Inhibition of pro-atherogenic trimethylamine production from choline 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

Elevated blood levels of trimethylamine N-oxide (TMAO) are linked to increased risk of atherosclerosis. TMAO is produced when gut bacteria metabolize quaternary amines such as choline to trimethylamine (TMA), which is converted to TMAO in the liver. Chlorogenic acid (CGA), a phenolic abundant in blueberries, inhibits TMA production. Blueberries may be a TMA- (and TMAO)-lowering food. CGA content in blueberries varies significantly. It remains unclear whether variations in CGA levels influence the TMA-lowering activity of different cultivars. We investigated the impact of blueberry CGA content on inhibition of choline-d_9 conversion to TMA-d_9 in our upper gastrointestinal and *in vitro* human fecal model. Preliminary experiments indicated near-total inhibition of TMA-d_9 production when whole blueberries were tested. Blueberry pulp and sugars recapitulated this complete inhibition, whereas blueberry skins and a fiber had more moderate inhibition. We proceeded with skins (to avoid interferences from sugar-rich pulp, which would not be present in the colon *in vivo*) from 20 highbush blueberry genotypes, chosen for extremes in CGA content. CGA in whole berries was 2.6-146 mg/100 g fresh weight, while CGA in skins was 13.6-975 mg/100 g fresh weight. No 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 when skin digesta were fermented with choline-d_9. However, significant differences were observed between all genotypes compared to blank digesta, with ~19.4.% reduction in TMA-d_9 AUCs, indicating that skins provides similar TMA-lowering benefits across genotypes. Levels of free CGA in fermenta of skin digesta were 0.05-0.3 μM, >1000-fold lower than the minimum effective dose we observed for pure CGA *in vitro*, suggesting that blueberry CGA content is not a crucial factor for lowering TMA. Fiber also does not account for most of the inhibitory activity of blueberry skins. Studies are needed to confirm this *in vitro* study and understand how blueberries inhibit TMA and potentially TMAO production *in vivo*.

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) from choline. 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

Due to the large number of different methods used in this study, we have provided complete and exhaustive methodological detail for all methods in **Supplementary Information.**

2.1 Chemicals: a full list of reagents and materials can be found in **Supplementary Information**. Fecal samples were obtained from OpenBiome (Cambridge, MA, USA). TMA- d_9 (CAS# 18856-86-5) was obtained from MilliporeSigma (Burlington, MA). 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 (Tewksbury, MA).

2.2 Blueberry Sources: Commercially available frozen (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 in March 2024, and frozen immediately at −80°C. A 1:1 mixture of these store-bought berries was used for method development, to represent a composite sample of typical blueberries available to consumers for the initial experiments while preserving the limited quantities of blueberry material from the unique genotypes with varying CGA levels for later experiments. Fruits from various blueberry genotypes were obtained from the 2019 and 2024 harvest of a diverse field collection through the United States Department of Agriculture Agricultural Research Service (USDA ARS) National Clonal Germplasm Repository (NCGR), Corvallis OR, USA. Fruit from a bi-parental population (Draper x Jewel, labled as DxJ) were obtained from plants grown by the USDA-ARS Berry Breeding Program at the Corvallis-based Horticultural Crops Production and Genetic Improvement Research Unit (HCPGIRU). Fruits from these two distinct populations were obtained: (1) accessions (labeled as Plant Introduction, PI) belonging to a diversity panel of cultivars/selections where the plants were not directly related and did not share genetic material from the same immediate parental sources, and (2) genotypes belonging to the DxJ bi-parental mapping population where plants shared genetic material from the same parental sources. The accessions and the DxJ genotypes were selected based on chlorogenic acid content evaluated in previous work [29] and verified in this study (see results). Also the genotypes from the mapping population were selected based on genotypic data, representing the dominant and recessive allele

149 at a major genetic locus associated with chrologenic acid content [29]. Blueberries were frozen,
150 shipped overnight on dry ice, and stored at −80°C immediately upon receipt prior to use.
151
152 *2.3 General Gastrointestinal Digestion:* Simulated *in vitro* upper gastrointestinal digestions were
153 performed to mimic oral, gastric, and small intestinal digestion following the methodology
154 described by Iglesias-Carres *et al.* [30,31]. Digestions were scaled from ~1 serving [150 g fresh
155 weight (FW)] in estimated human upper gastrointestinal volume (2 L) to an equivalent dose for
156 the *in vitro* digestion final volume (15 or 50 mL). "Blank" or "control" digesta was made with
157 saline in place of food material and treated the same as the other treatments. Further details of
158 digestion methods can be found in the **Supplementary Information.**
159
160 *2.4 Anaerobic Fecal Fermentation*
161
162 *2.4.1 Growth Media Preparation:* Anaerobic growth media were prepared following the
163 methodology described by Iglesias-Carres *et al.* [21]. Further information can be found in the
164 **Supplementary Information.**
165
166 *2.4.2 Fecal Slurry preparation:* De-identified fecal samples from healthy donors were obtained
167 through OpenBiome (Cambridge, MA, USA) and stored immediately at −80°C. Fecal slurries
168 were prepared 12 hrs before the start of fermentations (concentrations varied based on
169 experimental parameters). At least two different fecal samples were combined in a ratio of 1 mL
170 fecal sample to 9 mL anaerobic growth media and vortexed until homogenized. The fecal slurry
171 was left uncapped in the anaerobic chamber for at least 12 hrs prior to fermentations. Further
172 details can be found in the **Supplementary Information.**
173
174 *2.4.3 General Fermentation Conditions:* Fermentations took place inside an 855-ACB anaerobic
175 chamber (Plas-Labs, Lansing, MI, USA). The chamber was first purged of oxygen using N_2
176 (Airgas, Charlotte, NC, USA) and then mixed gas containing 5% H_2, 5% CO_2 and 90% N_2
177 (ARC3, Raleigh, NC, USA) until chamber conditions reached H_2 (2-3%) and O_2 levels below 20
178 ppm monitored with a CAM-12 anaerobic monitor (Coy Lab Products, Grass Lake, MI, USA).
179 Once anaerobic conditions were achieved, the heater inside the chamber was turned on to 37°C,

180 and a palladium catalyst (to consume residual O_2) was placed on top before leaving it to stabilize
181 overnight. Conditions were monitored throughout fermentations. Further information can be
182 found in the **Supplementary Information.**
183
184 *2.4.4 General Fermentation Procedure:* Fermentations were carried out per our established
185 methodology [21]. We focused exclusively on TMA production from choline using isotopically
186 labeled substrate to eliminate interference by endogenous TMA-lyase substrates. The anaerobic
187 chamber was prepared as described above. Choline-d_9 was added at a final concentration of 100
188 μM in all experiments, and choline-d_9 utilization and TMA-d_9 production were measured.
189 Digested samples were prepared as described in general gastrointestinal digestions, lyophilized
190 (to remove O_2) and reconstituted to 1X digesta concentration using filter-sterilized, overnight-
191 sparged PBS 1X inside the chamber. In 1.1 mL 96-well plates, 750 μL of growth media were
192 mixed with 90 μL of choline-d_9 stock solution (2 mM) in PBS 1X, 600 μL of reconstituted
193 digested samples and 360 μL fecal slurry (1:10 in PBS 1X). Final concentrations were thus 100
194 μM choline-d_9, 2% fecal matter, and 33.3 % digesta. Samples containing no digesta were brought
195 to volume with 600 μL of PBS 1X. The start of fermentation (time 0 h) began when fecal slurry
196 was inoculated into the reaction mixture with the substrate. From 0 up to 30 h, a 100 μL sample
197 was collected at various time points, combined with 100 μL of acetonitrile (ACN), and frozen
198 immediately at −80°C. Further details can be found in the **Supplementary Information.**
199
200 *2.8 Phenolic Fraction Extraction:* A phenolic extraction was performed to generate a blueberry
201 fraction representing phenolic compounds in an average serving of blueberries (~150 g) for use
202 in fermentation. Whole blueberries (1:1 mix of locally available store-bought fresh and frozen
203 berries) were homogenized, weighed, lyophilized, weighed and frozen at −80°C. Lyophilized
204 material representing a serving size of fresh weight blueberry was combined with an extraction
205 mixture containing acetone, water, and acetic acid (70:29.5:0.5) and vortexed, blended for 1 min
206 using a polytron (VWR 200), sonicated for 5 min in a water bath at 50 °C, and centrifuged (3248
207 x g, 10 min). The steps were repeated seven times until the supernatant was clear. The
208 supernatant was collected from the extractions, pooled, and concentrated using a rotary
209 evaporator at 45 °C. The extract was frozen at −80°C and lyophilized. The lyophilized extract
210 was weighed and resuspended in acidified mill-Q water (0.1% formic acid). The resuspended

211 extract material was eluted using Diaion HP-20 resin and a 24/40 column to remove and purify
212 phenolic compounds, then transferred into a round-bottom flask and concentrated using a rotary
213 evaporator. The extract material was frozen at −80°C and lyophilized. Further information can be
214 found in the **Supplementary Information.**
215
216 *2.9 Plant Phenolic Extraction and Analysis:* Extraction of blueberry material (skin, pulp and
217 whole fruit) was performed. The Folin assay was carried out to estimate total phenolics in the
218 plant extracts. To quantify the relative amounts of CGA in plant material, a solid phase extraction
219 (SPE) followed by analysis using UPLC-MS/MS was utilized following the methodology
220 described by Mengist *et al*.[32]. Further information can be found in the **Supplementary**
221 **Information.** CGA MS/MS parameters and a representative UPLC-MS/MS chromatogram are
222 shown in **Supplementary Table 1** and **Supplementary Figure 1**, respectively.
223
224 *2.10 Digesta and Fermenta CGA Analysis:* Digesta samples were combined 1:1 with 0.1%
225 formic acid in ACN, vortexed, sonicated for 5 min, then centrifuged (10 min at 17,000 x g). The
226 supernatant was filtered through a 0.2-micron PTFE filter before LC-MS analysis. Fermenta
227 samples were combined 1:1 with ACN after sample collection during fermentation. To acidify
228 these samples, 10 µL of 0.425% formic acid in ACN was mixed with 75 µL of the fermenta
229 sample. Samples were filtered through AcropreAdv 0.2 µm WWPTFE 96-well filtering plates
230 (Pall Corporation, Port Washington, NY, USA) using centrifugation (10 min at 3428 x g) before
231 LC-MS analysis. Further information can be found in **Supplementary Information.**
232
233 *2.11 BacTiter-Glo ATP Assay:* Bacterial viability was quantified by luminescence using a
234 commercial BacTiter-Glo Microbial Cell Viability Assay (Promega, USA). The reagent was
235 prepared by mixing equal amounts of the provided BacTiter-Glo substrate and BacTiter-Glo
236 buffer and leaving to rest for at least 15 min at room temperature. A total of 100 µL of the
237 fermenta sample was plated on a white 96-well opaque round-bottom plate and centrifuged (500
238 x g, 5 min) to remove interfering material. The pellet was resuspended in 100 µL of PBS 1X,
239 combined with 100 µL BacTiter-Glo reagent, and covered for 5 min before being placed in the
240 plate reader (SpectraMax iD3, Molecular Devices, USA). Luminescence was read (Shake: 10s,
241 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. One-way ANOVA was used for experiments with one independent variable (treatment effects). Two-way ANOVA was used for experiments with multiple independent variables, as well as choline-d_9 and TMA-d_9 kinetic curves (significant main effect/interaction). Mixed effects models were used instead of 2-way ANOVA in the event of missing values. If a significant treatment effect (1-way ANOVA) or main effect/interaction (2-way ANOVA) ($P < 0.05$) was noted, Tukey post hoc test or Sidak's multiple comparison test was used to assess the significance of the difference between means (in the case of 2-way ANOVA, or mixed effects models, one family per variable). If kinetic curve calculated values were negative, a zero was imputed for the areas-under-the-curve (AUCs) calculations. Significance was defined *a priori* as $\alpha < 0.05$.

3. Results

3.1 Experiment 1: Determination of TMA-d_9 lowering activities of blueberries with high and low CGA content. Based on previously reported CGA content in the DxJ blueberry bi-parental genetic mapping population (2017-2019 crop years, **Supplementary Figure 12**), eight blueberry

genotypes with large differences in CGA content were selected [27]. Four high CGA genotypes (170-250 mg/100 g FW) containing the dominant allele were compared to four low CGA genotypes (17-32 mg/100 g FW) containing the recessive allele were selected for further study. Simulated digestion was performed at a dose equivalent to 133 g of fresh blueberry consumed by an adult human. Digesta were diluted 3X and fermented for 24 hrs with fecal bacteria and choline-d_9. Near-complete inhibition of TMA-d_9 production was observed by all eight genotypes (**Figure 2A**), which we have not previously observed with coffee, tea, cocoa [30], artichokes or inulin [31]. Close examination of TMA-d_9 appearance kinetics (**Figure 2B**) suggests that differences between CGA genotypes may be present, which might increase if the assay were extended. Components such as sugar or fiber may also have reduced TMA-d_9 production *in vitro* [30,34]. Previous work by Bresciani *et al.* showed that sugar, which would not be present in the colon after upper gastrointestinal absorption, can significantly inhibit TMA formation *in vitro* [34]. This would be experimental interference, rather than a physiologically relevant effect.

3.2 Experiment 2: Determination of the impacts of CGA and major fruit components (sugar, fiber, skin, pulp) of blueberries on TMA production. We postulated that sugar, from blueberry pulp, may be responsible for the near-total inhibition of TMA-d_9 production by blueberries in Expeiment 1. Given the nature of our *in vitro* digestion model, removing sugar is not possible without also eliminating other water-soluble compounds, such as CGA, that are not well-absorbed (and would thus be present in the colon *in vivo*). To identify potential blueberry interferences, whole blueberry, sugar (mixture of glucose, fructose, and sucrose), fiber (mixture of cellulose, hemicellulose and pectin), and phenolic fractions (each matching the amount and composition in the whole berry, **Supplementary Table 4**) were digested and used in an extended fermentation (30 h). Further information can be found in the **Supplementary Information.**

BacTiter-Glo measurement of viable bacteria is presented in **Supplementary Figure 13A**. There is no evidence of broad cytotoxicity, but there were differences between treatments. Kinetics of choline-d_9 use and TMA-d_9 production from the fermentations are presented in **Figures 3A-B**, respectively. Whole blueberry digesta and sugar fraction digesta treatments exhibited essentially no choline-d_9 breakdown or TMA-d_9 production, as seen in areas-under-the-curve (AUCs) of the choline-d_9 (**Figure 3C**) and TMA-d_9 (**Figure 3D**) kinetics. This supports our hypothesis that sugar in the whole blueberry is responsible for the observed near-total TMA-

304 d_9 inhibition, as sugar (matched in content and composition) mimicked the behavior of whole
305 blueberry. Fiber (which would be present in the colon *in vivo*) did not affect choline-d_9 use or
306 TMA-d_9 production, suggesting that fiber does not interfere with the assay.
307
308 *3.3 Experiment 3: Identification of blueberry components with the greatest TMA inhibition*
309 *potential:* We attempted to further identify blueberry fractions with the most significant TMA-d_9
310 lowering effect. We unsuccessfully attempted to remove the sugar post-digestion using sucrose,
311 fructose, and glucose degrading enzymes (data not shown). We elected not to pursue this course
312 further due to the unknown interferences that might arise from sugar degradation products. We
313 then broke the whole blueberry into skin (fiber- and phenolic-rich) vs. pulp (sugar-rich) fractions
314 and compared their inhibitory effects to those of the sugar mixture (glucose, fructose, and
315 sucrose, matching the ratio and content in whole blueberry) and whole blueberry
316 (**Supplementary Table 5**). Total phenolics and CGA content analysis of whole blueberry, skin,
317 and pulp fractions (**Table 1**) confirmed that most phenolics and CGA are located in the skin, as
318 expected. BacTiter-Glo data for blueberry fraction fermentations are presented in
319 **Supplementary Figure 14**. Again, there is no evidence of broad cytotoxicity, but there were
320 differences between treatments. WB and pulp had significantly higher values at both 12 and 24 h.
321 It is interesting that whole blueberry and pulp, but not sugar, increased viable bacterial loads.
322 Kinetics of choline-d_9 use and TMA-d_9 production are presented in **Figure 4A-B** for
323 fermentations. Whole blueberry, pulp, and sugar demonstrated near-total inhibition of TMA-d_9
324 production. Digesta free, blank digesta, fiber, and skin treatments were significantly different
325 from whole blueberry, pulp, and sugar treatments, and each other. Digesta-free treatments
326 exhibited the quickest choline-d_9 utilization. Fiber digesta demonstrated the second quickest
327 choline-d_9 utilization. Blank digesta was similar to fiber. Skin digesta treatments exhibited low
328 choline-d_9 utilization, though not to the same extent as the whole blueberry, pulp, and sugar
329 treatments. Since the skin contains most of the fiber, the stronger inhibition observed with the
330 skin digeta compared to the matched fiber digesta suggests that some other skin component
331 contributes to inhibition of TMA-d_9 production.
332 AUCs of choline-d_9 and TMA-d_9 are presented in **Figure 4C** and **4D**, respectively. Whole
333 blueberry, pulp, and sugar digesta were not statistically different from each other for choline-d_9
334 or TMA-d_9. In contrast, digesta-free, blank, skin, and fiber digesta displayed statistically

335 significant differences in choline-d_9 and TMA-d_9 among themselves and were significantly lower
336 compared to whole blueberry, pulp, and sugar digesta. Although the skin contains most of the
337 fiber, distinct AUCs for choline-d_9 and TMA-d_9 for skin vs. fiber digesta suggest that a non-fiber
338 component in skin contributes to the observed inhibition. This experiment shows that the
339 extreme inhibitory effects of whole blueberries are mimicked by sugar-rich pulp and matched
340 sugar mixture, strongly suggesting a physiologically irrelevant interference due to sugar, which
341 would not be present in the colon *in vivo*. Therefore, we proceeded with skin-only digestions and
342 fermentations (at doses equivalent to the amount of skin obtained from 1 serving of whole
343 blueberries) to focus on indigestible, non-absorbed materials primarily in the skin.
344
345 *3.4 Experiment 4: Determining whether CGA content correlates with reduced TMA production in*
346 *multiple blueberry accessions from a genetic diversity population with a large spread of CGA in*
347 *our ex vivo-in vitro human fecal fermentation model.* Whole blueberry concentrations of CGA
348 from 20 blueberry genotypes (selected from 2017-19 data suggesting theywere particularly high
349 or low in CGA content) from the 2024 crop year fruit from two different populations (genetic
350 diversity panel and DxJ bi-parental mapping population) are presented in **Figure 5** and **Table 2**.
351 We selected eight genotypes for fermentation based on the mean CGA content of the whole
352 blueberries (four highest and four lowest genotypes). As skins were used for fermentation based
353 on Experiments 1-3, the eight selected genotypes were analyzed for skin CGA content. For
354 whole fruit (**Figure 6A**), CGA contents in the four highest genotypes (only PI 296339 was
355 different from DxJ 002) were significantly greater than the four lowest genotypes, which were
356 not different from each other. Differences were also observed in skin CGA content (**Figure 6B**),
357 where again the four genotypes with the highest whole fruit CGA had significantly greater skin
358 CGA than the four genotypes with the lowest whole fruit CGA. Interestingly, PI 296399 (the
359 cultivar with the highest whole fruit CGA content), had 3-4x greater skin CGA content than any
360 of the other high CGA genotypes, which were similar. The four lowest genotypes were not
361 different from each other. As the whole blueberry CGA content is thought to be mostly due to
362 CGA in skin, we plotted correlations of whole fruit CGA as a function of skin CGA (**Figure 6C**).
363 While a correlation was observed ($r^2 = 0.492$), it was skewed by PI 296399, and the slope
364 approached but did not reach significance ($p = 0.053$). Without PI 296399 (**Supplementary**
365 **Figure 15**), the correlation was strong ($r^2=0.945$, slope $p=0.0002$). Another factor determining

366 CGA content is average fruit size. Smaller fruits have more surface area (and thus more skin) per
367 mass unit of whole fruit. We plotted the CGA content as a function of mean fruit size for all 20
368 genotypes from the 2024 crop year (**Figure 6D**). While r^2 was moderate (0.2175), the correlation
369 was significant (slope p=0.0382). It warrants noting that fruits of all sizes (~5-20 mm mean
370 diameter) were high CGA and low CGA genotypes. Interestingly, the distribution of fruit
371 diameters of medium CGA genotypes was tight (~12-15 mm). These data suggest that skin CGA
372 content and fruit size determine whole fruit CGA content.

373 Digestions and fermentations of these eight genotypes were carried out (**Supplementary**
374 **Table 6**). BacTiter-Glo data are presented in **Supplementary Figure 16**. There is no evidence
375 suggesting that any treatment caused a cytotoxic effect that would lead to the perceived effect of
376 TMA-d_9 inhibition. All genotypes had lower viability values than the controls (but no differences
377 between each other) at 12 h. All genotypes had lower viability than digesta-free, but all were
378 similar to or higher than blank digesta, at 24 h.

379 Kinetics of choline-d_9 use and TMA-d_9 production are presented in **Figure 7A-B**,
380 respectively. Digesta-free treatments exhibited the quickest choline-d_9 utilization, due to the lack
381 of digesta or skin material to inhibit the reaction. Blank digesta demonstrated the second quickest
382 choline-d_9 utilization. Enexpectedly, choline-d_9 utilization was similar across all eight genotypes,
383 regardless of CGA content, with a significant decrease between 8 and 12 hr. While no differences
384 were observed among skins, their inhibition was higher than the controls.

385 AUCs of choline-d_9 and TMA-d_9 are presented in **Figure 7C-D**, respectively. Choline-d_9
386 AUCs again indicate that skins exhibited a greater inhibitory effect than controls. While there are
387 some differences in choline-d_9 AUCs between genotypes, these are small and do not suggest a
388 clear relationship to CGA content. TMA-d_9 AUCs for all genotypes are all lower than controls
389 but not different from one another, suggesting that CGA content does not play a significant role
390 but that all skins exhibited inhibition potential greater than the digestive background.

391 One confounding variable in our design is that we selected genotypes based on whole
392 fruit CGA content (**Figures 5** and **6A**), but we actually used skins from those genotypes (**Figure**
393 **6B**) as opposed to whole fruit in the digestion and fermentation, for the reasons explained above.
394 We employed the same mass of skin from each cultivar (~20 g skin from a 150 g FW serving of
395 whole fruit, based on experiments to determine the ratio of skin and pulp in whole berries,
396 **Supplementary Table 7**) despite differences in average fruit sizes between genotypes (and thus

397 different skin/pulp ratios, which would result in different amounts of skin between genotypes per
398 150 g serving of whole berries). This could have unintentionally affected the amount of CGA in
399 each digestion and fermentation. To further assess the potential role of blueberry CGA in
400 reducing bacterial TMA production, we performed linear regression analysis of choline-d_9 and
401 TMA-d_9 AUCs (representing total choline-d_9 and TMA-d_9 utilization and production,
402 respectively) as a function of both whole fruit CGA content (**Supplementary Figure 17A-B**) and
403 skin CGA content (**Supplementary Figure 17C-D**). Correlations of choline-d_9 and TMA-d_9
404 AUCs with whole fruit CGA are based on the premise that the majority of the CGA in the whole
405 fruit comes from the skin, but ignore the reality that we employed skin, not whole fruit, and that
406 various blueberry genotypes have distinct sizes and thus surface (skin) areas per 150 g serving.
407 Such correlations (**Supplementary Figure 17A-B**) were extremely poor, with both $r^2 < 0.1$.
408 Correlations of choline-d_9 and TMA-d_9 AUCs with skin CGA more accurately reflect the
409 experimental conditions, as equal masses of skin were used. Such correlations (**Supplementary**
410 **Figure 17C-D**) were stronger than for whole fruit CGA, but neither had $r^2 > 0.25$. It should be
411 noted that the distributions of skin CGA concentrations were different from those of whole fruit
412 (**Figure 6A-B**). However, our skin analysis from the eight genotypes selected for fermentations
413 confirms that there were major differences in skin CGA content (**Figure 6B**), albeit not as
414 different as amounts in whole fruit (**Figure 6A**). While whole fruit CGA content was somewhat
415 correlated to skin CGA content (r^2=0.492, **Figure 6C**), the relationship is not linear. This is due
416 to variations in both fruit size and skin CGA content (**Figure 6B, D**). PI 296399 had the highest
417 CGA content of all genotypes in both whole fruit and skin (**Figure 6A, B**), but its skin CGA
418 content was ~5x greater than that of any other genotypes. In summary, these correlations show
419 that CGA content was not a good predictor of *in vitro* choline-d_9 utilization or TMA-d_9
420 production. Given that the AUCs and kinetics were similar for all treatments (**Figure 7**), it is
421 unlikely to be a compositional variable that strongly predicts differences in activity.
422
423 *3.5 Experiment 5: Analysis of digesta and fermenta CGA content.* As our data do not support the
424 hypothesis that differences in CGA result in differences in inhibition of TMA-d_9 production, we
425 next attempted to identify factors that might have resulted in a lack of difference in CGA
426 available to interact with the bacteria in the fermentations. Bioaccessibility (digestive release
427 from bulk blueberry) and stability factors could have altered the amount of CGA free to interact

428 with TMA-producing bacteria so that differences in apparent CGA concentrations in digesta and
429 fermenta were not significantly different between treatments. Analyses of digesta and fermenta
430 were performed to determine the levels of free CGA. The relative CGA levels in digesta
431 generally mirrored those in whole fruit and skin, with high CGA genotypes containing more free
432 CGA in the digesta (**Figure 8A**). However, bioaccessibility data (percent of free CGA from the
433 skin released into the digesta) revealed that genotypes with lower skin CGA content had higher
434 % release (but still lower absolute levels) of free CGA compared to those with higher skin CGA
435 content (**Figure 8B**). CGA in the fermenta overall reflected that of the genotypes, with those
436 having higher CGA content also showing higher levels of CGA in fermenta, compared to
437 genotypes with lower CGA content (**Figure 9**). However, there was significant variation in free
438 CGA among the four high CGA genotypes, which did not reflect their skin CGA contents
439 (**Figure 6B**), although PI 296399 was the highest in both. There were no differences in free CGA
440 among the four low CGA genotypes, and all were lower than the four high CGA genotypes. The
441 % release, or bioaccessibility, of the genotypes showed that significant CGA is present in bound
442 forms or is not easily extractable, and a greater % release is generally obtained for low CGA
443 genotypes as opposed to high CGA genotypes. Fermenta samples from high CGA genotypes
444 exhibited higher CGA levels than fermenta of low CGA genotypes (**Figure 9**), although the
445 magnitude of differences was smaller than in skins (**Figure 6B**). All fermenta samples exhibited
446 a decline in CGA levels to essentially the same levels as fermentation progressed. Levels of free
447 CGA detected in fermenta of skin digesta were 0.05-0.3 μM, >1000-fold lower than the
448 minimum effective dose we observed for pure CGA *in vitro* (~2 mM) [21].
449

450 **4. Discussion**

451 Given the interest in plant bioactives for disease prevention and the mixtures of bioactive
452 phytochemicals in most plants, there is a need to understand which bioactives are essential for
453 the observed activities of a given plant food. One approach to link bioactives with activities is to
454 compare distinct genotypes of the same crop with a range of levels of the bioactive. In the
455 present study, we attempted to determine whether blueberries lower production of the pro-
456 atherogenic microbial metabolite TMA *in vitro*, and whether the level of CGA in blueberries
457 determined TMA-lowering activity. We employed sequential *in vitro* digestion and fecal
458 fermentation to assess conversion of choline-d_9 to TMA-d_9 by gut bacteria. We previously

459 studied isolated phenolic compounds [21,35], or foods rich in phenolics and/or fiber [30,31], but not
460 sugar. Optimizing fermentation parameters for high-fiber, sugar-rich fruits was crucial for
461 identifying the source of the inhibition in blueberries. This was particularly important since
462 Experiment 1 (Figure 2) highlighted the significant inhibitory effects of these highbush blueberry
463 genotypes, surpassing that of any food previously tested in our lab. In Experiment 2, we
464 demonstrated that sugar mimics near-total inhibition of TMA-d_9 production seen in whole
465 blueberry, but fiber and phenolic extract did not. This suggests, as others have shown [34], that
466 sugar interferes with the assay and that the apparent total inhibition by whole blueberry (an
467 initially promising finding) is not physiologically relevant to *in vivo* conditions. Additionally, this
468 showed that major fiber components provide little activity, and perplexingly, neither did a crude
469 phenolic extract. However, these store-bought highbush blueberries had relatively low CGA
470 content compared to the high CGA genotypes used later (**Tables 1-2**) and may have had low
471 levels of other phenolics. The lack of efficacy of the phenolic fraction in these berries may not be
472 indicative of a similar lack of phenolic activity in phenolic-rich berries. In Experiment 3, we
473 compared the activities of skin and pulp to whole blueberry, sugar, and fiber. While skin
474 exhibited less inhibition compared to the whole blueberry, pulp, and sugar treatments, it still
475 showed greater inhibition than the fiber and control treatments. Since the skin contains most of
476 the fiber, the greater inhibition observed with the skin compared to the fiber suggests another
477 skin component is contributing to inhibition of TMA-d_9 production. We then postulated that, as
478 skin contains most of the phenolics, including CGA, as well as most of the components that will
479 reach the colon *in vivo*, comparing skin of genetically diverse highbush blueberry genotypes with
480 varying CGA content could reveal whether CGA differences result in activity differences. In
481 Experiment 4, we compared skins from high- and low-CGA blueberries. Inhibition potential
482 among all skins was similar, and greater than the controls, but not different between each other.
483 The average reduction in TMA-d_9 AUC was 19.4% for the eight genotypes compared to blank
484 digesta. While TMA-d_9 levels from all genotypes eventually reached the same levels as the
485 controls, this was delayed by ~ 4 h. *In vivo*, this delay is critical, allowing choline to be absorbed,
486 metabolized to other metabolites, or pass out of the colon. Any of these alternatives will reduce
487 the amount of TMA absorbed and the resulting TMAO concentrations in circulation. Our data
488 suggest that CGA is not the primary factor driving the inhibition in blueberries. Free CGA in the
489 fermenta from physiologically relevant doses of blueberry skins (**Figure 9**) was 1,000-fold more

490 dilute than the minimum effective concentration of pure CGA we previously observed for
491 inhibiting TMA production. Experiment 5 sought to explain the lack of observed differences
492 based on concentrations of free CGA in digesta and fermenta, as the lack of differences in TMA
493 inhibition across genotypes may be due to the lack of variation in CGA concentration once it
494 reached the fermentation. Differences in CGA content in skins were reduced during digestion
495 and further reduced in fermentation. While higher CGA genotypes had higher concentrations in
496 digesta and fermenta, all concentrations were nearly the same by 8 hrs of fermentation.
497 Our data suggest that blueberry skin significantly reduces generation of TMA by gut
498 bacteria, but that CGA does not drive TMA-lowering activities of blueberries. The compounds
499 responsible for this activity remain unknown. Our data suggest that this inhibition is not due to
500 major blueberry skin fiber components (pectin, cellulose, and hemicellulose), as fiber did not
501 inhibit (but rather promoted) TMA production compared to controls. Thus, the responsible
502 compounds may be other minor fiber compounds or phenolics that we have not yet evaluated.
503 The likely candidates are anthocyanins. We have not yet evaluated the activities of anthocyanins,
504 which are abundant in blueberry skins. Instead of focusing on identifying compositional
505 differences and then testing activity differences based on these compositional differences one by
506 one, the best approach may be to gather a large number (~50-100) berry samples from many
507 different sources, profile their TMA-lowering activities, then perform broad phenolic profiling
508 on the 10-20% most vs. 10-20% least effective samples.
509 There are various mechanistic explanations for the observed inhibition of TMA
510 production. Broadly reducing the viability of gut bacteria in a non-specific fashion would lower
511 TMA production [36]. We employed an ATP-based bacterial activity assay to account for this. The
512 data do not suggest that any component of the blueberry caused cell death leading to TMA-d_9
513 inhibition. Furthermore, this is not a likely mechanism as blueberries are widely consumed and
514 are not associated with broad antibacterial activities or adverse gut effects. The observed TMA-
515 lowering activities are likely due to either direct or indirect effects on the activity of *cutC/D*.
516 Potential direct effects include inhibition of choline TMA lyase or reducing its expression.
517 Indirect effects include altering the composition of the gut microbiome by reducing the viability
518 or abundance of *cutC/D*-carrying bacteria. We have previously shown that long-term blueberry
519 supplementation alters the abundance of gut microbiota that are associated with circulating
520 TMAO in experimental mice [36]. However, given the acute nature of the present study in which

521 the fecal samples are only exposed to blueberry skins for 24 h (and most inhibition is observed
522 within 12 h), we believe that direct effects (reduced *cutC/D* expression and/or inhibition of
523 choline TMA lyase) are the most likely explanation. Both direct and indirect effects may be
524 observed *in vivo*. Future experiments must identify the mechanism of action. Reduced *cutC/D*
525 expression could be investigated with reverse-transcriptase (RT)-qPCR to detect *cutC/D* mRNA
526 or alternatively using Western blotting to detect choline TMA lyase protein. These studies will
527 likely require more enriched cultures of known *cutC/D* carriers, such as *Proteus mirabilis*, as
528 opposed to dilute *cutC/D* in fecal samples [15]. Studying the inhibition of choline TMA lyase
529 requires the elimination of all other potential mechanisms by excluding viable cells. This could
530 be done with recombinantly expressed enzyme or using a lysed, non-viable *cutC/D* carrier [15].
531 The present study has strengths that enhance its validity. First, the *in vitro* digestion
532 model incorporates physiologically relevant concentrations of digestive enzymes and conditions
533 designed to simulate upper gut digestion. The use of physiologically relevant concentrations of
534 test material maximizes translation portential. Our *in vitro* digestions used 1 serving of whole
535 berries (or equivalent amounts of phenolic extract, fiber, sugar, skin or pulp from 1 serving) per
536 2000 mL total upper gastrointestinal volume and scaled these ratios to our *in vitro* final volume.
537 When transferring digesta to fermentations, we employed digesta at 33% of the fermentation
538 volume. This is a conservative dilution, which still resulted in significant reductions in TMA-d_9
539 production. However, actual concentrations of inhibitory compounds in the colon may be higher,
540 as a critical function of the colon is to extract water from the digesta, thereby concentrating it [37].
541 The addition of digesta, rather than whole food, into the fermentation is more representative of a
542 real human system, as it accounts for the presence of digestive enzymes and related metabolites.
543 However, as discussed above, one limitation of this approach for macronutrient-rich foods is that
544 sugars, amino acids and fatty acids that would be absorbed *in vivo* in the stomach or small
545 intestine prior to colonic fermentation are not removed in this model. The *in vitro* colon-only
546 model allows for isolation of TMA production from other mechanisms that would affect
547 assessment of bioactivity, such as absorption of choline and TMA, and gut transit. The use of
548 isotopically labeled choline-d_9 substrate and measurement of TMA-d_9 reduces interference from
549 background substrates and products [21]. We employed fecal samples from OpenBiome, a trusted
550 provider of samples with a healthy and functioning microbiome. Since gut bacteria expressing
551 the *cutC*/D gene cluster can vary among individuals, pooling multiple OpenBiome fecal samples

552 ensures a more diverse population of gut bacteria for each TMA assay. The use of "blank"
553 digesta controls (containing digestion reagents and enzymes, but no blueberry) in the
554 fermentation accounts for background inhibition and endogenous choline from the digesta.
555 Additionally, use of an isotopically labeled substrate in fermentation ensures that the metabolites
556 of interest are accurately measured and tracked, considering the potential presence of
557 endogenous choline and TMA from the digesta. We employed sugar and fiber controls designed
558 to closely reflect the sugar and fiber composition of a serving size of blueberries. The sugar
559 mixture included sucrose, glucose, and fructose in ratios typical of those found in blueberries,
560 while the fiber blend contained hemicellulose, cellulose, and pectin in proportions similar to
561 those in a serving of the fruit. Although literature on blueberry fiber composition is limited, the
562 ratios used in these experiments were believed to accurately reflect the fiber content of
563 blueberries and closely replicate the sugar content. Finally, the key strength of this study is the
564 final experiment, in which eight genotypesof berries were assessed. Too often, experiments are
565 done in a single cultivar of unknown genetic background, and the translatability of the results to
566 consumers is unknown. The blueberries used in the final experiment represented a broad range of
567 highbush genotypes, reflecting a variety of phenolic compositions, sugar content, and fiber
568 levels. Any differences observed among these genotypes are likely due to genetic variations, as
569 they were all grown and harvested in the same climate by the USDA ARS NCGR in Corvallis,
570 OR, USA. Growing in the same climate helps eliminate potential differences caused by factors
571 like water availability, predators, or nutrient competition. The lack of variation in inhibition
572 potential across the peels of the four high and four low genotypes is encouraging for highbush
573 blueberry consumers, as it suggests that the benefits are not limited to a specific cultivar, making
574 it easier for people to access the same benefits regardless of the type of blueberry available.
575 Our findings should be considered in the context of the following limitations. This work
576 was conducted *in vitro*, which poses limitations. The absorption and conversion of TMA to
577 TMAO and TMAO excretion were not evaluated. These processes affect TMAO in circulation.
578 While we believe that inhibition of TMA formation is the key mechanistic target for lowering
579 TMA, *in vivo* studies are needed to account for all factors that control TMAO levels in
580 circulation. Furthermore, we studied production of TMA from choline exclusively (via *cutC/D*)
581 by adding exogenous labeled choline-d9 and monitoring TMA-d_9 production. Other precursors
582 (carnitine and betaine) are converted to TMA by TMA lyases encoded by the *cntA/B* and *yeaW/X*

583 gene clusters respectively[7,8,38,39]. Therefore, the present results are only applicable to TMA
584 arising from choline via *cutC/D*. The impact of blueberry skins on TMA levels due to other
585 precursors and their respective TMA lyases remains unknown and merits study in the future.
586 Other studies have previously examined the potential inhibition of TMA formation from
587 carnitine by dietary bioactive compounds[34,40,41], and the differences in TMA production from
588 various TMA lyases and their respective precursors could mean that a compound that is an
589 effective inhibitor for one pathway may not be effective for another.

590 As mentioned above, another major challenge is addressing the limitations of *in vitro*
591 digestion and fermentation when using whole fruits, as sugar is not absorbed in the model prior
592 to fermentation. As we and others have demonstrated [34], sugar almost completely inhibits
593 microbial TMA-d_9 generation, but this has little physiological relevance, as sugar would not be
594 present in the colon after upper gastrointestinal digestion. Including an absorption step (such as
595 passing digesta over Caco-2 cells in transwell inserts) between *in vitro* digestion and
596 fermentation could overcome this. Another potential solution to this is to use dialysis to remove
597 sugar and other water-soluble metabolites from the digesta [42], though this would result in loss of
598 water soluble (but poorly absorbed) phenolics like CGA. As a result of not modeling absorption,
599 the observed inhibition of TMA-d_9 may be due to the presence of compounds that would not
600 normally be in the colon, rather than phenolics, which have generally low bioavailability and
601 would be present during fermentation. We observed this issue in experiments with whole
602 blueberries, pulp, and sugar. However, this was addressed as much as possible by removing the
603 pulp, which contains most of the substrates not found in the colon, and focusing on inhibition
604 from the skin. We elected to use fiber and phenolic-rich skin for our multi-genotype experiment,
605 as this material is physiologically relevant to the colon and reduces interferences.

606 There are inherent limitations to ex *vivo* fecal fermentations. Fecal samples were sourced
607 from healthy donors, and their TMA production capacity compared to donors with CVD-
608 associated dysbiosis is unknown. TMA production depends on bacteria that produce TMA lyase.
609 By incorporating fecal samples from subjects with CVD, it may be possible to increase the
610 abundance of bacteria that metabolize choline, creating an environment that could be more
611 reflective of conditions seen in a state of CVD-associated dysbiosis. TMA production from
612 known high TMA-producing donors may better reflect the conditions of the population of
613 interest, as they likely include greater abundances of bacteria known to express cut*C/D* [24,43–47].

614 Additionally, fecal samples are kept frozen and reanimated before fermentation. However, some
615 bacteria may not grow outside the colon or survive the freeze-thaw and reanimation process,
616 although a cryoprotectant is added to the samples. Furthermore, fecal samples are not fully
617 representative of all regions of the colon. The use of pooled fecal samples is designed to create a
618 more representative sample. This approach is beneficial for introducing bacteria from individuals
619 with diverse lifestyles, living environments, and diets. However, it does not allow for an in-depth
620 examination of the individual variations in choline metabolism to TMA. An inter-individual
621 study could be conducted by keeping the OpenBiome fecal samples separate, to allow for
622 comparison of choline conversion to TMA across different individuals.

623 Animal models would help address limitations associated with *in vitro* models. *In vitro*
624 models tend to represent acute conditions, capturing short-term changes in enzyme production
625 rather than shifts in the microbiome, due to the brief observation period. Models like MiGut,
626 SHIME, and TIM-2 represent longer time frames *in vitro* and provide a more faithful simulation
627 of the gut environment. However, they are more cumbersome, costly, and have lower throughput
628 compared to our model [48–50]. Longer *in vivo* studies would capture microbial shifts over time.

629 The blueberries themselves posed several challenges. There is limited information in the
630 literature regarding blueberry fiber, making it difficult to determine exact ratios of the types of
631 fibers. To address this, fiber extraction from blueberries would be necessary; however, this is
632 time-consuming and outside the scope of this project. Removing the skin from the blueberries
633 was challenging due to their small and delicate nature. Frozen blueberries were particularly
634 difficult to blanch and separate the peel from the pulp without issues. Peeling by hand was a
635 time-intensive process that increased the risk of contamination, either by exposing the berries to
636 non-frozen temperatures for extended periods or by accidentally leaving peel in on the pulp.

637 The CGA content was measured for the whole blueberry across 20 different highbush
638 genotypes, rather than just the skin, due to time limitations and the lengthy process of peeling the
639 berries for the extracts used in SPE and LC-MS analysis. While previous experiments have
640 shown that the majority of phenolics and CGA are contained in the peel, this was not specifically
641 confirmed for the peels used in the final experiment. As a result, it is possible that the lack of
642 differentiation in the TMA-d_9 inhibition could be due to the smaller variations in the peel across
643 all genotypes, compared to the greater differences observed in the whole fruit. The variations in
644 the fruit could be attributed to the amount of peel rather than its concentration of CGA. Fruit

sizes varied between genotypes, with some being much smaller with higher peel to pulp ratios in smaller berries. This was not accounted for in the experiment, as the amount of peel used was the same for all genotypes. As a result, the amounts may not accurately reflect the actual serving size of whole berries for each genotype, as each fruit had a different peel-to-pulp ratio.

5. Conclusions

Our data suggest that although highbush blueberry skin inhibits the production of pro-atherogenic TMA by human gut bacteria, CGA does not drive this activity. Given the lack of available interventions to blunt TMAO production, the present data showing that blueberry skins at translatable human doses inhibit the key step in this process are provocative. Future studies are needed to elucidate the mechanism of inhibition, identify the compound(s) responsible, and assess interindividual variability in efficacy. Given the fact that blueberries are widely available and consumed, the translatability of this effect to humans is promising. Pilot clinical studies should be designed in individuals exhibiting a high TMAO production phenotype.

Author contributions

Ashley M. McAmis: methodology, validation, formal analysis, investigation, data curation, writing - original draft, writing - review & editing, visualization; **Michael G. Sweet:** methodology, formal analysis, investigation, writing - review & editing, visualization; **Sydney Chadwick-Corbin:** methodology, investigation, writing - review & editing; **Juanita G. Ratliff:** investigation, writing - review & editing; **Molla Fentie Mengist:** methodology, writing - review & editing; **Nahla V. Bassil:** resources, writing - review & editing; **Pon Velayutham Anandh Babu:** conceptualization, methodology, writing - review & editing, funding acquisition; **Massimo Iorizzo:** conceptualization, methodology, resources, writing - 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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794 **Figures**

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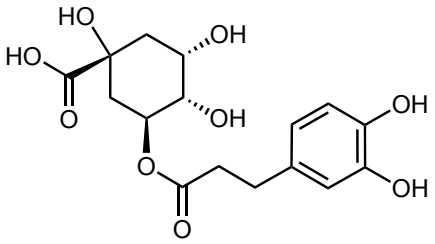

796

797 **Figure 1**. Structure of 5-caffeoyquinic acid (5-*O*-CQA), the most abundant form of chlorogenic
798 acid (CGA) found in highbush blueberries.

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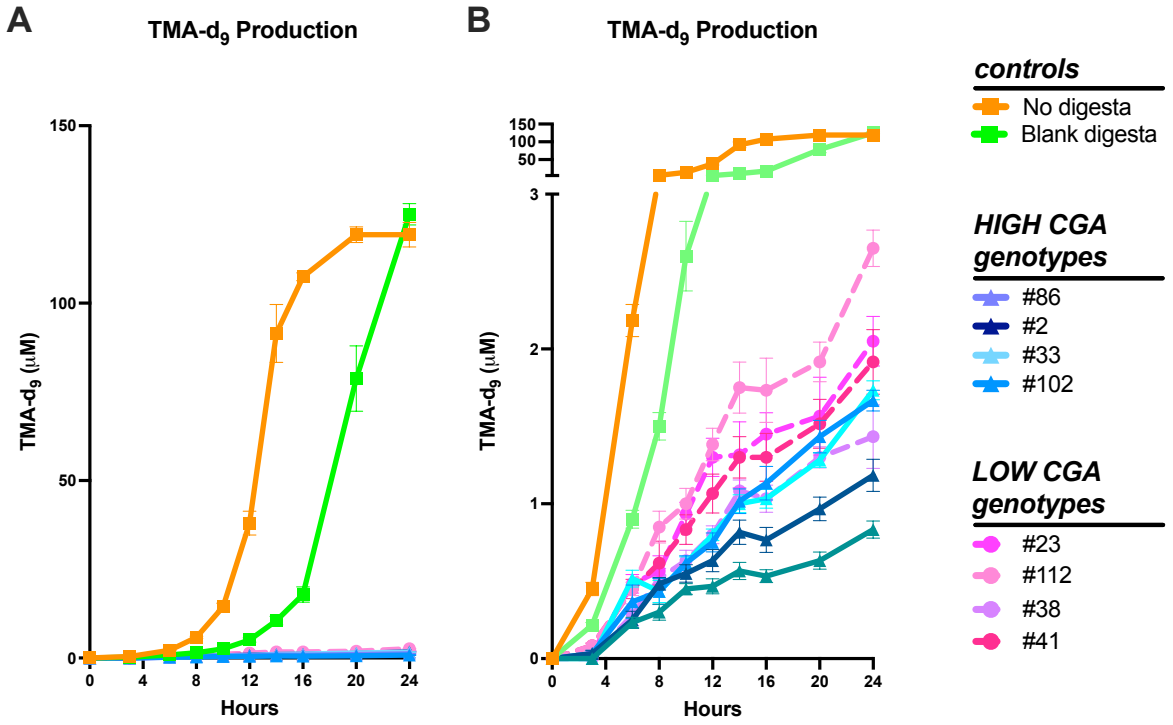


801

802 **Figure 2.** Kinetics of TMA-d_9 production in fecal fermentations with choline-d_9: No digesta,
803 Blank digesta, or Digesta from highbush blueberry genotypes from the bi-parental mapping
804 population were added. TMA production displayed without a broken axis (A) and with a broken
805 axis (B). Data represent mean ± SEM from *n*=6 replicates.

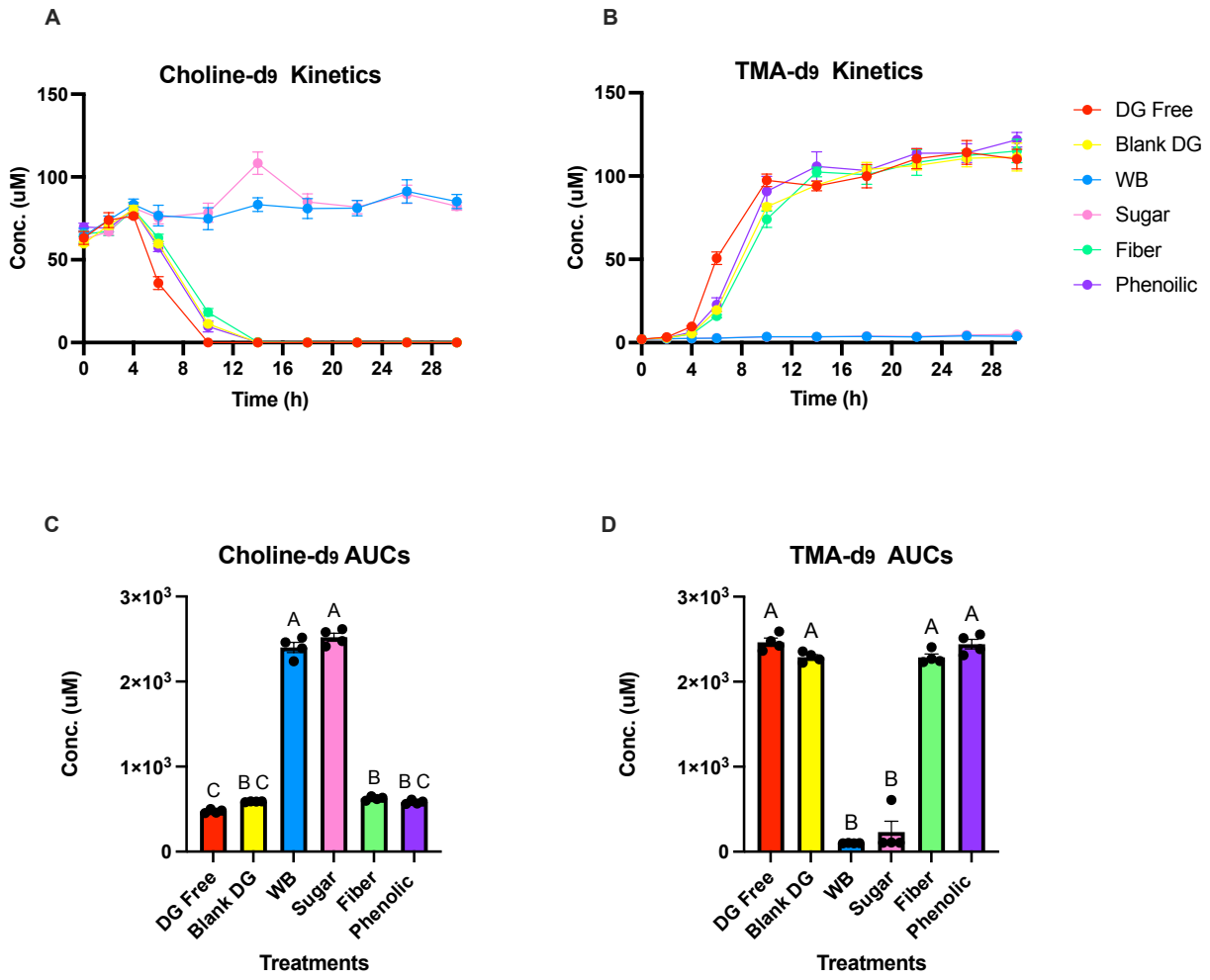


Figure 3. Kinetics of choline-d_9 use (A) and TMA-d_9 production (B), and areas under the curve (AUCs) for choline-d_9 utilization (C) and TMA-d_9 production (D) in fermentation (0-30 hr) for all treatments including: Digesta free (DG free), Blank DG (blank digesta), Whole blueberry (WB), Sugar, Fiber, and Phenolic fractions. 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.

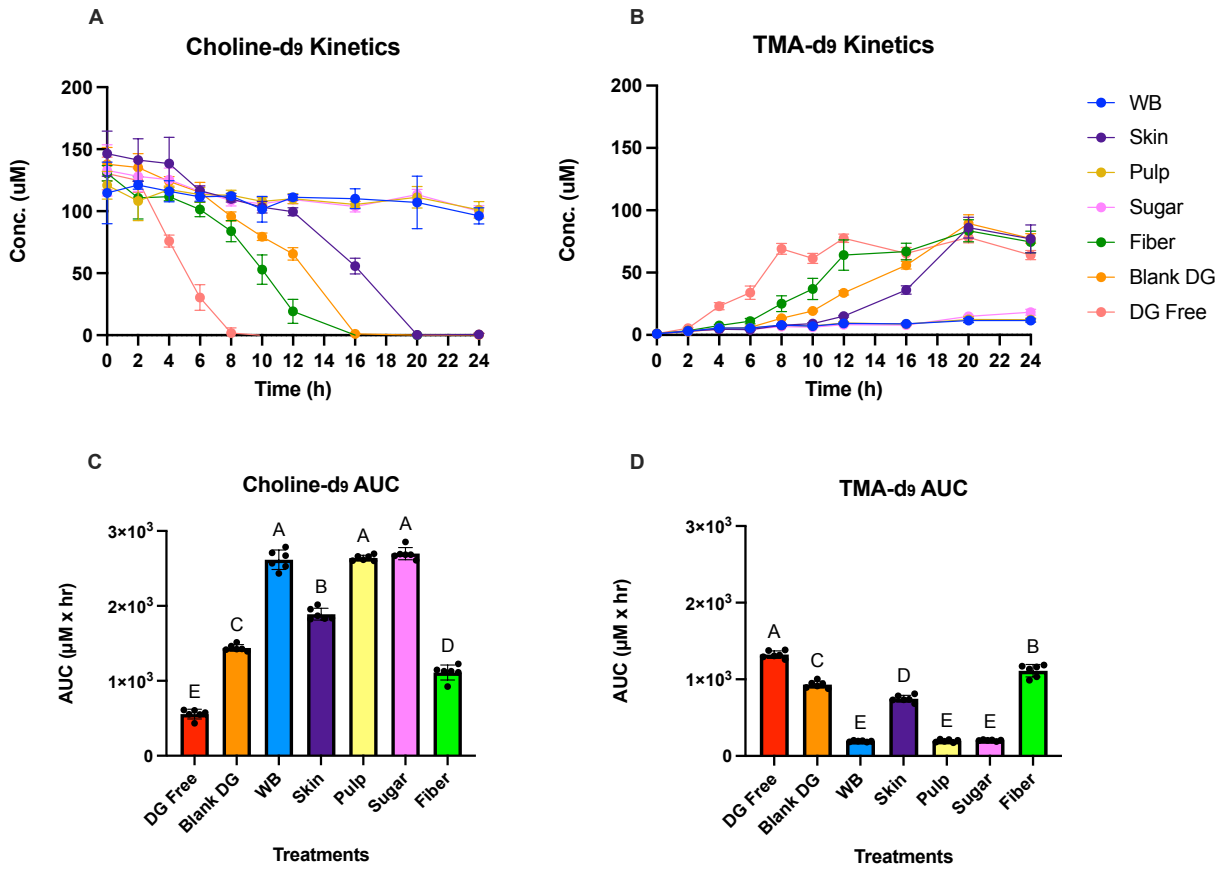


Figure 4 Kinetics of choline-d_9 use (A) and TMA-d_9 production (B), and areas under the curve (AUCs) for choline-d_9 utilization (C) and TMA-d_9 production (D) in fermentation (0-30 hr) for all treatments of digesta (DG), Whole blueberry (WB), Blank DG, DG free, WB, Peel, Pulp, Sugar, and Fiber. Using 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.

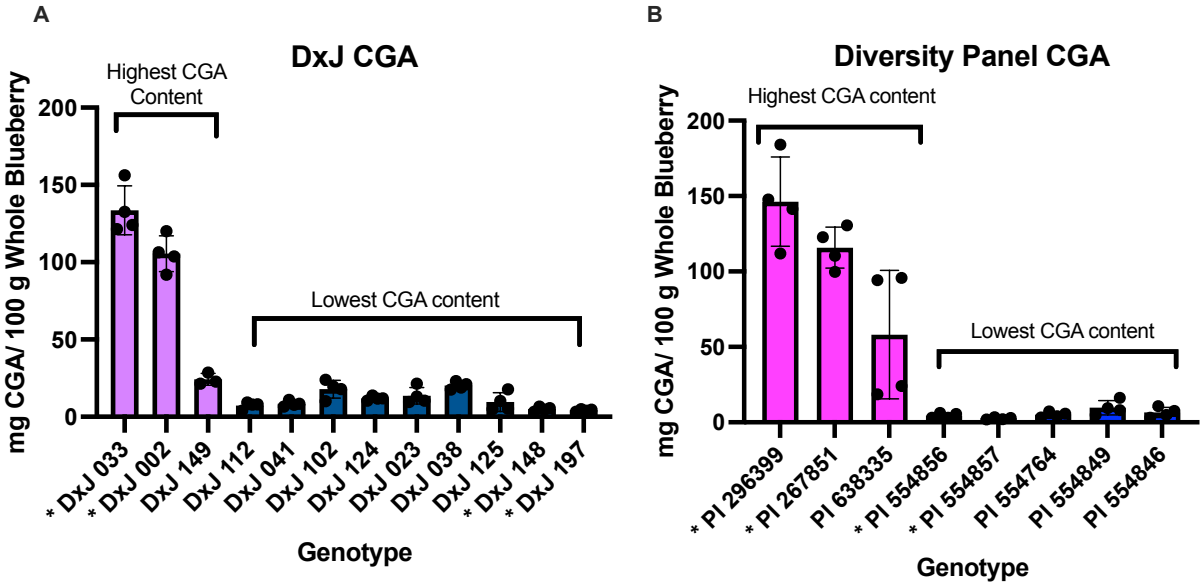


Figure 5. Chlorogenic acid (CGA) content of multiple genetically diverse highbush blueberry genotypes from the summer of 2024. Two (DxJ 197, DxJ 148) of the lowest CGA and two (DxJ 033, DxJ 002) of the highest CGA content blueberries from the biparental mapping population (A) and two (PI554857, PI 554856) of the lowest CGA and two (PI296399, PI 267851) 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.

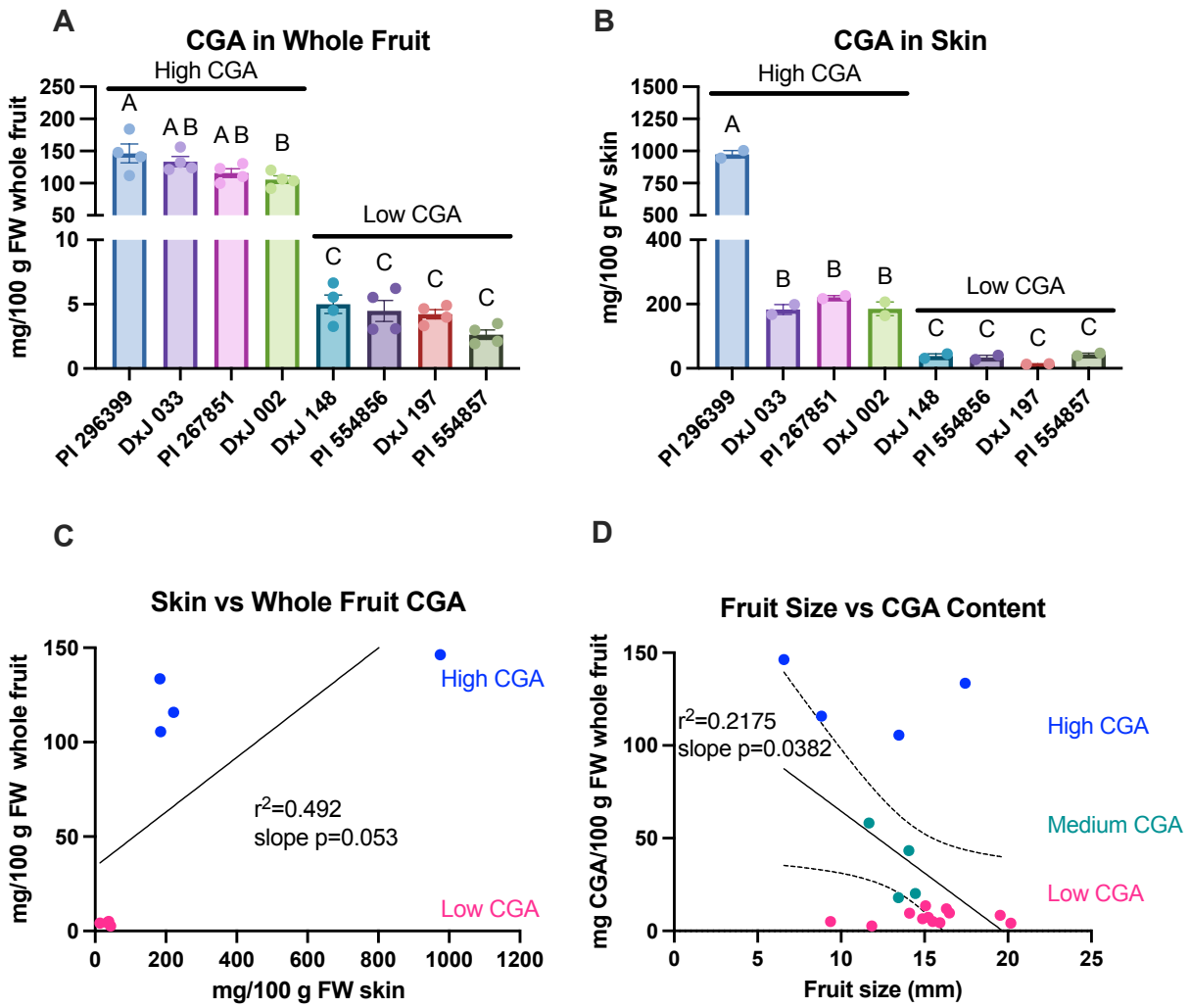


Figure 6. Chlorogenic acid (CGA) content in whole fruit (A) and skins (B) of highbush blueberry genotypes. Correlation of CGA content in skins and whole fruits in the 8 selected genotypes with extreme CGA content (C). Correlations of CGA content with fruit size in all genotypes. Data represents mean ± SEM for *n*=4 (A) or *n*=2 (B). Dots represent means (C and D). FW: fresh weight.

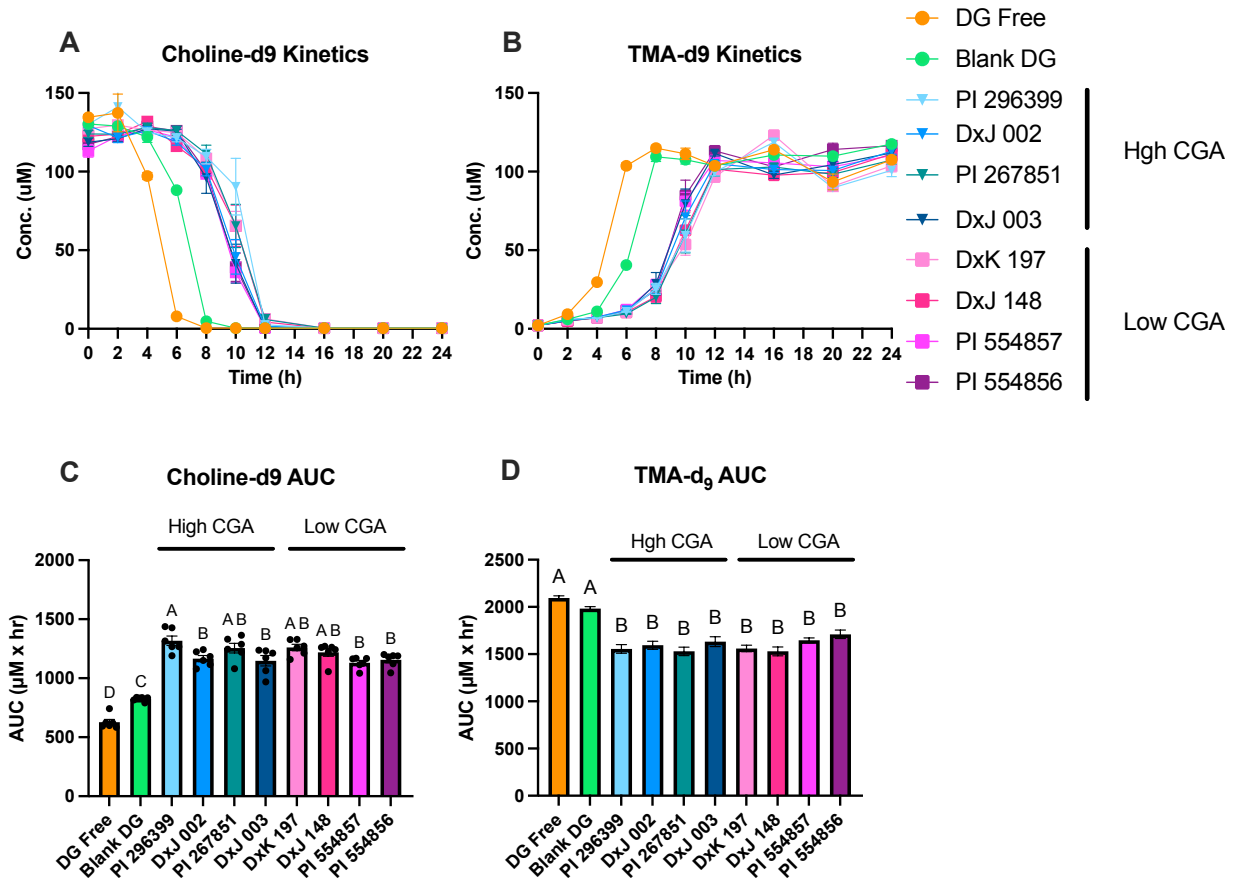


Figure 7. Kinetic for choline-d_9 utilization (A) and TMA-d_9 production (B) and area-under-the-curve (AUC) values for choline-d_9 utilization (C) and TMA-d_9 production (D) during *in vitro* fecal fermentation of choline-d_9 with *in vitro* digesta from highbush blueberry skins with varying chlorogenic acid (CGA) levels (0-24 hr). Treatments were compared using one-way ANOVA and Tukey's multiple comparison. Values not sharing a common superscript letter are significantly different ($P<0.05$). Data represent mean ± SEM from n=6.

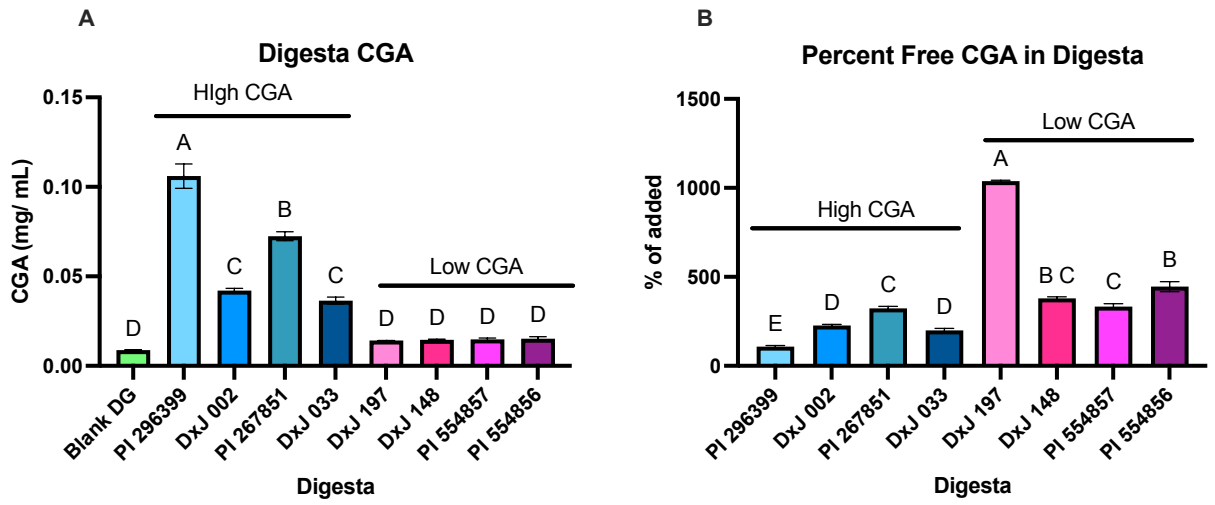
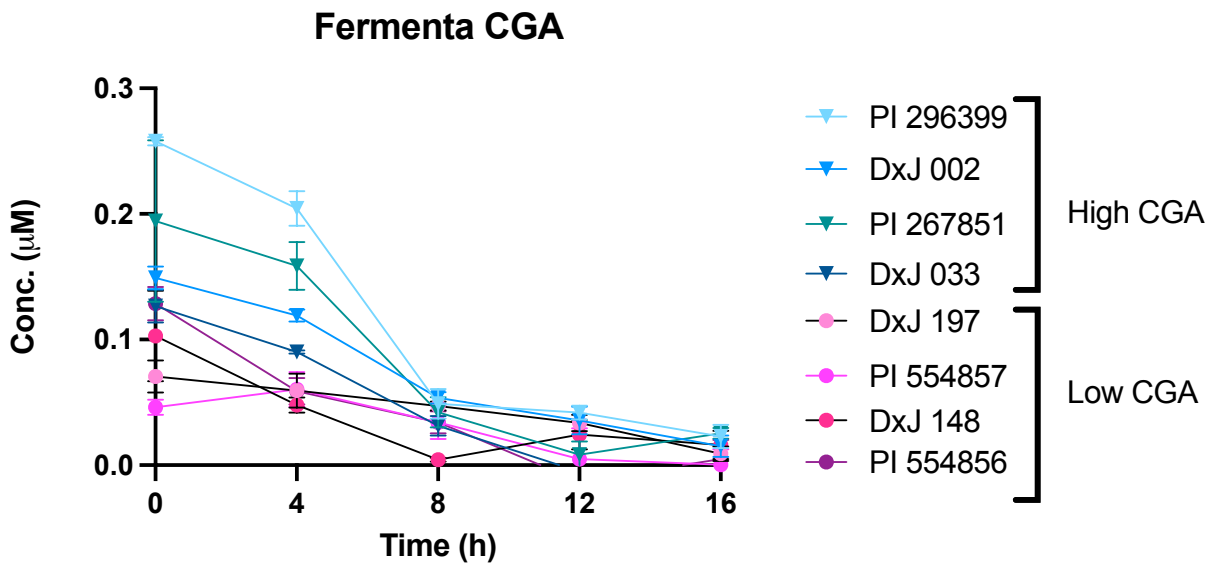


Figure 8. Concentration of free chlorogenic acid (CGA) in whole *in vitro* digesta of highbush blueberry skins (A) and percent CGA released (i.e. bioaccessibility) during digestion (B). Data represent mean ± SEM from *n*=2.



2-way ANOVA Summary	
Source of variation	**P-value**
Time	<0.0001
Genotype	<0.0001
Time x Genotype	0.0002

***Post hoc* tests per time point**					
Genotype	**Time (h)**				
	0	**4**	**8**	**12**	**16**
PI 296399	a	a	ab	a	a
DxJ 002	b	ab	a	ab	a
PI 267851	abc	ac	ab	ab	a
DxJ 033	bc	ac	ab	b	a
DxJ 197	bc	bc	a	ab	a
PI 554857	c	bc	ab	ab	a
DxJ 148	abc	c	b	ab	a
PI 554856	bc	bc	ab	b	a

Figure 9. Concentrations of free chlorogenic acid (CGA) in *in vitro* fecal fermenta from highbush blueberry skin digesta from 0-16 h. Data represent mean ± SEM from *n*=3. Tables show the significance of main effects and their interaction in a 2-2qy mixed effects model analysis, and differences among genotypes at each time point (genotypes not sharing a common superscript within the same time point are significantly different by Tukey's test, P<0.05).

858 **Tables**

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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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