Inhibition of pro-atherogenic trimethylamine production by human gut bacteria is not determined by varying chlorogenic acid content in highbush blueberries

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Abbreviations: acetonitrile (ACN); area-under-the-curve (AUC); cardiovascular disease (CVD); chlorogenic acid (CGA); Draper x Jewel (DxJ); flavin monooxygenase 3 (FMO3); fresh weight (FW); National Clonal Germplasm Repository (NCGR); solid phase extraction (SPE); trimethylamine (TMA); trimethylamine N-oxide (TMAO); trimethylaminuria (TMAU)

Abstract

Cardiovascular diseases (CVDs) account for ~32% of all deaths worldwide, and atherosclerosis-related conditions are the leading cause of death in the United States. Elevated blood levels of trimethylamine N-oxide (TMAO), a gut microbiota-derived metabolite, are linked to increased risk of atherosclerosis. TMAO is produced when gut bacteria metabolize dietary quaternary amines to trimethylamine (TMA), which is converted to TMAO in the liver. With no FDA-approved drugs available to reduce TMA or TMAO, dietary interventions present the most promising strategy. Chlorogenic acid (CGA), a phenolic abundant in blueberries, inhibits TMA production. Blueberries may thus be a TMA- (and TMAO)-lowering food. CGA content in highbush blueberries varies depending on cultivar, growth conditions, and storage conditions; it remains unclear whether these variations in CGA levels influence the TMA-lowering activity of different blueberry varieties. We investigated the impact of highbush blueberry CGA content on the inhibition of choline-d_9 conversion to TMA-d_9 in our *ex vivo-in vitro* human fecal fermentation model. We tested blueberry skins (to avoid interferences from sugar-rich pulp) from 20 genetically distinct highbush blueberry genotypes, chosen based on their high and low CGA content. CGA levels in whole berries ranged from 2.6-146 mg/100 g fresh weight (FW), while CGA concentrations in blueberry skins ranged from 0.14-9.7 mg/g. No significant differences were observed in TMA-d_9 production among the 4 highest and 4 lowest CGA genotypes in kinetic curves or area under the curve (AUC) values. However, significant differences were observed between all genotypes compared to controls, with ~19.4.% reduction in TMA-d_9 AUCs compared to blank digesta, indicating that phenolic-rich skin provides similar TMA-lowering benefits across blueberry varieties. This suggests that the CGA content of genotypes is not a crucial factor for lowering TMA. Preliminary evidence suggests that fiber is also not the primary driver of the inhibitory activities of blueberry skins. Future studies, including rodent and human studies, are needed to confirm this *in vitro* study and understand why blueberries may inhibit TMA and potentially TMAO, as *in vitro* studies have limitations, and the mechanism of TMA production remains unclear.

Key Words: Chlorogenic acid, cardiovascular disease, trimethylamine, cutC/D, Atherosclerosis

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 atherosclerosis, myocardial infarction, and stroke and represents a leading cause of morbidity and mortality [2]. Atherosclerosis linked diseases are the leading causes of death in the United States [3]. The prevalence of CVDs 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. 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 [4]. In particular, the metabolite trimethylamine (TMA), produced from choline, L-carnitine, and γ-butryobetaine by gut bacteria containing trimethylamine lyase (TMA-lyase) complexes, serves as a precursor to trimethylamine N-oxide (TMAO). Choline TMA-lyase, which metabolizes choline into TMA, is encoded by the *cutC/D* gene cluster found in specific gut microbiota [5,6].

Studies suggest that elevated concentrations of trimethylamine N-oxide (TMAO) in the blood are associated with increased risk of atherosclerosis [7,8]. 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 [9]. In addition, choline is metabolized to TMA by gut bacteria. 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 [10]. Once it reaches the liver, TMA is oxidized to TMAO by hepatic flavin monooxygenase 3 (FMO3) [11].

Currently, there is no FDA-approved drug to control or lower TMA or TMAO levels. 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 reducing TMA production [12]. Although the use of antibiotics effectively reduces TMA production by targeting bacteria with the cut*C/*D gene, it may also disturb beneficial commensal bacteria, potentially leading to adverse health effects [13,14]. Strategies such as non-lethal TMA lyase inhibition lower TMA and TMAO production and inhibit the onset of atherosclerosis in rodents [15]. Lowering FMO3 expression or inhibiting its action may provide an effective strategy to decrease TMAO production [16]. However, 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 [17–19]. The reduction or inhibition of FMO3 may result in a buildup of TMA, potentially leading to TMAU. Reducing dietary intake of choline proves to be an effective strategy to reduce TMA and TMAO levels [12]. However, this is not a viable strategy, as choline plays a crucial role in the synthesis of phospholipids and neurotransmitters.

There is a growing interest in the TMA- and TMAO-lowering activities of dietary phenolics [20,21]. 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. Due to the low oral bioavailability of many phenolics, unabsorbed phenolics accumulate in the colon's lumen, where *cutC/D* (and other TMA lyase) gene-complex containing bacteria are found [22–24]. Chlorogenic acid (CGA, or 5-caffeoyquinic acid) (**Figure 1**) is a phenolic commonly found in the diet, and our lab previously identified CGA as a potential inhibitor of TMA production [21]. CGA and CGA-rich foods exhibit anti-inflammatory, antihypertensive, and antioxidant properties that help reduce the risk of cardiovascular disease and improve overall health.

CGA stands out as the most predominant phenolic acid in blueberries, accounting for more than 95% of total phenolic acid content in some cases [25–27]. Although all blueberries contain some level of CGA, the difference between genotypes can vary by >20-fold [27]. We previously showed that CGA-rich blueberry supplementation reduces TMAO in mice [28]. However, blueberries contain varying amounts of CGA, so 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. Additionally, other components may drive the TMA- and TMAO-lowering activities of blueberries.

The overall aim of this study was to evaluate whether CGA is a key bioactive that enables blueberries to inhibit the formation of the gut microbial metabolite TMA (a precursor to the pro-atherogenic compound TMAO). We hypothesized that CGA content is a primary determinant of the TMA-lowering benefits of blueberries. To test the inhibition properties of CGA and other non-phenolic components of blueberries, an *ex vivo-in vitro* human fecal fermentation model was utilized to evaluate the inhibition of TMA production from choline.

2. Methods and Materials

2.1 Chemicals: Fecal samples were obtained from OpenBiome (Cambridge, MA, USA). Urea (CAS# 57-13-6), uric acid (CAS# 69-93-2), KCl (CAS# 7447-40-7), NaCl (CAS# 7647-14-5), $NaHCO_3$ (CAS# 144-55-8), Na_2HPO_4 (CAS# 7558-79-4), PBS (CAS# 7447-40-7, 7647-145-5), formic acid (CAS# 64-18-6), methanol (CAS# 67-56-1), acetone (CAS# 67-64-1), acetonitrile (CAS# 75-05-8), concentrated ammonia (CAS#1336-21-6, 7732-18-5), Na_3PO_4 (CAS# 7601-54-9), $ZnSO_4$ (CAS# 7446-20-0) and sodium acetate (CAS# 6131-90-4) were obtained from Thermo Fisher Scientific (Hampton, NH). Mucin (porcine stomach, CAS# 84082-62-4), α-amylase (porcine pancreas, CAS# 0009000902), Na_2SO_4 (CAS# 7757-82-6), pepsin powder (porcine gastric mucosa, CAS# 9001-75-6). NaOH (CAS# 1310-73-2), pancreatin (porcine pancreas, CAS# 8049-47-6), lipase (porcine pancreas, CAS# 9001-62-1), bile extract (porcine, CAS# 8008-63-7), peptone water, yeast extract (CAS# 8013-01-2), $MgSO_4 \times 7H_2O$ (CAS# 10034-99-8), $CaCl_2 \times 6H_2O$ (CAS# 7774-34-7), hemin (CAS# 1600-13-5), bile salts, Tween 80 (CAS# 9005-65-6), vitamin K1 (CAS# 84-80-0), resazurin (CAS# 62758-13-8), L-cysteine (CAS# 52-90-4), choline- d_9 (CAS# 61037-86-3), ethyl bromoacetate (CAS# 105-36-2), sodium carbonate (CAS# 497-19-8), Folin-Ciocalteu reagent, ammonium formate (CAS# 540-69-2), Diaion HP-20 resin (CAS# 9052-95-3), TMA- d_9 (CAS# 18856-86-5), pectin from apple (CAS# 9000-69-5), cellulose (microcrystalline, 20 μm, CAS# 9004-34-6), D-(−)-Fructose (CAS# 57-48-7), and D-(+)-Glucose (CAS#50-99-7) were obtained from MilliporeSigma (Burlington, MA). Acetic acid (CAS# 64-19-7) and D-(+)-Sucrose (CAS# 57-50-1) were obtained from VWR (Radnor, PA). Choline-1-^{13}C-1,1,2,2, -d_4, choline-1,2-$^{13}C_2$, TMA-$^{13}C_3$-^{15}N, and TMA-$^{13}C_3$-d_9 were obtained from Cambridge Isotope Laboratories. Hemicellulose (CAS#9034-32-6) was obtained from BOC Sciences (Shirley, NY). N_2 gas was obtained from Airgas, Charlotte, NC, USA.

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 method development were obtained from Food Lion, Kannapolis, NC, USA on March 4, 2024, and frozen immediately at −80°C. A 1:1 mixture of these store-bought berries was used for method development. Fruits from various blueberry genotypes were obtained from the 2019 and 2024 harvest of a diverse field collection through the United States Department of

149 Agriculture Agricultural Research Service (USDA ARS) National Clonal Germplasm Repository
150 (NCGR), Corvallis OR, USA. Fruit from a bi-parental population (Draper x Jewel, labled as
151 DxJ) were obtained from plants grown by the USDA-ARS Berry Breeding Program at the
152 Corvallis-based Horticultural Crops Production and Genetic Improvement Research Unit
153 (HCPGIRU). Fruits from these two distinct populations were obtained: (1) accessions (labeled as
154 Plant Introduction, PI) belonging to a diversity panel of cultivars/selections where the plants
155 were not directly related and did not share genetic material from the same immediate parental
156 sources, and (2) genotypes belonging to the DxJ bi-parental mapping population where plants
157 shared genetic material from the same parental sources. The accessions and the DxJ genotypes
158 were selected based on chlorogenic acid content evaluated in previous work [29] and verified in
159 this study (see results). Also the genotypes from the mapping population were selected based on
160 genotypic data, representing the dominant and recessive allele at a major genetic locus associated
161 with chrologenic acid content [29]. Blueberries were frozen, shipped overnight on dry ice, and
162 stored at −80°C immediately upon receipt prior to use.
163
164 *2.3 General Gastrointestinal Digestion:* Simulated *in vitro* upper gastrointestinal digestions were
165 performed to mimic oral, gastric, and small intestinal digestion following the methodology
166 described by Iglesias-Carres *et al.* [30,31]. Digestions were scaled from ~1 serving [150 g fresh
167 weight (FW)] in estimated human upper gastrointestinal volume (2 L) to an equivalent dose for
168 the *in vitro* digestion final volume (15 or 50 mL). "Blank" or "control" digesta was made with
169 saline in place of food material and treated the same as the other treatments. Further details of
170 digestion methods can be found in the **Supplementary Information.**
171
172 *2.4 Anaerobic Fecal Fermentation*
173
174 *2.4.1 Growth Media Preparation:* Anaerobic growth media were prepared following the
175 methodology described by Iglesias-Carres *et al.* [21]. Further information can be found in the
176 **Supplementary Information.**
177
178 *2.4.2 Fecal Slurry preparation:* Fecal samples collected from healthy donors and screened for
179 infectious diseases were obtained through OpenBiome (Cambridge, MA, USA) and stored

180 immediately upon arrival at −80°C. Fecal slurries were prepared 12 hrs before the start of
181 fermentations (concentrations varied based on individual experimental parameters). At least two
182 different fecal samples were combined in a ratio of 1 mL fecal sample to 9 mL anaerobic growth
183 media and vortexed until homogenized. The fecal slurry was left uncapped in the anaerobic
184 chamber for at least 12 hrs prior to fermentations. Further details can be found in the
185 **Supplementary Information.**
186
187 *2.4.3 General Fermentation Conditions:* Fermentations took place inside a 4-glove 855-ACB
188 anaerobic chamber (Plas-Labs, Lansing, MI, USA). The chamber was first purged of oxygen
189 using N_2 (Airgas, Charlotte, NC, USA) and then mixed gas containing 5% H_2, 5% CO_2 and 90%
190 N_2 (ARC3, Raleigh, NC, USA) until chamber conditions reached H_2 (2-3%) and O_2 levels below
191 20 ppm monitored with a CAM-12 anaerobic monitor (Coy Lab Products, Grass Lake, MI,
192 USA). Once anaerobic conditions were achieved, the heater inside the chamber was turned on to
193 37°C, and a palladium catalyst (to consume residual O_2) was placed on top before leaving it to
194 stabilize overnight. Conditions were monitored throughout the fermentation experiment. Further
195 information can be found in the **Supplementary Information.**
196
197 *2.4.4 General Fermentation Procedure:* Fermentations were carried out per the methodology
198 developed by Iglesias-Carres *et al.* [21]. The anaerobic chamber was prepared as described above.
199 To eliminate interferences by endogenous choline and other TMA-lyase substrates leading to
200 TMA metabolism in the absence of exogenous choline, choline-d_9 was added as a substrate at a
201 final concentration of 100 μM to all treatments. Choline-d_9 utilization and TMA-d_9 production
202 were measured to evaluate the effectiveness of treatments. Lyophilized, digested samples (if any)
203 were prepared as described in general gastrointestinal digestions, lyophilized (to remove O_2) and
204 reconstituted to 1X digesta concentration using filter-sterilized, overnight-sparged PBS 1X inside
205 the anaerobic chamber. In 1.1 mL 96-well plates, 750 μL of growth media were mixed with
206 90 μL of choline-d_9 stock solution (2 mM) in PBS 1X, 600 μL of reconstituted digested samples
207 and 360 μL fecal slurry (1:10 in PBS 1X). Final concentrations were thus 100 μM choline-d_9, 2%
208 fecal matter, and 33.3 % digesta. Samples containing no digesta material were brought to volume
209 with 600 μL of PBS 1X. The start of fermentation (time 0 h) began when fecal slurry was
210 inoculated into the reaction mixture with the substrate. From 0 up to 30 h, a 100 μL sample was

211 collected at various time points, combined with 100 µL of acetonitrile (ACN), and frozen
212 immediately at −80°C. Further information can be found in the **Supplementary Information.**
213
214 *2.8 Phenolic Fraction Extraction:* A phenolic extraction was performed to generate a blueberry
215 fraction representing phenolic compounds in an average serving of blueberries (~150 g) for use
216 in fermentation. Whole blueberries (1:1 mix of locally available store-bought fresh and frozen
217 berries) were homogenized, weighed, lyophilized, weighed and frozen at −80°C. Lyophilized
218 material representing a serving size of fresh weight blueberry was combined with an extraction
219 mixture containing acetone, water, and acetic acid (70:29.5:0.5) and vortexed, blended for 1 min
220 using a polytron (VWR 200), sonicated for 5 min in a water bath at 50 °C, and centrifuged (3248
221 x g, 10 min). The steps were repeated seven times until the supernatant was clear. The
222 supernatant was collected from the extractions, pooled, and concentrated using a rotary
223 evaporator at 45 °C. The extract was frozen at −80°C and lyophilized. The lyophilized extract
224 was weighed and resuspended in acidified mill-Q water (0.1% formic acid). The resuspended
225 extract material was eluted using Diaion HP-20 resin and a 24/40 column to remove and purify
226 phenolic compounds, then transferred into a round-bottom flask and concentrated using a rotary
227 evaporator. The extract material was frozen at −80°C and lyophilized. Further information can be
228 found in the **Supplementary Information.**
229
230 *2.9 Plant Phenolic Extraction and Analysis:* Extraction of blueberry material (skin, pulp and
231 whole fruit) was performed. The Folin assay was carried out to estimate total phenolics in the
232 plant extracts. To quantify the relative amounts of CGA in plant material, a solid phase extraction
233 (SPE) followed by analysis using UPLC-MS/MS was utilized following the methodology
234 described by Mengist *et al*.[32]. Further information can be found in the **Supplementary**
235 **Information.** CGA MS/MS parameters and a representative UPLC-MS/MS chromatogram are
236 shown in **Supplementary Table 1** and **Supplementary Figure 1**, respectively.
237
238 *2.10 Digesta and Fermenta CGA Analysis:* Digesta samples were combined 1:1 with 0.1%
239 formic acid in ACN, vortexed, sonicated for 5 min, then centrifuged (10 min at 17,000 x g). The
240 supernatant was filtered through a 0.2-micron PTFE filter before LC-MS analysis. Fermenta
241 samples were combined 1:1 with ACN after sample collection during fermentation. To acidify

these samples, 10 µL of 0.425% formic acid in ACN was mixed with 75 µL of the fermenta sample. Samples were filtered through Strata filter plates using centrifugation (10 min at 3428 x g) before LC-MS analysis. Further information can be found in the **Supplementary Information.**

2.11 BacTiter-Glo ATP Assay: Bacterial viability was quantified by luminescence using a commercial BacTiter-Glo Microbial Cell Viability Assay (Promega, USA). The reagent was prepared 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. A total of 100 µL of the fermenta sample was plated on a white 96-well opaque round-bottom plate and centrifuged (500 x g, 5 min) to remove interfering material. The pellet was resuspended in 100 µL of PBS 1X, combined with 100 µL BacTiter-Glo reagent, and covered for 5 min before being placed in the plate reader (SpectraMax iD3, Molecular Devices, USA). Luminescence was read (Shake: 10s, integration: 2000, read height: 0.50mm) and results recorded. 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. Validation of the assay is described in the **Supplementary Information**. BacTiter-Glo validation and performance data are shown in **Supplementary Table 2** and **Supplementary Figures 2-4.**

2.12 Extraction and Quantification of Choline-d_9 and TMA-d_9: Sample extraction and quantification of choline-d_9 and TMA-d_9 using isotope dilution were carried out as described by Iglesias-Carres et al. [33]. Two internal standards were available for use, with only one utilized per experiment (TMA-$^{13}C_3$-^{15}N or TMA-$^{13}C_3$-d_9, and choline-1-^{13}C-1,1,2,2, -d_4 or choline-1,2-$^{13}C_2$). Further information can be found in the **Supplementary Information.** Structure and reactions schemes for analytes and internal standards are shown in **Supplementary Figure 5.** MS/MS parameters and presesentative UPLC-MS/MS chromatograms for analytes and internal standards are shown in **Supplementary Table 3** and **Supplementary Figures 6-11**, respectively.

2.13 Statistical Analyses: Prism 10.4.1 (GraphPad, La Jolla, CA) was used for statistical analyses and graph creation. One-way ANOVA was used to evaluate statistical differences in experiments with one independent variable between control conditions and test solutions (treatment effects).

273 Two-way ANOVA was used to evaluate statistical differences in experiments with multiple
274 independent variables between control conditions and test solutions, as well as choline-d_9 and
275 TMA-d_9 kinetic curves (significant main effect/ interaction). If a significant difference ($P < 0.05$)
276 was noted, the Tukey post hoc test or Sidak's multiple comparison test was used to assess the
277 significance of the difference between means. If the kinetic curve calculated values were
278 negative, a zero was imputed for the areas-under-the-curve (AUCs) calculations. One-way
279 ANOVA was used to estimate differences in choline-d_9 and TMA-d_9 AUCs between treatments
280 and controls. For BacTiter-Glo data, one-way ANOVA test was used to evaluate statistical
281 differences. If a significant difference was noted ($P<0.05$), then a Tukey's post hoc multiple
282 comparison test was used to evaluate statistical differences between treatment means.
283
284 **3. Results**
285
286 *3.1 Experiment 1: Determination of TMA-d_9 lowering activities of blueberries with high and low*
287 *CGA content.* Based on the previously reported CGA content in the DxJ blueberry bi-parental
288 genetic mapping population (2017-2019 crop years, **Supplementary Figure 12**), eight blueberry
289 genotypes with large differences in CGA contents from previous years were selected [27]. Based on
290 fruit availability, four high CGA genotypes (170-250 mg/100 g FW in 2017-2019 crops)
291 containing the dominant allele were compared to four low CGA genotypes (17-32 mg/100 g FW
292 in 2017-2019 crops) containing the recessive allele were selected for further study. A simulated
293 gastric and small intestinal digestion was performed using the blueberries at a dose equivalent to
294 133 g of fresh blueberry consumed by an adult human (2 L upper gastrointestinal volume). The
295 blueberry digesta and control digesta were diluted 3X and fermented for 24 hrs with fecal
296 bacteria in the presence of choline-d_9. Near-complete inhibition of TMA-d_9 production was
297 observed by all eight genotypes tested (**Figure 2A**). These results indicated that blueberries are
298 the most potent TMA-lowering whole food tested in this model compared to previously
299 evaluated foods such as coffee, tea, cocoa [30], and artichokes [31]. The inhibition of TMA
300 production across all blueberry genotypes appeared to be nearly zero (Figure 2A). All eight
301 genotypes were effective in lowering TMA vs. control digesta. However, close examination of
302 TMA-d_9 appearance kinetics (**Figure 2B**) suggests that differences between high and low CGA
303 genotypes may be present (high CGA genotypes generally inhibited TMA-d_9 production more

304 than low CGA genotypes) and that these differences might increase if the assay period were
305 extended. Other components of blueberries such as sugar, fiber, or other non-phenolic
306 compounds may also have reduced TMA-d_9 production *in vitro* [30,34]. Previous work by Bresciani
307 *et al.* has shown that sugar, which would not be present in the colon after upper gastrointestinal
308 digestion, can significantly inhibit TMA formation in *in vitro* fecal models [34]. This inhibition is
309 more accurately described as experimental interference, rather than a physiologically relevant
310 effect, and would not be observed *in vivo*. Importantly, we have not previously observed such
311 strong inhibition of TMA-d_9 production from other fiber-rich products such as cocoa [30],
312 artichokes [31], or pure inulin [31].
313
314 *3.2 Experiment 2: Determination of the impacts of CGA and major fruit components (sugar,*
315 *fiber, skin, pulp) of blueberries on TMA production.* The data in Figure 2 revealed significant
316 (almost total) inhibition of TMA-d_9 generation compared to other foods previously studied in our
317 lab. Additionally, previous work by Bresciani *et al.* has shown that sugar (which would not be
318 present in the colon after upper gastrointestinal digestion) can significantly inhibit TMA
319 formation [34]. We postulated that sugar, located primarily in the pulp, may be responsible for the
320 observed near-total inhibition of TMA-d_9 production by blueberries *in vitro*. It was thus
321 necessary to investigate whether components of blueberries, such as sugar, were interfering with
322 the assay. Given the nature of our *in vitro* upper gastrointestinal digestion model, removing sugar
323 is not possible without also eliminating other water-soluble compounds, such as CGA, that are
324 not well-absorbed (and would thus be present in the colon *in vivo*). To identify what portion of
325 blueberry fruit tissue exhibits the greatest effect on TMA-lowering activities, whole blueberry,
326 sugar (mixture of glucose, fructose, and sucrose), fiber (mixture of cellulose, hemicellulose and
327 pectin), and phenolic fractions (each matching the amount and composition in the whole berry,
328 **Supplementary Table 4**) were digested and then used in an extended fermentation model (30 h).
329 Further information can be found in the **Supplementary Information.**
330 BacTiter-Glo luminescence measurement of viable bacterial load for these fermentations
331 is presented in **Supplementary Figure 13A**. There is no evidence suggesting that any treatment
332 caused a cytotoxic effect that would lead to the perceived effect of TMA-d_9 inhibition. However,
333 there were differences between treatments. Whole blueberry significantly elevated bacterial load
334 at 14 h, but significantly lowered it at 30 h, compared to other treatments. Digestions and

335 fermentations were carried out as described above, and the kinetics of choline-d_9 use and TMA-
336 d_9 production are presented in **Figures 3A-B**, respectively. Whole blueberry and sugar fraction
337 treatments exhibited significantly higher concentrations of choline-d_9 and lower concentrations
338 of TMA-d_9 throughout the 30 hr fermentation period (essentially no choline-d_9 breakdown)
339 compared to all other treatments. This is further displayed in areas-under-the-curve (AUCs) of
340 the choline-d_9 (**Figure 3C**) and TMA-d_9 (**Figure 3D**) kinetics. These data supported our
341 hypothesis that sugar in the whole blueberry might be responsible for the observed near-total
342 TMA-d_9 inhibition, as the behavior of sugar (matched in content and composition) mimicked the
343 behavior of the whole blueberry. Notably, fiber (which would be present in the colon *in vivo*) did
344 not seem to affect choline-d_9 use or TMA-d_9 production, suggesting that fiber does not interfere
345 with the assay.
346
347 *3.3 Experiment 3: Identification of blueberry components with the greatest TMA inhibition*
348 *potential:* Building on the results from Experiment 2, which showed that whole blueberry and
349 sugar fractions exhibited the greatest TMA-d_9 inhibition, a further experiment was conducted to
350 identify blueberry fractions and components with the most significant TMA-d_9 lowering effect.
351 We first attempted to remove the sugar post-digestion using sucrose, fructose, and glucose
352 degrading enzymes, which was unsuccessful (data not shown). We elected not to pursue this
353 course further due to the unknown interferences that would likely arise from sugar degradation
354 products. We then changed our strategy to isolate the sugar components sequentially. We broke
355 the whole blueberry into skin (fiber- and phenolic-rich) vs. pulp (sugar-rich) fractions and
356 compared their inhibitory effects to those of the sugar mixture (mixture of glucose, fructose, and
357 sucrose, matching the ratio and content in whole blueberry) and whole blueberry
358 (**Supplementary Table 5**). Total phenolics and CGA content for whole blueberry, skin, and pulp
359 fractions (**Table 1**) confirmed that the majority of total phenolics and CGA are indeed located in
360 the skin, as expected. BacTiter-Glo luminescence reading for treatments in blueberry fraction
361 fermentations at 12 hr (A) and 24 hr (B) are presented in **Supplementary Figure 14**. There is no
362 evidence suggesting that any treatment caused a cytotoxic effect that would lead to the perceived
363 effect of TMA-d_9 inhibition. However, WB and pulp had significantly higher values at both 12
364 and 24 h. It is interesting that whole blueberry and pulp, but not sugar, increased viable bacterial
365 loads. The reason for this is unclear.

366 Digestions and fermentations were carried out as described above and kinetics of choline-
367 d_9 use and TMA-d_9 production are presented in **Figure 4A-B** for fermentations. Whole blueberry,
368 pulp, and sugar demonstrated significant (near-total) inhibition of TMA-d_9 production
369 throughout the 24 hr fermentation period compared to all other treatments. Digesta free, blank
370 digesta, fiber, and skin treatments were different from the whole blueberry, pulp, and sugar
371 treatments, as well as from each other. Digesta-free treatments exhibited the quickest choline-d_9
372 utilization, with choline-d_9 concentrations decreasing and TMA-d_9 rising sharply by the 8 hr
373 mark. Fiber treatments demonstrated the second quickest choline-d_9 utilization, with a significant
374 decrease in choline-d_9 and corresponding increase in TMA-d_9 occurring between the 12 and 16
375 hrs. Blank digesta displayed similar results to the fiber treatments, with choline-d_9 concentrations
376 declining and TMA-d_9 rising by the 16 hr mark. Skin treatments exhibited low choline-d_9
377 utilization until the 18-20 hr time frame, though not to the same extent as the whole blueberry,
378 pulp, and sugar treatments. Since the skin contains most of the fiber, the stronger inhibition
379 observed with the skin compared to the matched fiber fraction suggests that some other
380 component of the skin is contributing to the inhibition of TMA-d9 production.
381 AUCs of choline-d_9 and TMA-d_9 kinetics from these fermentations are presented in
382 **Figure 4C** and **4D**, respectively. Whole blueberry, pulp, and sugar treatments did not show
383 statistically significant differences from each other in choline-d_9 or TMA-d_9 AUCs. In contrast,
384 digesta-free, blank digesta, skin, and fiber treatments displayed statistically significant
385 differences in choline-d_9 and TMA-d_9 AUCs both among themselves and were significantly
386 lower than compared to whole blueberry, pulp, and sugar AUCs. Although the skin contains most
387 of the fiber, its distinct AUCs for both choline-d_9 and TMA-d_9 suggest that another component in
388 the skin contributes to the observed inhibition. In summary, this experiment shows that the
389 extreme inhibitory effects of whole blueberries are mimicked by both the sugar-rich pulp and
390 matched sugar mixture, suggesting that this inhibition is a physiologically irrelevant interference
391 due to sugar, which would not be present in the colon *in vivo*. Therefore, as our focus was on
392 indigestible, non-absorbed materials primarily in the skin, we proceeded with skin-only
393 digestions and fermentations (at doses equivalent to the amount of skin obtained from 1 serving
394 of whole blueberries).
395

3.4 Experiment 4: Determining 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. Whole blueberry concentrations of CGA from 20 blueberry genotypes (selected from previous data suggesting these accessions were either particularly high or low in CGA content) from the 2024 crop year fruit from two different populations (genetic diversity panel and DxJ bi-parental mapping population) are presented in **Figure 5** and **Table 2**. From these populations, we selected eight cultivars for fermentation based on the mean CGA content of the whole blueberries (four highest and four lowest genotypes). As skins were used for fermentation based on the interference from whole blueberry, the highest four and lowest four CGA whole blueberry genotypes used in the fermentation were analyzed for skin CGA content. Significant differences were observed in both whole fruit and skin CGA content. For whole fruit (**Figure 6A**), CGA contents in the four highest genotypes (only PI 296339 was different from DxJ 002) were significantly greater than the four lowest genotypes, which were not significantly different from each other. Significant differences were also observed in skin CGA content (**Figure 6B**), where again the four genotypes with the highest whole fruit CGA had significantly greater skin CGA than the four genotypes with the lowest whole fruit CGA. Interestingly, PI 296399 (the cultivar with the highest whole fruit CGA content), had 3-4x greater skin CGA content than any of the other three high CGA genotypes, which were all similar. The four lowest genotypes were not significantly different from each other. As the whole blueberry CGA content is thought to be mostly due to CGA in skin, we plotted correlations of whole fruit CGA as a function of skin CGA (**Figure 6C**). While a correlation was observed ($r^2 = 0.492$), this was heavily skewed by PI 296399, and the slope approached but did not reach significance ($p = 0.053$). We plotted this correlation without PI 296399 (**Supplementary Figure 15**), and the correlation was extremely strong ($r^2=0.945$ and slope $p=0.0002$). Another factor determining CGA content is average fruit size. Smaller fruits have more surface area (and thus more skin) per mass unit of whole fruit. We plotted the CGA content as a function of mean fruit size for all 20 genotypes from the 2024 crop year (**Figure 6D**). While the r^2 value was moderate (0.2175), the correlation was significant (slope $p=0.0382$). It warrants noting that fruits of all sizes (~5-20 mm mean diameter) were high CGA and low CGA genotypes. Interestingly, the distribution of mean fruit diameters of medium CGA

426 genotypes was very tight (~12-15 mm). These data suggest that both skin CGA content and fruit
427 size play a role in determining whole fruit CGA content.
428 Digestions and fermentations of the genotypes highest and lowest in CGA were carried
429 out as described above (**Supplementary Table 6**). BacTiter-Glo luminescence readings are
430 presented in **Supplementary Figure 16** for the different genotypes in the fermentations at 12 hr
431 (A) and 24 hr (B). There is no evidence suggesting that any treatment caused a cytotoxic effect
432 that would lead to the perceived effect of TMA-d_9 inhibition. All genotypes had lower viability
433 values than the controls (but no differences between each other) at 12 h. All genotypes had lower
434 viability than digesta-free, but all were similar to or higher than blank digesta, at 24 h. Kinetics
435 of choline-d_9 use and TMA-d_9 production are presented in **Figure 7A** and **7B**, respectively.
436 Digesta-free treatments exhibited the quickest choline-d_9 utilization, with choline-d_9
437 concentrations decreasing between the 4 and 6 hr mark, which is expected due to the lack of any
438 digesta or skin material to potentially inhibit the reaction. Blank digesta treatments demonstrated
439 the second quickest choline-d_9 utilization, with a significant decrease in choline-d_9 occurring
440 between the 4 and 8 hr mark. Choline-d_9 utilization was similar across all eight genotypes,
441 regardless of CGA content, with a significant decrease observed between the 8 and 12 hr mark.
442 While no differences were observed among the skins, their inhibition potential remained notably
443 higher than that of the controls, indicating that the skin still possessed inhibitory activity.
444 AUCs of choline-d_9 and TMA-d_9 are presented in **Figure 7C** and **7D**, respectively. The
445 choline-d_9 and TMA-d_9 AUCs for all genotypes were statistically higher than the digesta-free and
446 blank digesta treatment AUCs, indicating that the skins exhibited a greater inhibitory effect than
447 the controls. While there are some statistical differences in the choline-d_9 AUCs between
448 genotypes, these differences are small and do not suggest distinct patterns or a clear relationship
449 to the variations in CGA content. This is further supported by the TMA-d_9 AUCs for all
450 genotypes, which are all lower than digesta-free or blank digesta controls but not statistically
451 different from one another, indicating no difference in the inhibition potential of the skins across
452 genotypes and suggesting that CGA content does not play a significant role. However, all TMA-
453 d_9 genotypes have significantly lower AUCs than the digesta-free and blank digesta controls,
454 demonstrating that the skins exhibited inhibition potential greater than the digestive background
455 controls.

456 One confounding variable in our design is that we selected genotypes for use based on
457 whole fruit CGA content (**Figures 5** and **6A**), but we actually used skins from those genotypes
458 (**Figure 6B**) as opposed to whole fruit in the digestion and fermentation, for the reasons
459 explained above. We employed the same mass of skin from each cultivar (~20 g skin from a 150
460 g FW serving of whole fruit, based on experiments to determine the ratio of skin and pulp in
461 whole berries, **Supplementary Table 7**) despite differences in average fruit sizes between
462 genotypes (and thus different skin/pulp ratios, which would result in different amounts of skin
463 between genotypes per 150 g serving of whole berries). This could have unintentionally affected
464 the amount of CGA in each digestion and fermentation. To further assess the potential role of
465 blueberry CGA in reducing bacterial TMA production, we performed linear regression analysis
466 of choline-d_9 and TMA-d_9 AUCs (representing total choline-d_9 and TMA-d_9 utilization and
467 production, respectively) as a function of both whole fruit CGA content (**Supplementary Figure**
468 **17A-B**) and skin CGA content (**Supplementary Figure 17C-D**). Correlations of choline-d_9 and
469 TMA-d_9 AUCs with whole fruit CGA are based on the premise that the majority of the CGA in
470 the whole fruit comes from the skin, but ignore the reality that we employed skin, not whole
471 fruit, and that various blueberry genotypes have distinct sizes and thus surface (skin) areas per
472 150 g serving. Such correlations (**Supplementary Figure 17A-B**) were extremely poor, with
473 both $r^2 < 0.1$. Correlations of choline-d_9 and TMA-d_9 AUCs with skin CGA more accurately
474 reflect the experimental conditions, as equal masses of skin were used. Such correlations
475 (**Supplementary Figure 17C-D**) were stronger than for whole fruit CGA, but neither had r^2
476 >0.25. It should be noted that the distributions of skin CGA concentrations were different from
477 those of whole fruit (**Figure 6A-B**). However, our skin analysis from the eight genotypes
478 selected for fermentations confirms that there were major differences in skin CGA content
479 (**Figure 6B**), albeit not as different as amounts in whole fruit (**Figure 6A**). While whole fruit
480 CGA content was somewhat correlated to skin CGA content (r^2=0.492, **Figure 6C**), the
481 relationship is not linear. This is due to variations in both fruit size and skin CGA content
482 (**Figure 6B, D**). PI 296399 had the highest CGA content of all genotypes in both whole fruit and
483 skin (**Figure 6A, B**), but its skin CGA content was ~5x greater than that of any other genotypes.
484 In summary, these correlations show that CGA content was not a good predictor of *in vitro*
485 choline-d_9 utilization or TMA-d_9 production. Given that the AUCs and kinetics were similar for

all treatments (**Figure 7**), it is unlikely to be a compositional variable that strongly predicts differences in activity.

3.5 Experiment 5: Analysis of digesta and fermenta CGA content. Given the initial data that was not supportive of our hypothesis that differences in CGA would create a differential biological activity in the form of t inhibition of TMA-d_9 production, we next attempted to identify factors that might have resulted in a lack of difference in CGA available to interact with the bacteria in the fermentations. Factors such as bioaccessibility (digestive release from bulk blueberry into the digestive milieu) and stability (breakdown of CGA) could have altered CGA contents interacting with TMA-producing bacteria so that differences in "effective" or "apparent" CGA concentrations in digesta and fermenta were not significantly different between treatments, resulting in similar inhibition of TMA-d_9 production. To determine whether the lack of differences in TMA production-d_9 production was associated with similar amounts of CGA in each digestion and fermentation, analyses of the digesta and fermenta were performed to determine the levels of free CGA. The relative CGA levels in the digesta generally mirrored those found in the whole fruit and skin, with genotypes containing higher CGA content in the skin also containing higher levels of free CGA in the digesta (**Figure 8A**). However, bioaccessibility data (percent of free CGA from the skin released into the digesta) revealed that genotypes with lower CGA content in the skin had higher % release (but still lower absolute levels) of free CGA compared to those with higher skin CGA content (**Figure 8B**). The CGA content in the fermenta overall reflected that of the genotypes, with those having higher CGA content in the genotypes also showing higher levels of CGA in the fermenta, compared to genotypes with lower CGA content (**Figure 9**). However, there was significant variation in free CGA among the four high CGA genotypes, which did not reflect their skin CGA contents (**Figure 6B**), although PI 296399 was the highest in both. There were no differences in free CGA among the four low CGA genotypes, and all were lower than the four high CGA genotypes. The % release, or bioaccessibility, of the genotypes showed that significant CGA is present in bound forms or is not easily extractable, and a greater % release is generally obtained for low CGA genotypes as opposed to high CGA genotypes. Fermenta samples from high CGA genotypes exhibited higher CGA levels than fermenta of low CGA genotypes (**Figure 9**), although the magnitude of differences was smaller than in skins (**Figure 6B**). All fermenta samples exhibited

517 a decline in CGA levels to essentially the same levels as the fermentation progressed. It should
518 be noted that the levels of free CGA detected in fermenta of blueberry skin digesta at
519 physiologically relevant doses were 0.05-0.3 μM, which is >1000-fold lower than the minimum
520 effective dose we previously observed for CGA *in vitro* (~2 mM) [21].
521
522 **4. Discussion**
523 Given the great interest in plant bioactives for disease prevention and the complex mixture of
524 bioactive phytochemicals in most plants, there is a need to understand which bioactives are
525 essential for the observed activities of a given plant food. One approach to link specific
526 bioactives with their activities is to compare the activities of distinct genotypes of the same crop
527 with a range of levels of the chosen bioactive(s). In the present study, we attempted to determine
528 whether blueberries lower the production of the pro-atherogenic gut microbial metabolite TMA
529 *in vitro*. We also endeavored to determine whether the level of CGA in blueberries was
530 associated with TMA-lowering activity.
531 Our method employs sequential *in vitro* digestion and *ex vivo-in vitro* fecal fermentation
532 to assess how various materials influence the conversion of choline-d_9 to TMA-d_9 by gut
533 bacteria. We have previously studied isolated phenolic compounds [21,35], or foods rich in
534 phenolics and/or fiber [30,31], but not sugar. Thus, this study required modification of our standard
535 experimental workflow. Optimizing fermentation parameters for high-fiber, sugar-rich fruits was
536 crucial for identifying the source of the inhibition in blueberries. This was particularly important
537 since data from Experiment 1 (Figure 2) highlighted the significant inhibitory effects of these
538 highbush blueberry genotypes, with their inhibition potential surpassing that of any food
539 previously tested in our lab. In Experiment 2, we demonstrated that sugar mimics the near-total
540 inhibition of TMA-d_9 production seen in whole blueberry, but fiber and phenolic extract did not.
541 This suggests that sugar interferes with the assay and that the apparent total inhibition by whole
542 blueberry in this model (an initially promising finding) is not physiologically relevant.
543 Additionally, this experiment showed that major fiber components provide little activity, and
544 perplexingly, neither did a crude phenolic extract. However, these store-bought highbush
545 blueberries had relatively low CGA content compared to the high CGA genotypes used later in
546 the study (**Tables 1-2**) and may have had lower levels of other phenolics. For this reason, the
547 lack of efficacy of the phenolic fraction in these berries may not be indicative of a similar lack of

548 phenolic activity in phenolic-rich berries. In Experiment 3, we compared the activities of skin
549 and pulp to whole blueberry, sugar, and fiber. While the skin exhibited less inhibition compared
550 to the whole blueberry, pulp, and sugar treatments, it still showed greater inhibition than the fiber
551 and control treatments. Since the skin contains most of the fiber, the greater inhibition observed
552 with the skin compared to the fiber fraction suggests that another component of the skin is
553 contributing to the inhibition of TMA-d_9 production. We then postulated that, as skin contains
554 most of the phenolics, including CGA, investigating the skin of genetically diverse highbush
555 blueberry genotypes with varying CGA content could reveal whether CGA differences result in
556 activity differences. In Experiment 4, we compared skins from four high-CGA and four low-
557 CGA content blueberries from a genetically diverse group of blueberries. Inhibition potential
558 among all skins was very similar, showing more inhibition than the controls, but no significant
559 differences between each other. The average reduction in TMA-d_9 AUC was 19.4% for the eight
560 genotypes tested compared to the blank digesta. While TMA-d_9 levels from all eight genotypes
561 eventually reached the same final levels as the controls, this was delayed by ~ 4 h. *In vivo*, this 4
562 h delay is critical, as it will allow native choline to be either absorbed, metabolized to less
563 deleterious metabolites, and/or pass out of the colon. Any of these alternatives will reduce the
564 amount of TMA that is absorbed and thus the resulting TMAO concentrations in circulation. Our
565 data suggest that CGA is not likely to be the primary factor driving the inhibition in blueberries.
566 Notably, the CGA content achieved in the fermenta from physiologically relevant doses of
567 blueberry skins (Figure 9) was 1,000-fold more dilute than the minimum effective concentration
568 of CGA we previously observed for inhibiting TMA production, further confirming that CGA is
569 likely not responsible for most if not all of the inhibitory activities observed for blueberry skins.
570 Experiment 5 sought to explain the lack of observed differences based on concentrations of free
571 CGA in digesta and fermenta, as the lack of differences in TMA inhibition across genotypes may
572 be due to the lack of variation in CGA concentration once it reached the fermentation. We
573 observed that differences in CGA content in skins were reduced during digestion and further
574 reduced when added to the fermentation. While higher CGA content genotypes had higher
575 concentrations in digesta and fermenta, all concentrations were nearly the same by the 8 hr
576 fermentation mark. It is possible, therefore, that significant differences in CGA intake could be
577 physiologically similar when inhibition of TMA is needed.

578 Based on the data from these experiments, evidence suggests that blueberry skin
579 possesses a significant ability to lower the generation of pro-atherogenic TMA by gut bacteria.
580 However, our data suggest that CGA does not drive TMA-lowering activities of blueberries, nor
581 do activities of high- and low-CGA blueberries differ. Therefore, the compound(s) responsible
582 for this activity remain unknown. Despite the lack of correlation with CGA, it is intriguing that
583 skin fractions from all eight genotypes tested inhibited TMA production. Our data suggest that
584 this inhibition is not due to major blueberry skin fiber components (pectin, cellulose, and
585 hemicellulose), as fiber did not inhibit (but rather promoted) TMA production compared to
586 controls. While CGA may not be the primary determinant of TMA inhibition, the skins
587 themselves show notable inhibitory potential compared to controls. Thus, the responsible
588 compounds may be other minor fiber compounds or phenolics that we have not yet evaluated.
589 The likely candidates are anthocyanins. While we have data on the TMA-lowering potential of
590 CGA and catechins, we have not yet evaluated the activities of anthocyanins, which are abundant
591 in blueberry skins. However, identifying these compounds will require the use of berries that
592 differ vastly in anthocyanin content. As the TMA-lowering properties of the eight genotypes
593 used in this study did not differ, correlating activity with any new compositional values will
594 likely be futile (showing correlation requires differences in both dependent and independent
595 variables). Instead of focusing on identifying compositional differences and then testing activity
596 differences based on these compositional differences one by one, the best approach may be to
597 gather a large number (~50-100) berry samples from many different sources, profile their TMA-
598 lowering activities, then perform broad phenolic profiling on the 10-20% most vs. 10-20% least
599 effective samples. There are various possible mechanistic explanations for our observations that
600 blueberry skins inhibit TMA production. Broadly killing or reducing the viability of gut bacteria
601 in a non-specific fashion would lower TMA production [36]. We employed an ATP-based bacterial
602 activity assay to account for this. The data do not suggest that any component of the blueberry
603 caused cell death leading to TMA-d_9 inhibition. Furthermore, this is not a likely mechanism as
604 blueberries are widely consumed and are not associated with broad antibacterial activities or
605 adverse gut effects. Thus, the observed TMA-lowering activities are likely due to either direct or
606 indirect effects on the activity of *cutC/D*. Potential direct effects, which do not alter the
607 composition of the gut microbiome, include inhibition of choline TMA lyase or reducing its
608 expression. Indirect effects would include altering the composition of the gut microbiome by

609 reducing the viability or abundance of *cutC/D*-carrying bacteria. We have previously shown that
610 long-term blueberry supplementation alters the abundance of gut microbiota that are associated
611 with circulating TMAO in experimental mice [36]. However, given the acute nature of the present
612 study in which the fecal samples are only exposed to blueberry skins for 24 h (and most
613 inhibition is observed within 12 h), we believe that direct effects (reduced *cutC/D* expression
614 and/or inhibition of choline TMA lyase) are the most likely explanation. Both direct and indirect
615 effects are thus likely to be observed *in vivo*. Future experiments are needed to identify the
616 mechanism of action. Reduced *cutC/D* expression could be investigated with reverse-
617 transcriptase (RT)-qPCR to detect *cutC/D* mRNA or alternatively using Western blotting to
618 detect choline TMA lyase protein. These studies will likely require more enriched cultures of
619 known *cutC/D* carriers, such as *Proteus mirabilis*, as opposed to dilute *cutC/D* in fecal samples
620 [15]. Studying the inhibition of choline TMA lyase requires the elimination of all other potential
621 mechanisms by excluding viable cells. This could be done with recombinantly expressed enzyme
622 and purifying it (an approach we are currently pursuing) or using a lysed, non-viable *cutC/D*
623 carrier [15].
624 The present study has several strengths that enhance its significance and validity. First,
625 the *in vitro* digestion model incorporates physiologically relevant concentrations of oral, gastric,
626 and small intestinal enzymes along with physiological conditions designed to simulate upper gut
627 digestion. The use of physiologically relevant concentrations of test material is a key parameter
628 for maximizing the *in vivo* relevance of *in vitro* experiments. We designed our *in vitro* digestions
629 to have 1 serving of whole blueberries (or the equivalent amount of phenolic extract, fiber, sugar,
630 skin or pulp that would be obtained from 1 serving of whole blueberries) per 2000 mL total
631 upper gastrointestinal (stomach + small intestinal) volume and then scaled these ratios to our *in*
632 *vitro* digestion final volume. When transferring digesta to fermentations, we employed digesta at
633 33% of the fermentation volume. This is a conservative dilution, which still resulted in
634 significant reductions in TMA-d_9 production. However, actual concentrations of compounds that
635 reduce TMA-d_9 production in the colon may be higher than those used in this study, as a critical
636 function of the colon is to extract water from the digesta, thereby concentrating it [37]. The addition
637 of digesta material, rather than whole food, into the fermentation is more representative of a real
638 human system, as it accounts for the presence of digestive enzymes and related metabolites that
639 would be involved in the breakdown of food. However, as discussed above, one limitation of this

640 approach for macronutrient-rich foods is that sugars, amino acids and fatty acids that would be
641 absorbed *in vivo* prior to colonic fermentation are not removed in this model. The *in vitro colon-*
642 *only* model allows for the isolation of TMA production from other mechanisms that would affect
643 assessment of bioactivity, such as absorption of choline and TMA, and gut transit. The use of
644 isotopically labeled choline-d_9 substrate and measurement of TMA-d_9 reduces interference from
645 background substrates and products [21]. We employed fecal samples sourced from OpenBiome, a
646 trusted provider known for supplying samples that are screened and approved for clinical use to
647 recolonize the gut with a healthy, normal, and functioning microbiome. The donors of these
648 samples undergo screening for 30 infectious diseases, ensuring that our model starts with a
649 reliable and diverse seed of bacteria to measure TMA production. Since gut bacteria expressing
650 the *cutC*/D gene cluster can vary among individuals, pooling multiple OpenBiome fecal samples
651 ensures a more diverse population of gut bacteria for each TMA assay. The use of "blank"
652 digesta controls (containing all digestion reagents and enzymes, but no blueberry) in the
653 fermentation accounts for background inhibition and endogenous choline from the digesta.
654 Additionally, the use of an isotopically labeled substrate in fermentation ensures that the
655 metabolites of interest are accurately measured and tracked, considering the potential presence of
656 endogenous choline and TMA from the digesta. We employed sugar and fiber controls designed
657 to closely reflect the sugar and fiber composition of a serving size of blueberries. The sugar
658 mixture included sucrose, glucose, and fructose in ratios typical of those found in blueberries,
659 while the fiber blend contained hemicellulose, cellulose, and pectin in proportions similar to
660 those in a serving of the fruit. Although literature on blueberry fiber composition is limited, the
661 ratios used in these experiments were believed to accurately reflect the fiber content of
662 blueberries and closely replicate the sugar content. Finally, the key strength of this study is the
663 final experiment, in which eight genotypesof berries were assessed. Too often, experiments are
664 done in a single cultivar of unknown genetic background, and the translatability of the results to
665 consumers is unknown. The blueberries used in the final experiment represented a broad range of
666 highbush genotypes, reflecting a variety of phenolic compositions, sugar content, and fiber
667 levels. Any differences observed among these genotypes are likely due to genetic variations, as
668 they were all grown and harvested in the same climate by the USDA ARS NCGR in Corvallis,
669 OR, USA. Growing in the same climate helps eliminate potential differences caused by factors
670 like water availability, predators, or nutrient competition. The lack of variation in inhibition

potential across the peels of the four high and four low genotypes is encouraging for highbush 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.

Our findings should be considered in the context of the following limitations. This work was conducted *in vitro*, which poses limitations due to the nature of the model. The absorption and conversion of TMA to TMAO and TMAO excretion were not evaluated. These processes affect TMAO in circulation. While we believe that inhibition of TMA formation is the key mechanistic target for lowering TMA, *in vivo* studies are needed to account for all factors that control TMAO levels in circulation.

As mentioned above, another major challenge is addressing the limitations of *in vitro* digestion and fermentation when using whole fruits, as sugar is not absorbed in the model prior to fermentation. As we and others have demonstrated [34], sugar almost completely inhibits microbial TMA-d_9 generation, but this effect has little physiological relevance, as sugar would not typically be present in the colon after upper gastrointestinal digestion. Including an absorption step (such as passing digesta over Caco-2 cells in transwell inserts) between *in vitro* digestion and fecal fermentation could overcome this approach. Another potential solution to this is to use dialysis to remove sugar and other water-soluble metabolites from the digesta [38], though this would also result in the loss of water soluble but poorly absorbed phenolics like CGA. As a result of not modeling absorption, the observed inhibition of TMA-d_9 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. We clearly observed this issue in experiments with whole blueberries, pulp, and sugar. 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 skin. We elected to use fiber and phenolic-rich skin for our multi-cultivar experiment, as this material is physiologically relevant to the colon and reduces interferences. Obviously, utilizing *in vivo* studies would be a useful step in reducing sugar interferences.

The ex *vivo* fermentation model, which utilizes human fecal samples in a controlled anaerobic environment, aims to mimic the conditions of the colon during *in vivo* fermentation. 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

702 most of the TMA production is in gut microbiomes that are in a state of “dysbiosis”. TMA
703 production depends on bacteria that produce TMA lyase. By incorporating a larger variety of
704 fecal samples, including those from subjects with CVD, it may be possible to increase the
705 abundance of bacteria that metabolize choline, creating an environment that could be more
706 reflective of conditions seen in a state of "dysbiosis". TMA production from known high TMA-
707 producing donors may better reflect the conditions of the population of interest, as they likely
708 include greater abundances of bacteria known to express cut*C/D,* such as *Desulfovibrio*, *Proteus*,
709 and *Klebsiella* [24,39–43]. Additionally, fecal samples are frozen and then reanimated 12 hours
710 before fermentation using a growth media. However, some bacteria may not grow outside the
711 colon, let alone survive the freeze-thaw and reanimation process, although a cryoprotectant is
712 added to the samples. Furthermore, these samples are not fully representative of all regions of the
713 large intestine. *In vivo*, the gut microbiome is never frozen rather it is constantly active,
714 metabolizing substrates. The reanimation process may alter the behavior of the bacteria,
715 potentially causing them to act differently than they would in the colon. Finally, choline is
716 typically consumed as a part of a complex meal, not as a pure substrate as in the fermentation
717 model. The complexity of meals, which typically contains a variety of foods with different
718 molecular compositions, may impact TMA production in ways that differ from the effects
719 observed with a single food type, as used in the fermentation model.
720 The use of pooled OpenBiome fecal samples for fermentation experiments is designed to
721 create a more representative sample of the human population by incorporating a variety of
722 bacteria that reflect a broader population. This approach is beneficial for introducing bacteria
723 from individuals with diverse lifestyles, living environments, and diets. However, it does not
724 allow for an in-depth examination of the individual variations in choline metabolism to TMA.
725 Some individuals harbor high TMA-producing bacteria, while others do not. By combining these
726 samples in the fermentation, it may appear that all people have TMA-producing bacteria, when
727 the results could be a mixture of one person with high TMA-producing bacteria and others
728 without. To address this, an inter-individual study could be conducted by keeping the reanimated
729 OpenBiome fecal samples separate, with each representing an individual, while all other
730 experimental conditions remain consistent. This would allow for the comparison of the rate of
731 choline conversion to TMA across different individuals.

732 Using animal models to track the metabolism of choline, TMA, and TMAO would help
733 address the limitations associated with *in vitro* models. In an animal model, the gut microbes
734 remain alive and active, eliminating the need for reanimation. While the diet of animal models is
735 typically less diverse than that of humans, which may limit its representation of the population of
736 interest, it is easier to control and modify, allowing for more realistic incorporation of different
737 foods compared to *in vitro* experiments. *In vitro* models tend to represent acute conditions,
738 capturing short-term changes in enzyme production rather than shifts in the microbiome, due to
739 the brief observation period. Models like MiGut, SHIME, and TIM-2 represent longer time
740 frames *in vitro* and provide a more faithful simulation of the gut environment. However, they are
741 more cumbersome, costly, and have lower throughput compared to our model [44–46]. Longer *in*
742 *vivo* studies, over the course of a week or longer, would likely capture both changes in enzymatic
743 activity and microbial shifts over time. *In vivo* experiments could offer insight into how TMA
744 and TMAO are absorbed and metabolized in the body, helping to determine whether inhibiting
745 TMA also prevents the formation of TMAO or if TMA follows an alternative metabolic pathway.
746 The blueberries themselves posed several challenges. There is limited information in the
747 literature regarding the fiber content of blueberries, making it difficult to determine the exact
748 ratios of the different types of fiber present. This is likely due to the variation between highbush
749 blueberry genotypes and the lack of research on the fruit. To address this, a fiber extraction from
750 a serving size of blueberries would be necessary; however, this process is time-consuming and
751 outside the scope of this project. Removing the skin from the blueberries was also challenging
752 due to their small and delicate nature. Frozen blueberries were particularly difficult to blanch and
753 separate the peel from the pulp without issues. Peeling by hand was a time-intensive process that
754 increased the risk of contamination, either by exposing the berries to non-frozen temperatures for
755 extended periods or by accidentally leaving peel in on the pulp.
756 The CGA content was measured for the whole blueberry across 20 different highbush
757 genotypes, rather than just the skin, due to time limitations and the lengthy process of peeling the
758 berries for the extracts used in SPE and LC-MS analysis. While previous experiments have
759 shown that the majority of phenolics and CGA are contained in the peel, this was not specifically
760 confirmed for the peels used in the final experiment. As a result, it is possible that the lack of
761 differentiation in the TMA-d_9 inhibition could be due to the smaller variations in the peel across
762 all genotypes, compared to the greater differences observed in the whole fruit. The variations in

763 the fruit could be attributed to the amount of peel rather than its concentration of CGA. The fruit
764 sizes varied between genotypes, with some being much smaller than others, resulting in a higher
765 peel to pulp ratio in smaller berries. This was not accounted for in the experiment, as the amount
766 of peel used was the same for all genotypes, regardless of fruit size. As a result, the amounts may
767 not accurately reflect the actual serving size of whole berries for each specific cultivar, as each
768 fruit had a different peel-to-pulp ratio. Although the genotypes used in this project were
769 genetically diverse, they were all grown and harvested in Corvallis, OR, under the same
770 environmental conditions, thereby eliminating potential differences that may arise if they were
771 sourced from varying climates and regions in the world.
772

773 **5. Conclusions**

774 The present data suggest that although highbush blueberry skin inhibits the production of pro-
775 atherogenic TMA by human gut bacteria, CGA is not the driving factor for this activity. Given
776 the lack of available interventions to blunt TMAO production, the present data showing that
777 blueberry skins at reasonable, translatable human doses could inhibit the key step in this process
778 are provocative. Future studies are needed to elucidate the mechanism of this inhibition, identify
779 the compound(s) responsible, and assess interindividual variability in efficacy. Given the fact
780 that blueberry skins are widely available and consumed (via whole blueberries), the potential
781 translatability of this effect to humans is promising. Pilot clinical studies should be designed in
782 individuals specifically exhibiting a high TMAO production phenotype.
783

784 **Author contributions**

785 **Ashley M. McAmis:** methodology, validation, formal analysis, investigation, data curation,
786 writing - original draft, writing - review & editing, visualization; **Michael G. Sweet:**
787 methodology, formal analysis, investigation, writing - review & editing, visualization; **Sydney**
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789 **Ratliff:** investigation, writing - review & editing; **Molla Fentie Mengist:** methodology,
790 writing - review & editing; **Nahla V. Bassil:** resources, writing - review & editing; **Pon**
791 **Velayutham Anandh Babu:** conceptualization, methodology, writing - review & editing,
792 funding acquisition; **Massimo Iorizzo:** conceptualization, methodology, resources, writing -
793 review & editing, funding acquisition; **Andrew P. Neilson:** conceptualization, methodology,

formal analysis, data curation, writing - review & editing, visualization, supervision, project administration, funding acquisition.

Conflict of interest

There are no conflicts to declare

Data availability

The data supporting this article have been presented in the figures and tables (including supplementary figures and tables). Raw data are available upon request from the authors.

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References

1 Cardiovascular diseases (CVDs), https://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds), (accessed January 30, 2024).

2 What is Cardiovascular Disease?, https://www.heart.org/en/health-topics/consumer-healthcare/what-is-cardiovascular-disease, (accessed January 30, 2024).

3 Atherosclerosis - What Is Atherosclerosis?, https://www.nhlbi.nih.gov/health/atherosclerosis, (accessed January 30, 2024).
4 N. Yoshida, T. Yamashita and K. Hirata, *Diseases*, 2018, **6**, 56.
5 Y. Zhu, Q. Li and H. Jiang, *APMIS*, 2020, **128**, 353–366.
6 L. Iglesias-Carres, M. D. Hughes, C. N. Steele, M. A. Ponder, K. P. Davy and A. P. Neilson, *The Journal of Nutritional Biochemistry*, 2021, **91**, 108600.
7 R. A. Koeth, Z. Wang, B. S. Levison, J. A. Buffa, E. Org, B. T. Sheehy, E. B. Britt, X. Fu, Y. Wu, L. Li, J. D. Smith, J. A. DiDonato, J. Chen, H. Li, G. D. Wu, J. D. Lewis, M. Warrier, J. M. Brown, R. M. Krauss, W. H. W. Tang, F. D. Bushman, A. J. Lusis and S. L. Hazen, *Nat Med*, 2013, **19**, 576–585.
8 J. R. Ussher, G. D. Lopaschuk and A. Arduini, *Atherosclerosis*, 2013, **231**, 456–461.
9 S. H. Zeisel and K.-A. da Costa, *Nutrition Reviews*, 2009, **67**, 615–623.
10 Anatomy & Function of Your Portal Vein, https://my.clevelandclinic.org/health/body/25048-portal-vein, (accessed March 2, 2024).
11 X. Li, J. Hong, Y. Wang, M. Pei, L. Wang and Z. Gong, *Frontiers in Molecular Biosciences*, DOI:10.3389/fmolb.2021.733507.
12 L. Jing, H. Zhang, Q. Xiang, L. Shen, X. Guo, C. Zhai and H. Hu, *Front Cardiovasc Med*, 2022, **9**, 864600.
13 A. A. Amara and A. Shibl, *Saudi Pharm J*, 2015, **23**, 107–114.
14 H. Bodke and S. Jogdand, *Cureus*, **14**, e31313.
15 Z. Wang, A. B. Roberts, J. A. Buffa, B. S. Levison, W. Zhu, E. Org, X. Gu, Y. Huang, M. Zamanian-Daryoush and M. K. Culley, *Cell*, 2015, **163**, 1585–1595.
16 Flavin monooxygenase 3, the host hepatic enzyme in the metaorganismal trimethylamine N-oxide-generating pathway, modulates platelet responsiveness and thrombosis risk - ScienceDirect, https://www.sciencedirect.com/science/article/pii/S1538783622023698, (accessed March 27, 2024).
17 Trimethylaminuria ('fish odour syndrome'), https://www.nhs.uk/conditions/trimethylaminuria/, (accessed March 25, 2024).
18 J. Messenger, S. Clark, S. Massick and M. Bechtel, *J Clin Aesthet Dermatol*, 2013, **6**, 45–48.
19 A. C. Schmidt and J.-C. Leroux, *Drug Discovery Today*, 2020, **25**, 1710–1717.

20 L. Iglesias-Carres, M. D. Hughes, C. N. Steele, M. A. Ponder, K. P. Davy and A. P. Neilson, *The Journal of Nutritional Biochemistry*, 2021, **91**, 108600.

21 L. Iglesias-Carres, L. A. Essenmacher, K. C. Racine and A. P. Neilson, *Nutrients*, 2021, **13**, 1466.

22 Bioaccessibility and bioavailability of phenolic compounds, https://www.sciopen.com/article/10.31665/JFB.2018.4162, (accessed January 18, 2025).

23 Are you absorbing the nutrients you eat?, https://www.canr.msu.edu/news/are_you_absorbing_the_nutrients_you_eat, (accessed January 13, 2025).

24 S. Rath, B. Heidrich, D. H. Pieper and M. Vital, *Microbiome*, 2017, **5**, 54.

25 A. Rodriguez-Mateos, T. Cifuentes-Gomez, S. Tabatabaee, C. Lecras and J. P. E. Spencer, *J Agric Food Chem*, 2012, **60**, 5772–5778.

26 V. Gavrilova, M. Kajdžanoska, V. Gjamovski and M. Stefova, *J. Agric. Food Chem.*, 2011, **59**, 4009–4018.

27 M. F. Mengist, M. H. Grace, J. Xiong, C. D. Kay, N. Bassil, K. Hummer, M. G. Ferruzzi, M. A. Lila and M. Iorizzo, *Front Plant Sci*, 2020, **11**, 370.

28 A. K. Satheesh Babu, C. Petersen, L. Iglesias-Carres, H. A. Paz, U. D. Wankhade, A. P. Neilson and P. V. Anandh Babu, *BioFactors*, DOI:10.1002/biof.2014.

29 M. F. Mengist, M. H. Grace, T. Mackey, B. Munoz, B. Pucker, N. Bassil, C. Luby, M. Ferruzzi, M. A. Lila and M. Iorizzo, *Frontiers in Plant Science*.

30 L. Iglesias-Carres, K. C. Racine and A. P. Neilson, *Food Funct.*, 2022, **13**, 8022–8037.

31 L. Iglesias-Carres, A. Bruno, I. D'Antuono, V. Linsalata, A. Cardinali and A. P. Neilson, *Journal of Functional Foods*, 2023, **107**, 105674.

32 Development of a genetic framework to improve the efficiency of bioactive delivery from blueberry - PubMed, https://pubmed.ncbi.nlm.nih.gov/33057109/, (accessed January 1, 2025).

33 In vitro evidences of the globe artichoke antioxidant, cardioprotective and neuroprotective effects - ScienceDirect, https://www.sciencedirect.com/science/article/pii/S1756464623002748?via%3Dihub, (accessed January 10, 2024).

34 L. Bresciani, M. Dall'Asta, C. Favari, L. Calani, D. D. Rio and F. Brighenti, *Food Funct.*, 2018, **9**, 6470–6483.

35 L. Iglesias-Carres, E. S. Krueger, J. A. Herring, J. S. Tessem and A. P. Neilson, *J. Agric. Food Chem.*, 2022, **70**, 3207–3218.

36 A. K. Satheesh Babu, C. Petersen, L. Iglesias-Carres, H. A. Paz, U. D. Wankhade, A. P. Neilson and P. V. Anandh Babu, *BioFactors*, 2024, **50**, 392–404.

37 G. I. Sandle, *Gut*, 1998, **43**, 294–299.

38 Q. Yang, M. Van Haute, N. Korth, S. E. Sattler, J. Toy, D. J. Rose, J. C. Schnable and A. K. Benson, *Nat Commun*, 2022, **13**, 5641.

39 J. Chen, B. Yu, D. Chen, P. Zheng, Y. Luo, Z. Huang, J. Luo, X. Mao, J. Yu and J. He, *Appl Microbiol Biotechnol*, 2019, **103**, 8157–8168.

40 Z. Wang, K. Lam, J. Hu, S. Ge, A. Zhou, B. Zheng, S. Zeng and S. Lin, *Food Sci Nutr*, 2019, **7**, 579–588.

41 Anaerobic choline metabolism in microcompartments promotes growth and swarming of P roteus mirabilis - PMC, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5026066/, (accessed March 28, 2024).

42 J. Song, N. Zhou, W. Ma, X. Gu, B. Chen, Y. Zeng, L. Yang and M. Zhou, *Food Funct.*, 2019, **10**, 2947–2957.

43 Chlorogenic Acid-Induced Gut Microbiota Improves Metabolic Endotoxemia - PMC, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8716487/, (accessed March 28, 2024).

44 T. Van de Wiele, P. Van den Abbeele, W. Ossieur, S. Possemiers and M. Marzorati, in *The Impact of Food Bioactives on Health: in vitro and ex vivo models*, eds. K. Verhoeckx, P. Cotter, I. López-Expósito, C. Kleiveland, T. Lea, A. Mackie, T. Requena, D. Swiatecka and H. Wichers, Springer, Cham (CH), 2015.

45 W. A. Davis Birch, I. B. Moura, D. J. Ewin, M. H. Wilcox, A. M. Buckley, P. R. Culmer and N. Kapur, *Microb Biotechnol*, 2023, **16**, 1312–1324.

46 E. Maas, J. Penders and K. Venema, *Journal of Fungi*, 2023, **9**, 104.

Figure Captions

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915 **Figure 2.** Kinetics of TMA-d_9 production in fecal fermentations with choline-d_9: No digesta,
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937 (A) and two (PI554857, PI 554856) of the lowest CGA and two (PI296399, PI 267851) of the
938 highest CGA content blueberries from the diversity panel (B) were selected and noted with an
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945 D).

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953

954 **Figure 8.** Concentration of free chlorogenic acid (CGA) in whole *in vitro* digesta of highbush
955 blueberry skins (A) and percent CGA released (i.e. bioaccessibility) during digestion (B). Data
956 represent mean ± SEM from n=2.

957

958 **Figure 9.** Concentrations of free chlorogenic acid (CGA) in *in vitro* fecal fermenta from
959 highbush blueberry skin digesta from 0-16 h. Data represent mean ± SEM from n=3.

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961

962 **Tables**

963

Table 1. Mean and standard error of the mean (SEM) for commercially available highbush blueberry fractions: Folin Assay (n=3) and chlorogenic acid (CGA) content after solid phase extraction (SPE) and LC-MS analysis (n=2)

	Folin Assay		**SPE and LC-MS Analysis**	
Material	**mg GAE/ 100 g whole blueberry[1]**	**SEM**	**mg CGA/ 100 g whole blueberry[1]**	**SEM**
Peel	122.76	2.60	11.86	0.04
Pulp	40.77	0.17	6.32	0.50
Whole Blueberry	180.05	1.29	8.07	1.23

[1]Whole blueberry values were measured for the bottom row and calculated for the top two rows based on ratios of peel and pulp in the whole blueberry

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Table 2. Average chlorogenic acid (CGA) content in 20 highbush blueberry genotypes (*n*=4)

Genotype[1]	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

[1]DxJ: Biparental mapping population, PI: genetic diversity panel population

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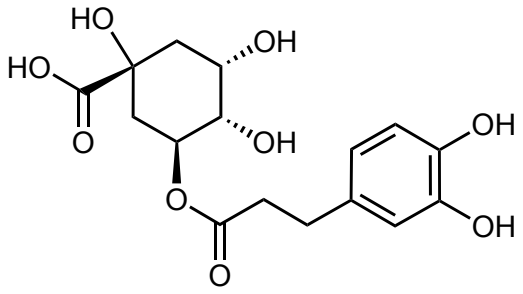
Figures



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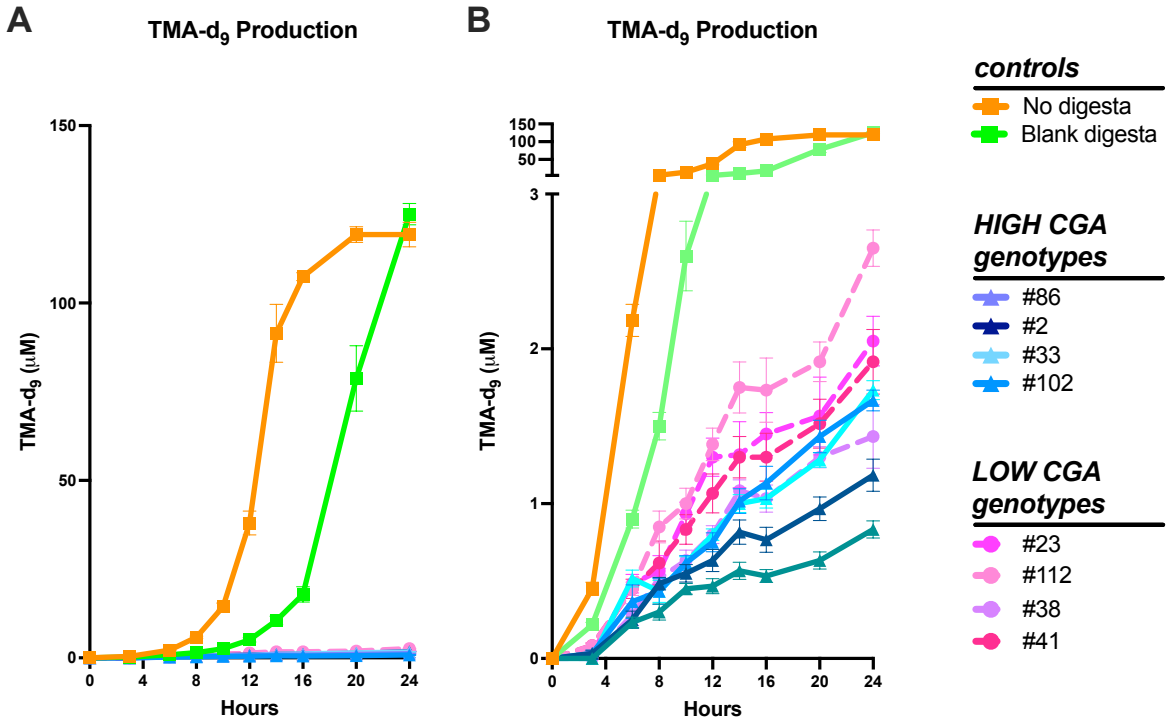

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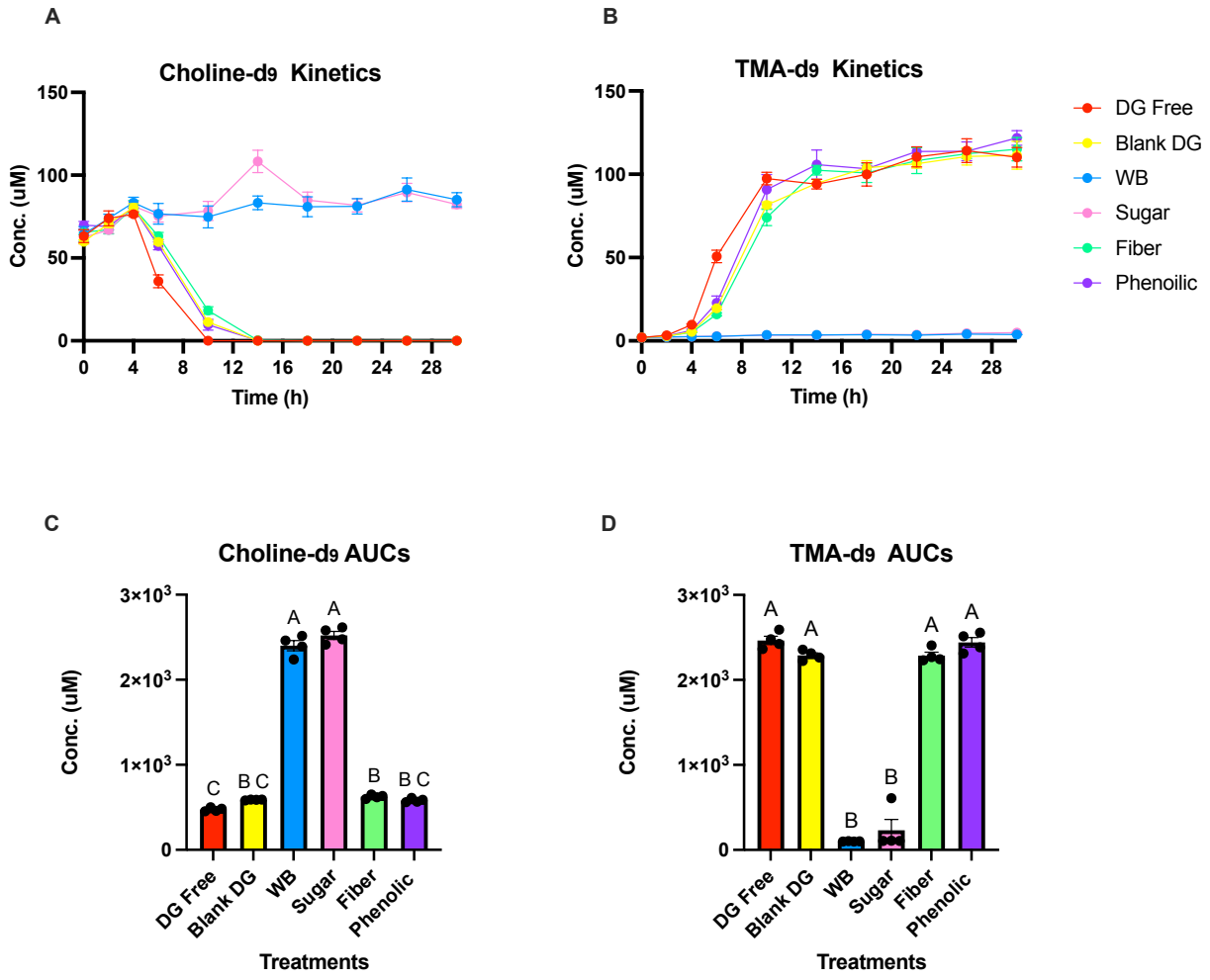

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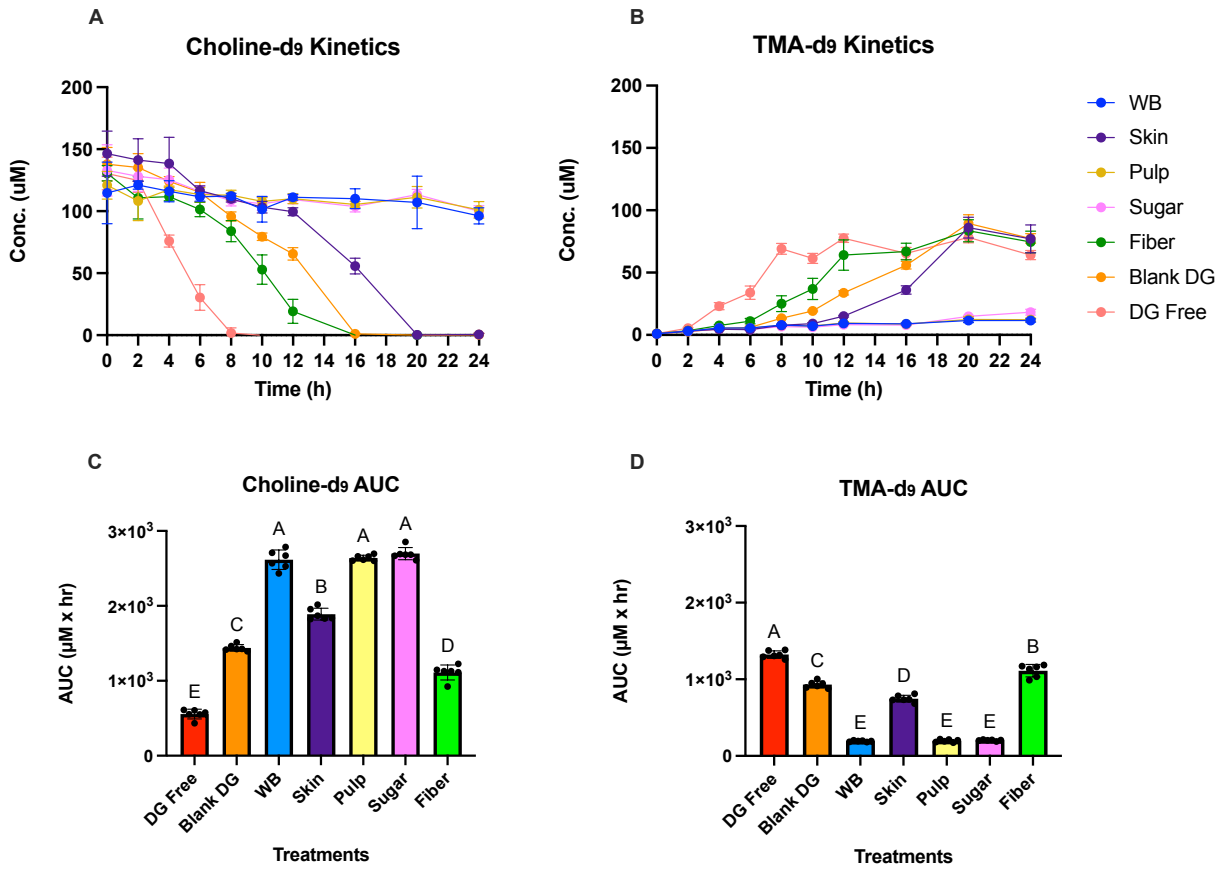

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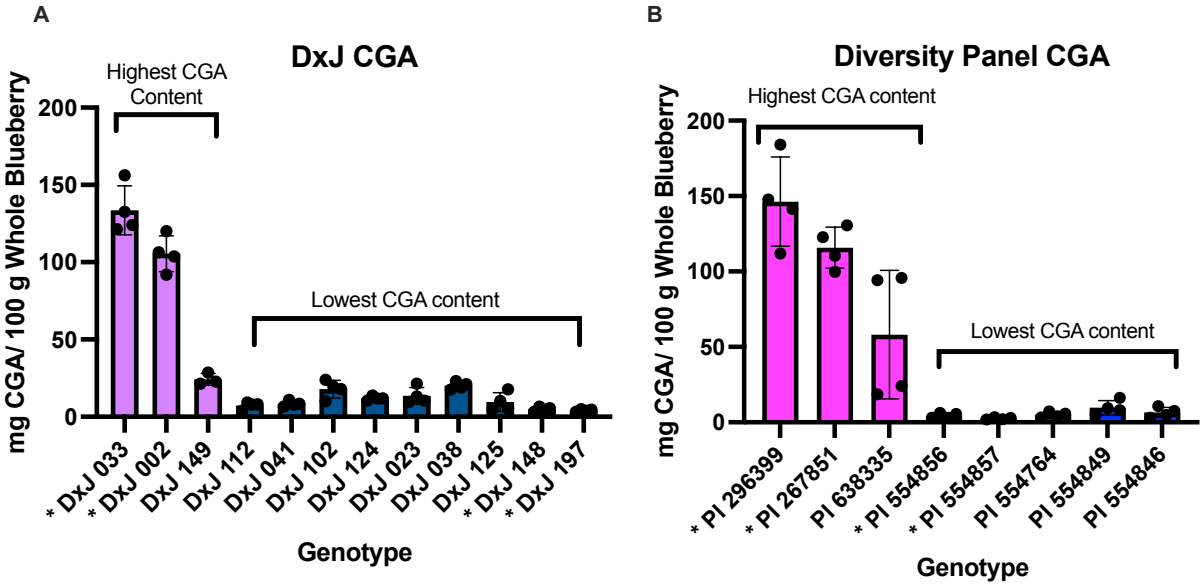

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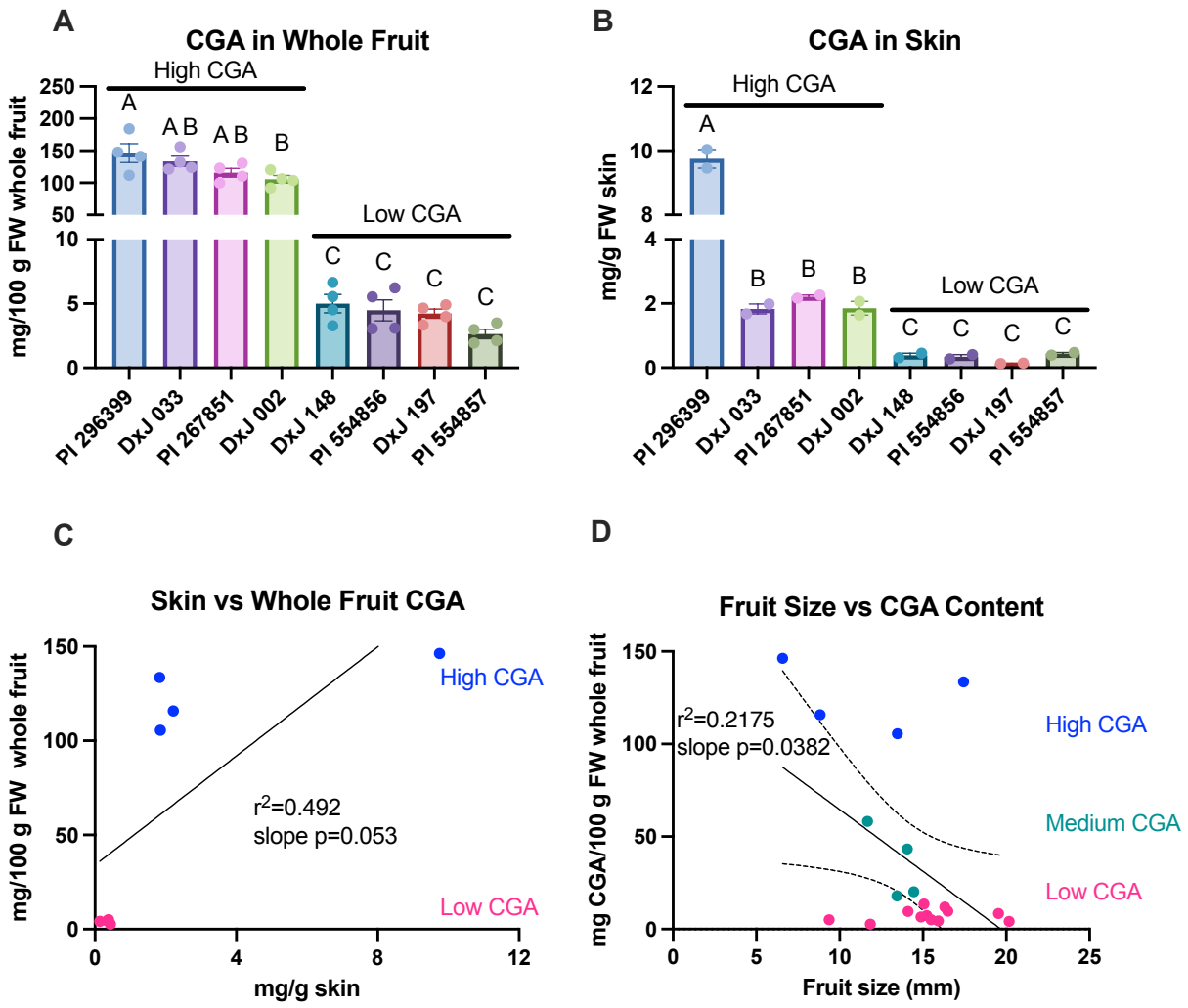

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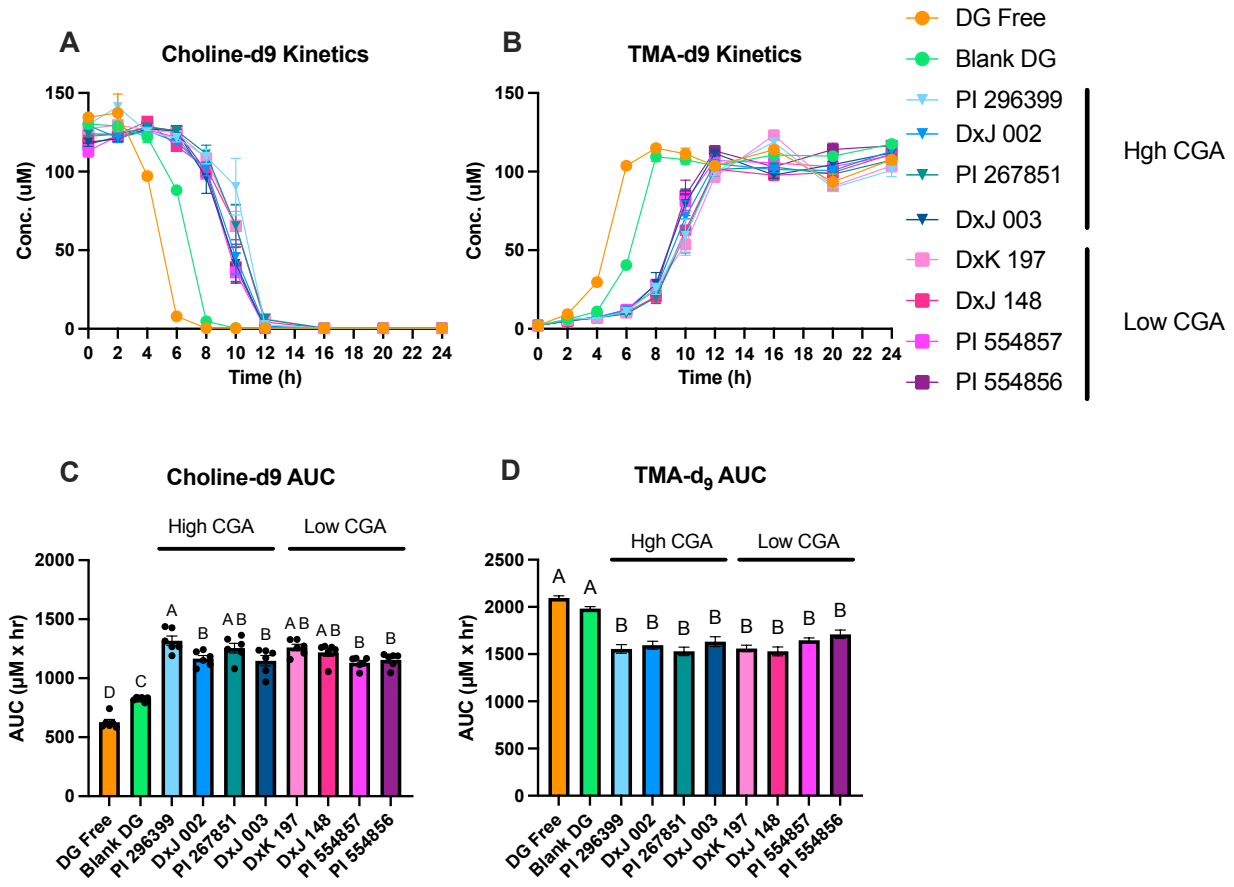

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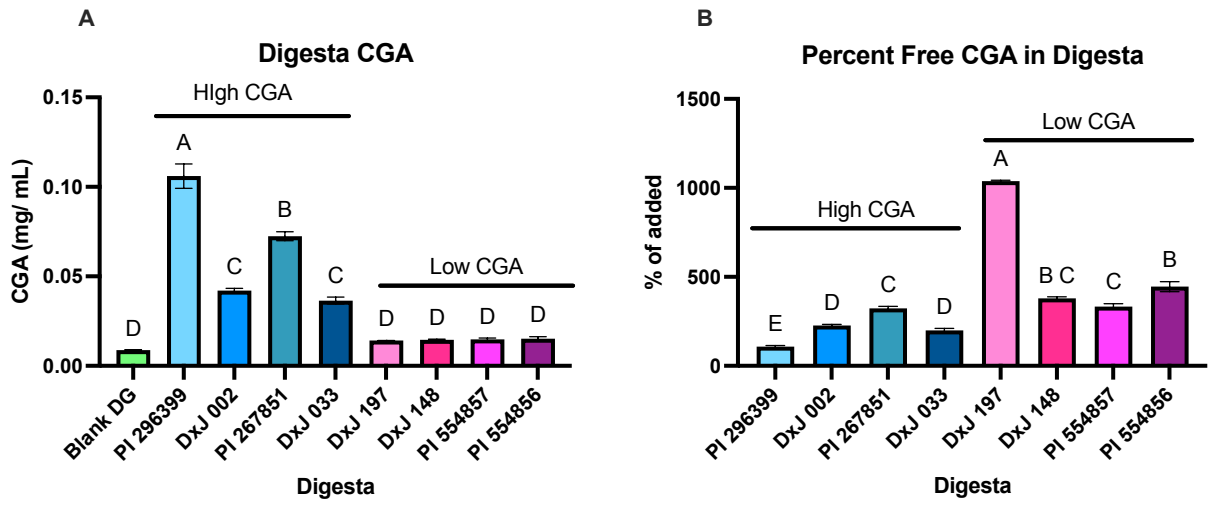

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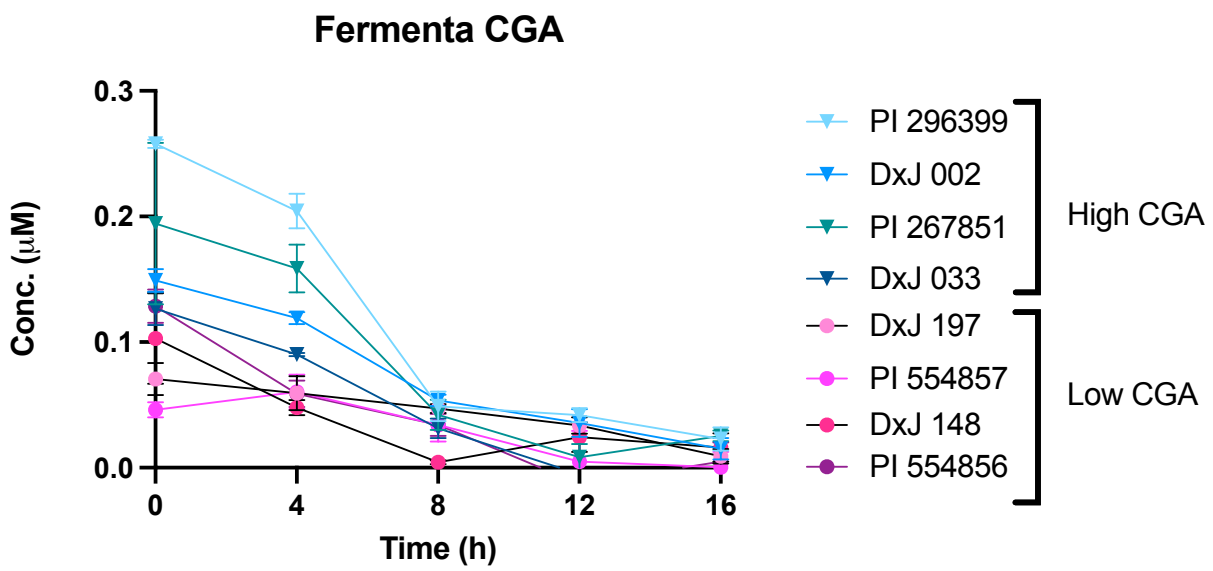

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