and previous harvest years. Similarly, cultivars like PI 554764, PI 554857, PI 554856, DxJ 197, DxJ 148, and DxJ 112 consistently show the lowest CGA content in both the 2024 and previous harvests. For the subsequent fermentation, the highest four and lowest four CGA-content cultivars from the populations were selected to examine the effects of varying CGA levels (labeled with an asterisk in Figure 4. 12 and presented in table 4.6).

Table 4.6 Average Chlorogenic Acid (CGA) Content in Cultivars Chosen for Fermentation (*n*=4)			
Cultivar	**CGA Level**	**mg CGA/ g whole blueberry**	**SEM**
DxJ 197	Low	4.22	0.36
DxJ 148	Low	5.00	0.72
DxJ 002	High	105.56	5.79
DxJ 033	High	133.52	7.92
PI 554857	Low	2.63	036
PI 554856	Low	4.47	0.82
PI 267851	High	115.83	6.79
PI 296399	High	146.29	14.82
DxJ: Biparental Mapping Population PI: Diversity Panel Population			

Average cultivar CGA content for the eight cultivars selected for the fermentation are presented in table 4.6. Cultivars for fermentation were selected based on the mean CGA content of the whole blueberries. From the 20 cultivars analyzed, the highest four CGA content (two from each population) and the lowest four CGA content (two from each population) were chosen. Comparing these high and low cultivars allowed for the documentation of TMA inhibition potential. With ~70-fold difference in CGA content between the highest and lowest cultivars, differences in inhibition may be attributed to the varying CGA levels.

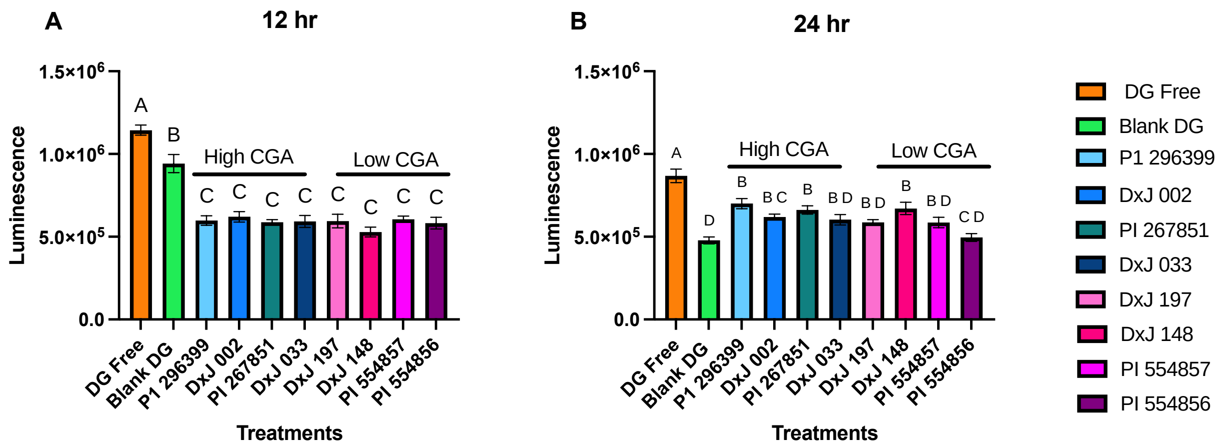


Figure 4.13. BacTiter-Glo luminescence for kinetic curves of choline-d_9 concentration (A) and TMA-d_9 (B) production in fermentation (0-24 hr) for all treatments using one-way ANOVA and Tukey's multiple comparison to test for treatment effects: digesta (DG): DG free, Blank DG, PI 296399 (high CGA), DxJ 002 (High CGA), PI 267851 (High CGA), DxJ 033 (High CGA), DxJ 197 (Low CGA), DxJ 148 (Low CGA), PI 554857 (Low CGA), PI 554856 (Low CGA). Data represent mean ± SEM from *n*=6.

BacTiter-Glo luminescence readings are presented in Figure 4.13 for the different cultivars in the fermentations at 12 hr (A) and 24 hr (B). A reduction in bacterial growth would be indicated by a very low or absent luminescence signal. Compared to the controls there is no evidence suggesting that any treatment caused a cytotoxic effect that would lead to the perceived effect of TMA-d_9 inhibition. At the 12 hr time point, luminescence readings for peels from all cultivars are not statistically different, with DG free and blank digesta exhibiting slightly higher readings. The slightly lower readings of the peels compared to the controls is likely due to the color of the digesta interfering slightly with the signal, even with washing. By the 24 hr time point, there is slight variation in luminescence readings among the cultivars, though none suggest toxicity as the cause of the lower signal.

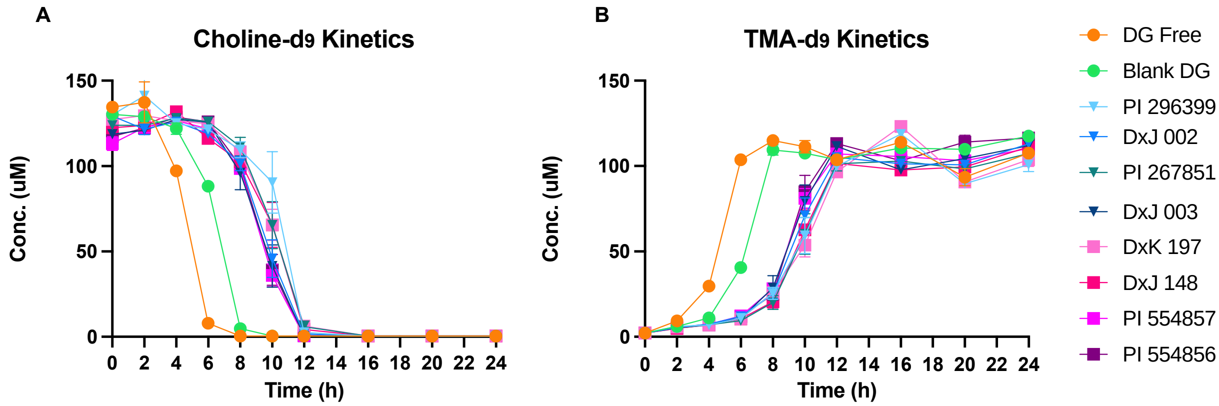


Figure 4.14 Kinetic curve for choline-d_9 concentration (A) and TMA-d_9 production (B) in fermentation (0-24 hr) for all treatments: digesta (DG): DG free, Blank DG, PI 296399 (high CGA), DxJ 002 (High CGA), PI 267851 (High CGA), DxJ 033 (High CGA), DxJ 197 (Low CGA), DxJ 148 (Low CGA), PI 554857 (Low CGA), PI 554856 (Low CGA). Data represent mean ± SEM from *n*=6.

Kinetic curves of choline-d_9 concentration(A) and TMA-d_9 production (B) are presented in Figure 4.14 for fermentations with digesta free, blank digesta, and peel fractions from 8 genetically different cultivars. Digesta free treatments exhibited the quickest choline-d_9 utilization, with choline-d_9 concentrations decreasing between the 4 and 6 hr mark. Blank DG treatments demonstrated the second quickest choline-d_9 utilization, with a significant decrease in choline-d_9 occurring between the 4 and 8 hr mark. These controls represent the inhibition of the digesta background and reflect what is being metabolized by the bacteria without the presence of food. Choline-d_9 utilization was similar across all eight cultivars, with a significant decrease observed between the 8 and 12 hr mark. The kinetic curves for the peels of both high and low CGA content cultivars were very similar, suggesting that there is no significant difference in inhibition due to CGA or other components in the peel. This is further supported by the strong inhibition seen in all cultivars in figure 2.6. While no differences were observed among the peels, their inhibition potential remained notably higher than that of the controls, indicating that the peel still possessed inhibitory activity.

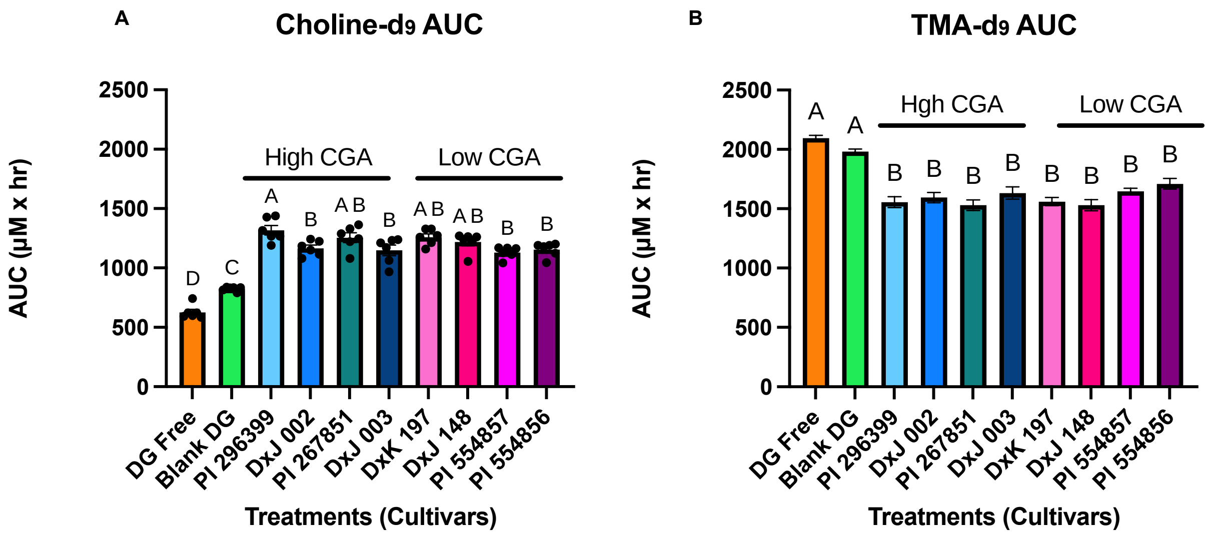


Figure 4.15. AUC for choline-d$_9$ utilization (A) and TMA-d$_9$ (B) production in fermentation (0-24 hr) for all treatments using one-way ANOVA and Tukey's multiple comparison to test for treatment effects digesta (DG): DG free, Blank DG, PI 296399 (high CGA), DxJ 002 (High CGA), PI 267851 (High CGA), DxJ 033 (High CGA), DxJ 197 (Low CGA), DxJ 148 (Low CGA), PI 554857 (Low CGA), PI 554856 (Low CGA). Data represent mean ± SEM from *n*=6.

Area under the curve (AUC) of choline-d$_9$ (A) and TMA-d$_9$ (B) kinetic curves are presented in Figure 4.15 obtained from fermentations with choline-d$_9$ 100 µM, fecal slurry 1:10 in PBS 1X, and digested treatments. The choline-d$_9$ and TMA-d$_9$ AUCs for all cultivars were statistically higher than the DG-free and blank DG AUCs, indicating that the peels exhibited a greater inhibitory effect than the controls. While there are some statistical differences in the choline-d$_9$ AUCs between cultivars, these differences are small and do not suggest distinct patterns or a clear relationship to the variations in CGA content. This is further supported by the TMA-d$_9$ AUCs for all cultivars, which are not statistically different from one another, indicating no difference in the inhibition potential of the peels across cultivars and suggesting that CGA content does not play a significant role. However, all TMA-d$_9$ cultivars have significantly lower AUCs than the DG-free and blank DG controls, demonstrating that the peels exhibited inhibition potential greater than the digestive background controls. Of note, the AUCs are dependent on the selected time frame. The AUCs presented here represent the full 24 hr fermentation period. If the time frame had been chosen to capture when the first control began showing TMA-d9 and ended when the TMA-d9 in the last sample stopped changing, the differences between the treatments and controls would likely be even more significant. This alternative approach would exclude the period after the 12-14 hr time point, where all samples showed roughly the same amount of TMA-d9.

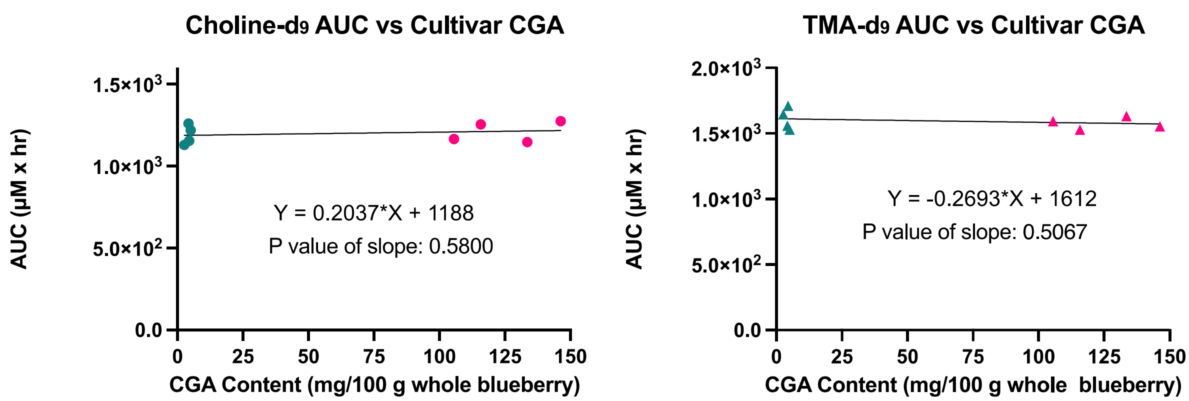


Figure 4.16. Mean area under the curve (AUC) vs mean chlorogenic acid (CGA) content of blueberry cultivars: PI554856, DxJ 003, PI 554857, PI 267851, DxJ 002, DxJ 148, DxJ 197, PI 296399 using a simple linear regression with line equation.

The relationship between mean AUC vs CGA content for choline-d9 and TMA-d9 plots are presented in Figure 4.16. The choline-d9 vs. CGA content plot (A) shows an equation with a slope close to zero and a nonsignificant p-value for the slope, indicating a weak correlation between CGA content and TMA-d9 inhibitory activity. This is further supported by the TMA-d9 vs. CGA content plot (B), where the equation's slope is near zero and the p-value is also not significant. There is a lack of correlation between CGA content and TMA inhibitory activity, as the slope shows minimal variation in activity.

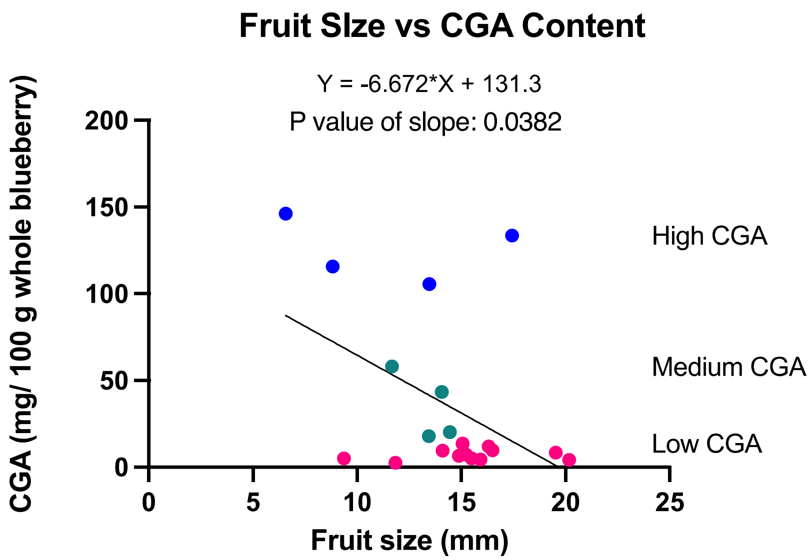


Figure 4.17 Mean chlorogenic acid (CGA) content vs mean size of blueberry fruit from eight genotypes, PI554856, DxJ 003, PI 554857, PI 267851, DxJ 002, DxJ 148, DxJ 197, PI 296399 using simple linear regression.

The relationship between mean chlorogenic acid vs mean fruit size is presented in Figure 4.17. Fruit size was measured to assess whether it correlates with CGA content, helping to determine if CGA levels are influenced by the size of the fruit (due to the fact that smaller fruits have more surface area, or peel, per serving). If CGA content is size-dependent, this could impact the amount ingested based on recommended serving sizes. The negative slope value indicates an inverse relationship between CGA content and fruit size, meaning that as size increases, CGA content decreases. The significant p-value suggests that this relationship is meaningful and not due to chance. However, it's important to note that both the highest and lowest CGA content cultivars vary in fruit size, representing both smaller and larger berries. While the correlation between size and CGA content is evident, size does not necessarily determine CGA content in these cultivars.

4.4 Discussion

4.4.1 Experiment 1: BacTiter-Glo Optimization

The first BacTiter-Glo optimization experiment gave insight on the best plate color for maximizing luminescence signal. ATP standard curves were mixed with various components of the final fermentation mixture and plated on both white 96-well round-bottom plates and black 96-well clear-bottom plates to assess the impact of plate color on the luminescence. The white plates consistently produced higher luminescence readings compared to the same treatments plated on the black plates. This is due to the black color absorbing most of the emitted luminescence signal emitted by the treatments, whereas the white color reflects the signal, allowing for a more accurate reading. Additionally, the white plates improved the differentiation between treatments, making it easier to identify interferences in the fermentation mixture. Notably, blueberry digesta exhibited a lower luminescence signal compared to other treatments, while blank digesta showed a smaller luminesce, though less pronounced than blueberry digesta. This is due to the digesta background, made up of digestive enzymes and food material that contribute to a brown color, interferes with and quenches the luminescence signal. This finding is relevant for subsequent experiments where digesta is present in almost all treatments. Other treatments, such as CGA or choline-d_9, did not reduce the luminescence signal as significantly as the digesta samples. These data provide insight into the potential factors that could lead to either reduced or increased luminescence signals. This information was crucial for interpreting BacTiter-Glo luminescence results, helping to distinguish whether reduced signals were due to color interference or actual cytotoxicity. To address the interferences present in nearly all samples, a "washing" procedure was tested to minimize the impact of the fermenta background and focus specifically on the bacterial cells.

The second BacTiter-Glo optimization experiment aimed to reduce interferences, particularly from the digesta background, which were identified in the "plate color determination" experiment. By testing the effect of "washing" the fecal cells to remove the fermenta background, the fermenta matrix responsible for these interferences could be eliminated, allowing the luminescence signal to reflect the fecal cells themselves rather than the color of the fermenta background. “Washing” involved centrifuging the fermenta samples at a low speed to sperate fecal cells from the fermenta matrix. The supernatant was discarded, and the fecal cells were resuspended in PBS 1X before being mixed with the BacTiter-Glo reagent. The “unwashed” treatments followed the same procedure, except the cells were resuspended in the original solution rather than removing the supernatant. Luminescence signals showed that the “washed” treatments with fecal slurry had noticeably higher luminescence signals than the “unwashed” treatments with the same slurry. At the 12 hr mark, a statistically significant difference was observed between the “washed” and “unwashed” blueberry digesta treatments. While there was not a significant difference for the other treatments at this time point, eliminating the blueberry digesta background, (which had the most reduced signal when mixed with the ATP standard curve) greatly improved the luminescence signal by eliminating the color associated with the blueberry digesta. This is important in subsequent experiments containing blueberry digesta, as minimizing interferences is critical to obtaining accurate readings. At the 24 hr mark, statistical differences were observed between the “washed” and “unwashed” treatments for media+ fecal and blank+ fecal, but no differences were seen for any other treatments. This could be due to the lower concentration of fecal material in these samples at the later time point. By 24 hours, it's reasonable to assume that most of the growth media or digesta background has been metabolized, leaving the fecal concentration lower than the start of the fermentation. With these low concentrations and the remaining fermenta background still interfering, the washing step helped make the signal from the lower concentration bacteria more noticeable, leading to statistical differences. This is important for future experiments where fecal concentration might be low but still detectable, as washing could help determine whether there is true cytotoxicity. There were no significant differences in luminescence across all the treatments at the 24 hr time point. By this time, most of the digesta, and its associated color had been metabolized by the bacteria, likely causing the samples to appear similar and produce comparable signals. Washing the cells was implemented in all subsequent experiments, as it enhanced the signal by minimizing interference from the fermenta matrix.

The third optimization experiment was conducted to explore the relationship between cell concentration and BacTiter-Glo luminescence, serial dilutions of a concentrated fecal slurry were tested. The results showed a generally linear response between cell concentration and luminescence, up to the highest concentration (5-fold dilution), where the signal began to plateau. The most diluted sample (160-fold dilution) produced the lowest signal, with signal gradually increasing as the dilutions became less concentrated. This was helpful to determine the relative fecal concentrations at different time points. It is reasonable to assume that by the end of a fermentation, most of the growth media or digesta background has been metabolized, unless the food's serving size is larger which would provide more material to support bacterial growth for a longer period, resulting in lower bacterial concentrations. Based on this data, it is understood that if the concentration decreases by a certain factor, the luminescence signal also decreases by the same factor.

4.4.2 Experiment 2: Accounting for Assay Interferences

Optimizing fermentation parameters for high-fiber, sugar-rich fruits was crucial for identifying the source of the inhibition in blueberries. This was particularly important since unpublished preliminary data documented in Figure 2.6 by Iglesias *et al*. highlighted the significant inhibitory effects of these blueberry cultivars, with their inhibition potential surpassing that of any food previously tested. In this experiment, treatments were inoculated with either fecal slurry pre-fed with unlabeled choline or untreated fecal slurry. The fermentation process was extended to 30 hrs, compared to the usual 24 hrs. Treatments included whole blueberry, sugar fraction, fiber fraction, and phenolic fraction along with blank digesta and no digesta as controls. The results showed no statistical significance in BacTiter-Glo luminescence between the pre-fed and non-pre-fed treatments. However, there were significant differences between whole blueberry and the other treatments, likely due to the color of the blueberry digesta. At the 30 hr mark, no statistical differences were observed between pre-fed and non-pre-fed within treatments. Whole blueberry showed a significantly lower luminescence compared to other treatments, likely due to the metabolism of the blueberry digesta, and associated color, by fecal bacteria. The luminescence signals from the treatments do not signify any cell toxicity would be associated with TMA-d_9 inhibition. For all subsequent experiments, pre-feeding was not continued as it did not provide a difference in luminescence.

The kinetic curves displayed concentrations of both choline-d_9 and TMA- d_9 over the 30 hr fermentation period. Notably, whole blueberry and sugar (both pre-fed and non-pre-fed) exhibited the greatest inhibition of TMA- d_9 throughout the fermentation. This was reflected in the choline-d_9 AUCs, where whole blueberry and sugar showed statistically larger AUCs, while the TMA-d_9 AUCs were statistically smaller compared to all other treatments. Within treatments, there was no statistical difference between pre-fed and non-pre-fed, reinforcing that pre-feeding did not alter bacterial metabolism of choline before other components like sugar. The digestion model does not simulate absorption, which means compounds typically absorbed in the small intestine, like sugar, remain in the fermentation process. Since sugar would not be present in the colon after upper gastrointestinal digestion, the inhibition seen in this fermentation by the sugar fraction is unlikely to be observed *in-vivo*. Whole blueberry contains the pulp, which holds most of the fruit's sugar[100]. Therefore, the observed inhibition by both the whole blueberry and sugar may be due to the sugar itself, rather than chlorogenic acid or other compounds. Given the fermentation model used in this assay, removing water-soluble sugar through dialysis before the fermentation, as demonstrated by Yang *et al.*[131], would likely eliminate water-soluble chlorogenic acid. An enzymatic sugar removal method was attempted to eliminate sugar while preserving the chlorogenic acid in the digesta. Sucrase enzymes were added to reduce the sugar content. However, analysis of the digesta after enzyme addition showed minimal degradation (data not shown), indicating that the method was not effective enough for use in future experiments. Instead of removing the sugar and potentially losing the analyte of interest, separating the peel from the pulp may provide a clearer way to pinpoint the source of inhibition.

4.4.3 Experiment 3: Identifying Blueberry Components with the Greatest TMA Inhibition Potential:

Building on the results from the previous experiment, where whole blueberries and sugar demonstrated the greatest inhibition potential compared to other treatments, this experiment further investigated the source of inhibition through the breakdown of the blueberry into its components. Since most of the sugar in whole blueberries is found in the pulp, which would not be present in the colon after upper gastrointestinal digestion, separating the peel from the pulp allowed for a clearer understanding of where the inhibition is coming from. Folin and SPE with LC-MS analysis were used to identify the distribution of the phenolics and chlorogenic acid in blueberries. The results revealed that the peel contains the majority of these compounds in a whole blueberry. Additionally, it was noted that the store-bought blueberries in this experiment had relatively low CGA content compared to some cultivars documented in Figure 2.4, suggesting that the inhibition observed with low CGA content fruit might be even more pronounced in blueberries with higher CGA levels.

The treatments in this experiment included whole blueberry, peel fraction, pulp fraction, sugar fraction, fiber fraction, as well as blank digesta and no digesta as controls. BacTiter-Glo luminesces readings revealed significantly higher readings for whole blueberry and pulp readings compared to all other treatments at the 12 hr and 24 hr time points. This is likely due to increased bacterial growth resulting from the greater food content and readily available substrates, such as sugar. It's also worth noting that luminescence readings for both whole blueberry and peel treatments may be lower due to the darker color of the digesta, which could mask the true concentration of the bacteria. Although the readings for the other treatments were lower than those for whole blueberry and pulp, this difference is not suggesting cell toxicity. Kinetic curves for choline-d_9 and TMA-d_9, showed similar inhibition for whole blueberry, sugar, and pulp throughout the fermentation period. While the peel exhibited less inhibition compared to the whole blueberry, pulp, and sugar treatments, it still showed greater inhibition than the fiber and control treatments. Fiber appeared to accelerate the conversion of choline-d_9 to TMA-d_9, which could be due the bacteria's quick metabolism of fiber, a substrate they are accustomed to, before moving on to the choline. It is also important to note that carbohydrates, such as fiber, can be metabolized by gut bacteria into simple sugars. These sugar byproducts may exhibit similar inhibitory effects as the whole blueberry, pulp, and sugar treatments. Since the peel contains most of the fiber, the greater inhibition observed with the peel compared to the fiber fraction suggests another component of the peel is contributing to the inhibition of TMA-d_9 production.

Peel contains most of the polyphenolics, including CGA, so investigating the peel of genetically diverse blueberry cultivars with varying CGA content may help explain differences in inhibition. In addition to analyzing CGA content, it is important to examine other phenolic compounds present in the skin. While CGA is the most prevalent phenolic, the total composition of other phenolics could potentially surpass that of CGA. As noted earlier, sugar would not be present in the colon, so most of the inhibition observed from the whole blueberry, sugar, and pulp is likely due to bacterial metabolism of the easily digestible sugar molecules rather than choline-d_9. Comparing the peel of blueberries with high and low CGA content could clarify whether the inhibition is primarily attributed to phenolics rather than sugar.

4.4.4 Experiment 4: Determination of TMA inhibition potential of varying levels of CGA content in genetically different blueberries

The final experiment focused on comparing four high-CGA and four low-CGA content blueberries from a genetically diverse group of blueberries. To minimize sugar interference from the pulp, all blueberries were peeled, and the peels, equivalent to one serving of whole blueberries, were used in the fermentation to assess TMA-d_9 inhibition. BacTiter-Glo luminescence readings showed no significant differences in signal for any of the peel treatments at the 12 hr mark, and only a few minor statistical differences at the 24 hr mark, with no indication of cell toxicity from treatments. The luminescence from the peels was lower than the controls, likely due to the color of the peel digesta interfering with the luminescence signal.

Inhibition potential among all peels were very similar, showing more inhibition than the controls but no significant differences between each other. This pattern was further confirmed in the AUCs for Choline-d_9, where all the peels treatments showed statistically higher AUCs compared to the controls, with only minor statistical differences between peels. For TMA-d_9, the AUCs for the peel treatments were statistically smaller than those for the controls, with no statistical differences observed between peels. These findings were also reflected in the AUCs vs CGA content plots in Figure 4.16, which showed a slope close to zero, with P-values that are not significant. This suggests that CGA may not be the primary factor driving the inhibition in blueberries, or if it is, the concentration of CGA in blueberries does not need to be high to have an effect. It's important to note that the cultivars varied in size, with some being significantly smaller than others. This may affect the amount of CGA in a serving, as the peel-to-pulp ratio differs with fruit size, potentially leading to higher or lower CGA levels per serving. The relationship between average CGA content and average cultivar size shown in Figure 4.17 exhibits a slightly negative slope, indicating an inverse relationship where larger fruits tend to have lower CGA content. The significant P-value suggests this relationship is not due to chance. However, both the highest and lowest CGA content cultivars used in the fermentation include both smaller and larger berries. While a correlation between size and CGA content exists, size alone does not determine CGA content in these cultivars, suggesting that the lack of differences in peel inhibition may not be predominantly due to berry size.

Although the hypothesis that CGA content drives the TMA-inhibiting effect of blueberries was not confirmed, the data are promising for consumers. They suggest that peels, where most phenolics reside, from all blueberries, regardless of CGA content, seem to provide similar benefits and eliminate the need to selectively choose varieties that may be less accessible.

Since the peels contain most of the fruit's phenolic compounds, and inhibition across all peel treatments were similar, it suggests that variations in the content of other phenolics may not have a significant impact either.

4.5 Limitations

This project was conducted exclusively *in vitro*, which posed some limitations due to the nature of the model. While the conversion of choline-d_9 to TMA-d_9 was measured, the absorption and conversion of TMA to TMAO were not evaluated, as they were beyond the scope of this study. *In vivo* studies would address the absorption and conversion of TMA to TMAO, offering a more accurate understanding of the true conversion rate and circulating TMAO levels in the plasma from dietary choline.

Although inhibition from different blueberry fractions was measured and some were found to be higher than others, it remains unclear which specific component within those fractions is responsible for the inhibitory effect. Without a clear understanding of the mechanism by which choline is converted to TMA, identifying the compound responsible for the inhibition is challenging. The data presented in the results suggest that it may not be CGA or any other component in the peel causing stronger inhibition. This is because, despite varying levels of phenolic compounds in the blueberries, they all exhibited the same inhibition potential.

There are some limitations to the digestion and fermentation model used in this project. First, our digestion model does not simulate absorption, which means compounds typically absorbed in the small intestine remain in the fermentation process. One potential solution to this is to use dialysis to remove sugar and other water-soluble metabolites from the digesta, though this would also result in the loss of water soluble but poorly absorbed phenolics like CGA.

Another option could be adding the digesta to Caco-2 intestinal epithelial cells to remove these substrates, but this approach is beyond the scope of this project. As a result, the inhibition of TMA-d9 observed may be due to the presence of compounds that would not normally be in the colon, rather than phenolics, which have generally low bioavailability and would typically be present during fermentation. However, this was addressed as much as possible by removing the pulp, which contains most of the substrates not found in the colon, and focusing on measuring the inhibition from the peel.

The ex *vivo* fermentation model, using human fecal samples in a controlled anaerobic environment, aims to mimic the conditions of the colon during *in vivo* fermentation conditions. However, there are some limitations to this approach. The fecal samples used in this project are sourced from OpenBiome, where donors are considered healthy, but it is important to note that most of TMA production is in gut microbiomes that are in a state of “dysbiosis”. TMA production depends on bacteria that produce TMA lyase. By incorporating a larger variety of fecal samples, it may be possible to increase the abundance of bacteria that metabolize choline, creating an environment more reflective of conditions seen in a state of "dysbiosis". TMA production from known high TMA-producing donors may better reflect the conditions of the population of interest as they likely include greater abundances of bacteria known to express cut*C/D* such as *Desulfovibrio*, *Proteus*, and *Klebseilla*[8,152,153,155–157]. Additionally, fecal samples are frozen and then reanimated 12 hours before fermentation using a growth media. However, some bacteria may not grow outside the colon, let alone survive the freeze-thaw and reanimation process, although a cryoprotectant is added to the samples. Furthermore, these samples are not fully representative of all regions of the large intestine. Additionally, fecal samples are frozen and then reanimated 12 hrs before fermentation using a growth media. *In vivo*, the gut microbiome is never frozen rather it is constantly active, metabolizing substrates. The reanimation process may alter the behavior of the bacteria, potentially causing them to act differently than they would in the colon. Finally, choline is typically consumed as a part of a complex meal, not as a pure substrate as in the fermentation model. The complexity of meals, which typically contains a variety of foods with different molecular compositions, may impact TMA production in ways that differ from the effects observed with a single food type, as used in the fermentation model.

The use of two or more OpenBiome fecal samples per fermentation experiment aims to create a more representative sample of the human population by incorporating a variety of bacteria that reflect a broader population. This approach is beneficial for introducing bacteria from individuals with diverse lifestyles, living environments, and diets. However, it does not allow for an in-depth examination of the individual variations in choline metabolism to TMA. Some individuals harbor high TMA-producing bacteria, while others do not. By combining these samples in the fermentation, it may appear that all people have TMA-producing bacteria when the results could be a mixture of one person with high TMA-producing bacteria and others without. To address this, an inter-individual study could be conducted by keeping the reanimated OpenBiome fecal samples separate, with each representing an individual, while all other experimental conditions remain consistent. This would allow for the comparison of the rate of choline conversion to TMA across different individuals.

Using animal models to track the metabolism of choline, TMA, and TMAO would help address the limitations associated with *in vitro* models. In an animal model, the gut microbes remain alive and active, eliminating the need for reanimation. While the diet of animal models is typically less diverse than that of humans, which may limit its representation of the population of interest, it is easier to control and modify, allowing for more realistic incorporation of different foods compared to *in vitro* experiments. *In vitro* models tend to represent acute conditions, capturing short-term changes in enzyme production rather than shifts in the microbiome, due to the brief observation period. Models like MiGut, SHIME, and TIM-2 represent longer time frames *in vitro* and provide a more faithful simulation of the gut environment. However, they are more cumbersome, costly, and lower throughput compared to our model[70,71,168]. Longer *in vivo* studies, over the course of a week or longer, would likely capture both changes in enzymatic activity and microbial shifts overtime. *In vivo* experiments could offer insight into how TMA and TMAO are absorbed and metabolized in the body, helping to determine whether inhibiting TMA also prevents the formation of TMAO, or if TMA follows an alternative metabolic pathway.

The blueberries themselves posed several challenges. There is limited information in the literature regarding fiber content of blueberries, making it difficult to determine the exact ratios of the different types of fiber present. This is likely due to the variation between blueberry cultivars and the lack of research on the fruit. To address this, a fiber extraction from a serving size of blueberries would be necessary; however, this process is time-consuming and outside the scope of this project. Removing the peel from the blueberries was also challenging due to their small and delicate nature. Frozen blueberries were particularly difficult to blanch and separate the peel from the pulp without issues. Peeling by hand was a time-intensive process that increased the risk of contamination, either by exposing the berries to non-frozen temperatures for extended periods or by accidentally leaving peel in on the pulp.

The CGA content was measured for the whole blueberry across 20 different cultivars, rather than just the peel, due to time limitations and the lengthy process of peeling the berries for the extracts used in SPE and LC-MS analysis. While previous experiments have shown that the majority of phenolics and CGA are contained in the peel, this was not specifically confirmed for the peels used in the final experiment. As a result, it is possible that the lack of differentiation in the TMA-d_9 inhibition could be due to the smaller variations in the peel across all cultivars, compared to the greater differences observed in the whole fruit. The variations in the fruit could be attributed to the amount of peel rather than its concentration of CGA. The fruit sizes varied between cultivars, with some being much smaller than others, resulting in a higher peel to pulp ratio in smaller berries. This was not accounted for in the experiment, as the amount of peel used was the same for all cultivars, regardless of fruit size. As a result, the amounts may not accurately reflect the actual serving size of whole berries for each specific cultivar, as each fruit had a different peel-to-pulp ratio. Although the cultivars used in this project were genetically diverse, they were all grown and harvested in Corvallis, OR, under the same environmental conditions, removing the potential differences that may arise if they were sourced from varying climates and regions in the world.

4.6 Strengths

The research presented in this thesis has several strengths that enhance its significance and validity. First, the *in vitro* digestion model incorporates accurate concentrations of oral, gastric, and small intestinal enzymes along with physiological conditions designed to simulate upper gut digestion. The enzymes used are representative of those found at each stage of digestion, and the shifting pH levels and incubation periods are timed to reflect the duration food spends in each section of the human digestive system. The addition of digesta material, rather than whole food, in the fermentation process is more representative of a real human system, as it accounts for the presence of digestive enzymes and related metabolites that would be involved in the breakdown of food.

The *in vivo* model allows for the isolation of TMA production using isotopically labeled choline-d_9 and TMA-d_9. This approach measured only the exogenously added choline and its metabolism to TMA, helping to control for other variables present *in vivo*, such as absorption or an alternative metabolic pathway. This ensures accurate quantification of the conversion rate of these metabolites by bacteria, specifically in the presence of potential inhibitors

The fermentation process occurs in an anaerobic chamber, with conditions continuously monitored to ensure they simulate a human colon environment. Fecal samples used in the fermentation are sourced from OpenBiome, a trusted provider known for supplying samples that are screened and approved for clinical use to recolonize the gut with a healthy, normal, and functioning microbiome. The donors of these samples undergo screening for potential health risks, ensuring that our model starts with a reliable and diverse seed of bacteria to measure TMA production. Since gut bacteria expressing the *cutC*/D gene cluster can vary among individuals, the use and combination of multiple OpenBiome fecal samples helps to simulate a more diverse population of gut bacteria for TMA assay. Notably, similar inhibition was observed for whole blueberry, pulp, and sugar across different fermentations using various fecal samples. This suggest that combining multiple fecal samples helps to create a more diverse environment, reflecting the variability in gut bacteria. Digesta controls in the fermentation account for the background inhibition and endogenous choline from the digesta. Additionally, the inclusion of labeled substrates in the fermentation ensures that the metabolites of interest are accurately measured and tracked, considering the potential presence of endogenous choline and TMA from the digesta.

The sugar and fiber ratios used in the experiments were designed to accurately reflect the composition of a serving size of blueberries. The sugar mixture included sucrose, glucose, and fructose in ratios typical of those found in blueberries, while the fiber blend contained hemicellulose, cellulose, and pectin in proportions similar to those in a serving of the fruit. Although literature on blueberry fiber composition is limited, the ratios used in these experiments were believed to accurately reflect the fiber content of blueberries and closely replicate the sugar content. The blueberries used in the final experiment represented a broad range of highbush cultivars, reflecting a variety of phenolic compositions, sugar content, and fiber levels. Any differences observed among these cultivars are likely due to genetic variations, as they were all grown and harvested in the same climate by the National Clonal Germplasm Repository (NCGR), Corvallis OR, USA. Growing in the same climate helps eliminates potential differences caused by factors like water availability, predators, or nutrient competition. The lack of variation in inhibition potential across the peels of the four high and four low cultivars is encouraging for blueberry consumers, as it suggests that the benefits are not limited to a specific cultivar, making it easier for people to access the same benefits regardless of the type of blueberry available.

CHAPTER 5 CONCLUSION AND FUTURE WORK

5.1 Conclusion

This project explored a novel aspect of the relationship between blueberries and their potential to inhibit TMA-d_9 *in vitro*. The significance of this research lies in the limited exploration of the impact of fruit, specifically blueberry, on dietary choline metabolism and TMA production in the gut, with no studies specifically examining the fruit's individual components. The depth of this project adds further significance, as most studies focus on the whole food in the gut rather than isolating and analyzing specific components. Since most of a food's composition may not reach the colon intact after upper gastrointestinal digestion, the inhibition of TMA production observed may not occur in the absence of the whole fruit. This project accounted for these variables, using methods designed to minimize interferences. This work highlights the potential TMA-lowering and cardiovascular health benefits of blueberries and helps to identify the source of their inhibitory effects, providing a deeper understanding of how they interact with gut bacteria.

I failed to reject the null hypothesis, which proposed that chlorogenic acid (CGA) content is not a primary determinant of the TMA-lowering benefits of blueberries. No significant differences were observed between cultivars with high CGA content and those with low CGA content in kinetic curves or AUCs for choline-d_9 and TMA- d_9 inhibition. However, it was observed that peel fractions showed significantly different AUCs for choline-d_9 and TMA- d_9 compared to fiber content and controls. While CGA may not be the primary determinant of TMA inhibition, the peels themselves show notable inhibitory potential compared to controls. If CGA is not the main factor responsible for the TMA-lowering benefits of blueberries, consumers could enjoy a wider variety of blueberries and still reap the benefits, without needing to specifically seek out a particular cultivar with high CGA content.

BacTiter-Glo optimization experiments provided a strong foundation for ATP assays used in subsequent experiments, helping to determine whether the treatments were toxic and causing inhibition, rather than inhibiting through a non-toxic mechanism. Once optimized for complex fermenta matrices, the ATP assay produced clear, readable signals, making it easy to distinguish differences between treatments. At no point did the data suggest that any component of the blueberry caused cell death leading to TMA-d_9 inhibition. In experiment 2, it was found that sugar components of blueberries may be the primary source of the strong, near-total TMA-d_9 inhibition of whole blueberries. The pulp of whole blueberry contains most of the fruit's sugar, as both whole blueberry and sugar treatments inhibited TMA-d_9 production for the full 30 hr fermentation period. This was further supported in experiment 3, where blueberries were separated into peel and pulp components and tested against whole blueberry, fiber, and sugar. Whole blueberry, pulp, and sugar showed similar inhibition compared to all other treatments for the 24 hr fermentation period, reinforcing the idea that sugar is the main contributor to inhibition. However, since sugar would not remain in the colon after upper gastrointestinal digestion *in vivo*, this inhibition is physiologically irrelevant. While peel exhibited less inhibition than whole blueberry, pulp and sugar, it still showed a stronger inhibition than fiber, suggesting there is an additional component or components in the peel contributing to the effect. The final experiment, which focused solely on the peels of four high and four low CGA blueberries, revealed no significant difference in inhibition between cultivars. Although there was greater inhibition compared to controls, the lack of difference between cultivars pointed to sugar, or another non-phenolic component of the whole blueberry, as the likely cause of the observed inhibition differences.

5.2 Future Work:

This thesis takes a proactive approach by exploring the impact of specific components of blueberries, paving the way for future studies involving animals and humans. Before these studies can take place, some *in vitro* research is still needed to help bridge the gap and provide further insight as to why blueberries may have TMA and potentially TMAO inhibiting effects.

In relation to the work presented in this thesis, it is crucial to measure the CGA content in the peels of the eight cultivars used in the fermentation process. Additionally, the CGA content and its hydrolyzed components, quinic and caffeic acid, in the final digesta and in the fermenta at various time points should be assessed to determine whether the lack of difference observed between cultivars was due to CGA not being released or if the CGA is primarily concentrated in the pulp of some of the whole fruits. Once the CGA levels are determined, it will be easier to identify whether any inhibition is linked to actual CGA (or quinic and caffeic acid) concentration in the fermenta or another factor. Since fruit size varies between cultivars, the peels from each cultivar should be standardized to represent the different peel masses of one serving, based on the cultivar rather than using the same masses across all cultivars. Furthermore, a broad screening of other phenolic compounds, coupled with in-depth phenolic compound analysis in the peel, can help determine whether different cultivars contain higher levels of specific phenolics that may contribute to the TMA-inhibiting benefits. In addition to evaluating the TMA-lowering activities of a variety of phenolic acids separately, conducting activity-guided fractionation would be valuable to explore phenolics with different polarities. This approach would further aid in identifying active compounds or groups of compounds, which could serve as the focus of future studies. The method used to measure CGA content in the whole blueberry also captured other phenolic compounds that will be analyzed at a later date. The isolation and purification of these compounds can be used in future studies to determine their effect on the TMA assay. The inhibition potential of these fruits on choline metabolism should also be examined in relation to other dietary substrates such as L-carnitine or betaine. This would help determine whether the same blueberry inhibition potential affects other genes, such as cnt*A/B* and yea*W/*X, or if its effects are specifically limited to the cutC/D gene.

A major challenge is addressing the limitations of *in vitro* digestion and fermentation when using whole fruits. Sugar is a primary concern in this assay, as it shows significant TMA-d_9 inhibition potential, but it would not remain in the colon after upper gastrointestinal digestion. This step can be particularly valuable as a preliminary method to identify if compounds are water-soluble before continuing with a dialysis procedure. Dialysis, as demonstrated by Yang *et al.*.[131], can simulate absorption and remove compounds before fermentation, especially if the analyte of interest, or the component intended to be in the fermentation, is not water-soluble or small enough to pass through the dialysis membrane. Another approach could involve a standard addition method, where varying levels of CGA are added to the same concentration of blueberry digesta to evaluate the effects of increasing CGA levels. Additionally, varying serving sizes of blueberries in digesta can help assess the impact of inhibition and the amount of fruit consumed.

Varying fecal samples could also provide valuable insight into the differences in the inhibition potential of blueberries. By using fecal samples from donors with a gut microbiome in a state of dysbiosis or those screened for bacteria that are high TMA producers, it is possible to create an environment where inhibition is more easily detectable, as it would contain the species of interest. In addition, interindividual experiments would be useful for tracking variations across a diverse group of individuals. This would help to determine whether differences in inhibition potential are influenced by genetic factors, environmental conditions, or dietary habits.

The primary focus of future research should be to investigate the mechanism by which choline and other dietary substrates are metabolized into TMA. Currently, the mechanism remains unidentified, and it is unclear whether inhibition of TMA production is caused by the inhibition of an enzyme produced by the bacteria, or if it results from changes in the bacterial community structure, bacterial viability, gene copy numbers, or gene expression. Potential *in vitro* experiments to identify the mechanism of choline metabolism to TMA could involve using non-viable bacteria cells that still contain active TMA lyase enzymes. If choline metabolism to TMA is not inhibited by the treatments, it would suggest that the mechanism is not due to enzymatic inhibition. Additionally, removing a component of the reaction, such as the inhibitory compound from the blueberry, during a choline challenge *in vivo* could offer insight into the mechanism behind choline conversion to TMA. Blueberry could be chronically administered and then removed from the diet before a choline challenge with subsequent TMA measurement. Chronic blueberry consumption might influence the bacteria by either reducing the population of TMA-producing bacteria or altering the gene expression of the TMA lyase enzyme. Observing TMA inhibition, or lack thereof, in the absence of blueberry will help clarify how blueberry inhibits TMA production and shed light on the mechanism of TMA synthesis. Tracking gene expression in the presence of an inhibitory compound could help determine whether protein expression is suppressed by the inhibitor. *In vivo* experiments can help assess the rate of absorption and compare it to circulating plasma levels of TMA and TMAO, allowing researchers to determine whether all prerequisite substrates contribute to TMAO, or if some are further metabolized by other bacteria or redirected into different metabolic pathways. Future work should focus on animal models and clinical studies, as *in vivo* experiments address many of the limitations presented in this study. These experiments take into account the absorption of food components in the upper gastrointestinal tract, bioavailability of phenolic compounds, the effect of chronic and acute exposure to substrates, and the absorption and metabolism of TMA and TMAO. In vivo studies leverage the body's natural processes, offering a more comprehensive understanding of how blueberries might inhibit TMAO formation.

This thesis takes a proactive approach by investigating the components of blueberries that contribute to their inhibitory potential, specifically focused on blueberry peels and their TMA inhibition. The work lays the foundation for future *in vivo* and clinical studies to better understand the mechanisms behind this metabolic process, while reinforcing the potential of blueberries in improving cardiovascular health.

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APPENDICES

Appendix A: Protocols

<u>BacTiter-Glo Protocol</u>

Purpose: To confirm the treatments did not have a cytotoxic or cytostatic effect on fecal bacteria cells. A luminescent assay is used to measure the presence of ATP and monitor the number of viable microbial cells during fermentation. BacTiter-Glo Microbial Cell Viability Assay (Promega, USA)

**This assay is done at room temperature, and BacTiter-Glo reagent should be brought to room temp before use

1.) <u>Prep BacTiter-Glo reagent</u>: mix equal amounts of the provided BacTiter-Glo substrate and BacTiter-Glo buffer and leave to rest for at least 15 min at room temperature before use. (This reconstituted reagent should be stored in the fridge throughout fermentation and can be used for both the 12 hr and 24 hr test).
2.) Collect 100 uL of fermenta sample in a white 96-well opaque plate
3.) Centrifuge the plate for 5 minutes at 4°C at 500 x g
4.) After centrifugation, remove and discard the supernatant
5.) Add 100 uL of PBS 1X to all wells and resuspended the pellet
6.) Add 100 uL of the reconstituted BacTiter-Glo reagent to all wells and aspirate to mix
7.) Place the 96-well plate in the plate reader for 5 min to reduce luminesce signal from the light
8.) Read luminescence
9.) Place pate in bleach water before discarding in biohazard

Storage Conditions

Reconstituted reagent is good for <u>8 hrs at room temp</u>., <u>4 days at 4°C</u>, <u>1 week at -20°C</u>, or <u>1 month at -80°C</u>

Three Stage In vitro Digestion Protocol

Preparation

Stock Solutions**:**
0.9% NaCl
1.0 M HCl
0.1M HCl
1.0M $NaHCO_3$
0.1M $NaHCO_3$

Gastric and Small Intestinal Phase Solutions**:**
20 mg/mL Pepsin in 0.1M HCl
Pancreatin-Lipase Solution
20 mg/mL Pancreatin (in 0.1M $NaHCO_3$)
10 mg/mL Lipase (in 0.1M $NaHCO_3$)
Bile Solution
30 mg/mL Bile Extract (in 0.1M $NaHCO_3$)

Preparation of Oral Phase**:**
Base Solution (q.s. to 1 L with Dl water):
- Potassium Chloride 1.792g
- Sodium Phosphate 1.776g
- Sodium Sulfate 1.140g
- Sodium Chloride 0.596g
- Sodium Bicarbonate 3.388g

Begin preparation by calculation amount of oral phase solution to be prepared (6mL's / reaction tube, + 1 additional for pipetting errors)

1. Pipette over calculated amount of base solution into a beaker.
2. Add 0.4 mg urea per ml base solution.
3. Add 0.03 mg uric acid per mL base solution.
4. Add l0.6 mg α-amylase per mL base solution.
   a. 10.6mg **per g** food digested. (Must adjust this amount if a value other than 1 g of food per reaction).
5. Add 0.05 mg mucin per **mL** base solution.
6. Mix well (at least 15 minutes) before using.

α-amylase –Sigma, A3176. The activity is 15.8 units/mg of solid at pH 6.9 of food to be digested (=500 units per 3 g food)
OPM = oscillations per minute

~When placing samples in the incubator they will be horizontal. If you are not using the Sarstedt red cap tubes, you may need to use parafilm to help ensure there is no leaking.

Procedure:

Start Up.
1. Weigh 0.5 to 3 g of food to be digested into a 50 mL centrifuge tube.

Oral Phase
2. Add 6 mL oral phase per reaction tube (see above for preparation of oral phase) Vortex (1 minute).

Three Stage In vitro Digestion Protocol Cont.

3. Blanket with nitrogen gas, cap tightly.
4. Place in 37 °C incubator. leave at 120 opm for **10 minutes.**

Gastric Phase

5. Remove from incubator, place immediately on ice.
6. Bring to 30 mL with saline. (Assume 1g food material = 1mL)
7. Add *2* mL pepsin
8. Adjust pH to equal 2.5±0.1 using 1.0 M HCI. Measure in increments of 0.1 and 0.5 mL recording pH and volume after every addition.
9. Bring to 40 mL with Saline
10. Blanket with nitrogen gas, cap tightly and place in 37 °C incubator. leave at 120 opm for **1 hour.**

Intestinal Phase

11. Remove from incubator, place immediately on ice.
12. Add 2 mL Pancreatin/Lipase solution
13. Add 3 mL Bile Solution
14. pH to 6.5 ± 0.1 using 1M $NaHCO_3$. record volume and pH at each step.
15. Bring to 50 mL with saline.

Blanket with nitrogen gas, cap tightly and place in 37 °C incubator. leave at 120 opm for **2 hours.**

Fermentation Protocol

Fermentation (In anaerobic chamber):

- Mix growth media A+B
- Dilute 4 mL of choline-d9 4 mM with a volume of 4 mL PBS 1x to get choline-d9 2mM solution.

12 h reanimation**:** Make a 10X dilution of the original fecal slurry with media (3 g fecal + 27 mL media) using 3 different open biome samples. (2 of the solid fecal samples +1 of the liquid fecal sample)

- Mix fecal samples in 25 mL of media. Allow to sit for 12 h in open 50 mL tube covered with foil before starting fermentation.
- Filter Fecal slurry right before use, use the remaining media (2mL) to rinse residual fecal through filter

We are going to run the experimental conditions (see table) in *n*=6, in open 96-well plate, 2mL waters plate, 1800µL total volume.

Experimental Conditions (µL) 12 h Reanimation					
n=6	Growth Media	FS (10X diluted)	Choline-d9 (2mM)	Digesta	PBS (1X)
DG Free (“control”)	750	360	90	0	600
Blank DG	750	360	90	600	0
Digesta Treatments	750	360	90	600	0

- Take 100uL sample at 0, 2, 4, 6,8, 10, 12, 16, 20, and 24 hr
- Add 100uL ACN.
- BacTiter-glo at 12 h and 24 hr: 100 uL of fermenta sample centrifuged at 500 x g for 5 min, supernatant removed, pellet resuspended in 100 µL PBS 1X, mixed with 100 uL BacTiter-Glo reagent, placed in plate reader for 5 min before reading LUM
- Transfer sample to storage plate, and freeze -80 C.