Potential of phenolic compounds and their gut microbiota-derived metabolites to reduce microbial TMA formation: Application of an *in vitro* fermentation high throughput screening model.

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Abstract:

Trimethylamine *N*-oxide (TMAO) is a pro-atherosclerotic product of dietary choline metabolism generated by a microbiome-host axis. The first step in this pathway is enzymatic metabolism of choline to trimethylamine (TMA) by the gut microbiota. This reaction could be targeted to reduce atherosclerosis risk. We aimed to evaluate potential inhibitory effects of select dietary phenolics and their relevant gut microbial metabolites on TMA production via a human *ex vivo-in vitro* fermentation model. Various phenolics inhibited choline use and TMA production, especially larger compounds or their larger metabolites, without altering cell respiration or cell growth. However, inhibitory effects decreased at late fermentation times, which suggest that these compounds delay choline metabolism rather than completely inhibiting TMA formation. Overall,

caffeic acid, catechin and epicatechin were the most effective non-cytotoxic inhibitors of choline use and TMA production. Thus, these compounds are proposed as lead bioactives to test *in vivo*.

Keywords: Atherosclerosis; Food bioactives; Gut microbiota metabolites; Phenolic compounds; TMAO.

1. Introduction:

Cardiovascular disease (CVD) is the leading cause of mortality worldwide, accounting for 17.9 million deaths a year [1]. Arteriosclerosis development, characterized by the accumulation of a fibrofatty plaque in arterial walls, is a risk factor for CVD [2]. Several factors can trigger atherosclerosis development, such endothelial dysfunction, dyslipidemia or elevated cytokine levels [3,4]. Recently, trimethylamine *N*-oxide (TMAO) has been identified as a biomarker of atherosclerosis development and CVD [5].

Quaternary amines from the diet, such as choline and L-carnitine, can be metabolized by the gut microbiota to trimethylamine (TMA) [6,7], which is absorbed into circulation and further metabolized by host hepatic flavin-containing monooxygenase 3 (FMO3) into TMAO [8]. Bacteria must contain the putative choline utilization gene cluster (CutC) to efficiently transform choline into TMA, along with a glycyl radical activating protein gene (CutD) [6]. There is extensive interest in developing effective strategies to prevent or treat elevated TMAO levels in order to reduce subsequent CVD risk. Phenolic compounds are plant secondary metabolites with a vast array of biological activities, including well-established cardioprotective functions [9]. Several phytochemicals have reported promising potential to reduce TMAO formation in humans [5]. Among phenolic compounds, flavan-3-ols stand out due to their high content in foodstuffs (i.e., tea, cocoa and grapes) and their health-promoting properties [10–13]. Hydroxycinnamic acids (i.e., chlorogenic acid) have also reported relevant cardioprotective functions, and are present in beverages such as coffee and tea, and fruits such as cherries [14]. The absolute absorption of phenolic compounds in the small intestine is < 5-10 % (and often much less), and thus these compounds remain in the gut at high concentrations where they are subjected to gut microbial fermentation [15]. Due to poor systemic bioavailability, one theory is that the compounds primarily responsible for

some observed bioactivities of native phenolics may in fact be their gut microbial metabolites rather than the naturally-occurring compounds *per se* [16,17]. Microbial metabolites of flavan-3-ols include low molecular weight phenolic acids such as phenylpropionic, phenylacetic and benzoic acid derivates [11,15,18]. Microbial metabolites of hydroxycinnamic acids include caffeic, ferulic and *p*-coumaric acids [14].

We previously developed a high-throughput 96-well *ex vivo* anaerobic fecal fermentation assay to assess inhibition of TMA production from choline [19]. Using this assay, we are now capable of screening large numbers of compounds to identify promising lead compounds for subsequent focused animal studies. Due to the reported cardioprotective benefits of flavan-3-ols, hydroxycinnamic acids and other phenolic compounds from foods such as cocoa [14,20,21], these compounds are of particular interest as potential TMA- and TMAO-lowering agents. Indeed, we found that chlorogenic acid was able to reduce TMA formation in our *ex vivo-in vitro* fermentation model [19]. The aim of this study was therefore to evaluate the potential of *p*-coumaric acid, caffeic acid, ferulic acid, catechin, epicatechin (major hydroxycinnamic acids and flavan-3-ols in the diet) and their gut-derived metabolites to inhibit microbial conversion of choline to TMA.

2. Materials and methods:

2.1. Chemicals and reagents:

Glucose, peptone water, yeast extract, KCl, NaCl, Na_2HPO_4, KH_2PO_4, $MgSO_4 \times 7H_2O$, $CaCl_2 \times 6H_2O$, $ZnSO_4 \times 7H_2O$, $NaHCO_3$, ammonium formate, hemin, bile salts, Tween 80, vitamin K1, resazurin, L-cysteine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, ammonia, ethyl bromoacetate, choline, choline-d_9, choline-1-^{13}C-1,1,2,2-d_4, TMA, TMA-d_9, TMA-$^{13}C_3$-^{15}N, 3,3-dimethyl-1-butanol (DMB), catechin, epicatechin, *p*-coumaric acid, caffeic acid, ferulic acid, hippuric acid, 5-phenylvaleric acid, vanillic acid, homovanillic acid, 3-(3,4-dihydroxyphenyl)propionic acid, 3-(4-hydroxyphenyl)propionic acid, 3-(3-hydroxyphenyl)propionic acid, 3-phenylpropionic acid, 3,4-dihydroxyphenylacetic acid, 4-hydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, phenylacetic acid, 3,4-dihydroxybenzoic acid and 3-hydroxybenzoic acid were purchased from Sigma-Aldrich/Millipore (St. Louis, MO, USA). Acetonitrile and water (HPLC grade) as well as dimethyl sulfoxide (DMSO; reagent grade) were purchased from VWR International (Suwanee, GA, USA). Fecal samples from different

healthy donors were obtained from OpenBiome (Cambridge, MA, USA). These fecal samples were from healthy donors and rigorously screened for 30 infectious diseases by OpenBiome. Health histories, clinical data, pathogen screen results and 16S rDNA sequences are available at OpenBiome. Samples are processed by OpenBiome in sterile 12.5% glycerol and 0.9% saline buffer at 2.5 ml of buffer per gram of stool, and filtered through a 330 μm filter to remove large particulate matter, and frozen at −80°C until use.

2.2. Culture media preparation:

Fermentation media was prepared according to our optimized methodology [19], which was originally adapted from Alqurashi *et al.* [22]. The composition of 1 L of growth medium was 2 g peptone water, 2 g yeast extract, 0.1 g NaCl, 40 mg Na_2HPO_4, 40 mg KH_2PO_4, 10 mg $MgSO_4 \cdot 7H_2O$, 10 mg $CaCl_2 \cdot 6H_2O$, 2 g $NaHCO_3$, 50 mg hemin, 0.5 g bile salts, 2 mL Tween 80, 10 μL vitamin K1, 1 mg resazurin, and 0.5 g L-cysteine. Two different 500 mL solutions were prepared at 2X final concentration. Solution A included all components except for resazurin and L-cysteine, which were included in Solution B. The pH of both solutions was adjusted to 6.8, and solutions were filter-sterilized separately through a 0.22 μm sterile filtering system (Corning, Corning, NY, USA). Solution B was then boiled for 10 min. Thereafter, both solutions were sparged overnight (minimum of 8 h) with N_2 (g) under agitation and were then combined 1:1 in the anaerobic chamber (O_2 < 15 ppm) to the final 1X concentration. This growth media mixture was used to grow bacteria in fecal slurries under different experimental conditions. The PBS 1X solution was filter-sterilized (22 μm sterile filtering system, Corning, Corning, NY, USA) and sparged overnight with N_2 (g) to maintain sterile and anaerobic working conditions.

2.3. Anaerobic chamber conditions:

All fermentations were carried out inside a 4-glove 855-ACB anaerobic chamber (Plas-Labs, Lansing, MI, USA). filled with a mixed gas composed of 5 % H_2, 5 % CO_2 and 90 % N_2 (Airgas, Durham, NC, USA). H_2 (typically 2 – 3 %) and O_2 (typically < 15 ppm) levels were monitored with a CAM-12 anaerobic monitor (Coy Lab Products, Grass Lake, MI, USA). Temperature was set at 37 °C, and it was maintained constant (recorded values within 36 – 38 °C) throughout the fermentation procedure. Humidity ranged between 40 and 50 % during all experiments.

2.4. Fermentation experiments:

Due to the presence of endogenous choline and other TMA lyase substrates, and resulting production of TMA in the absence of exogenous choline [19], choline-d_9 at a final concentration of 100 μM was added as the substrate, and the resulting TMA-d_9 was quantified in order to reduce the impact of background TMA production on assay results. To screen for the potential effects of our target compounds and their metabolites, fermentations were carried out in 96-well plates following the optimized procedure described by Iglesias-Carres *et al.* [19]. A stock solution of growth media (60 %) and PBS 1X (40 %) was prepared with filter-sterilized, overnight-sparged individual solutions inside the anaerobic chamber. This solution was used to dissolve test compounds at a final concentration of 2.67 mM (except DMB, 13.35 mM) and filter-sterilized again through a PTFE (0.45 μM) MicroSolv AQ syringe filter inside the anaerobic chamber. In 1.1 mL 96-well plates, 675 μL of test compound solutions were mixed with 45 μL of choline-d_9 stock solution (2 mM) in PBS 1X and 180 μL fecal slurry (1:10 in PBS 1X,) to reach a final well volume of 900 μL containing test compound at 2 mM (except for DMB, at 10 mM), choline-d_9 at 100 μM and original fecal slurry at 2 %. These conditions were optimized previously [19]. All solutions were pre-heated at 37 °C, and fermentation was considered to start (time 0 h) when fecal slurry was inoculated into the reaction mixture containing substrate. Aliquots from two different donors were used as fecal slurries, with reference numbers 0105-0003-11 and 0128-0001-01. A total volume of 100 μL was sampled from 0 to 12 h in 2 h intervals, analyzed for cell density (see section 2.7), then combined with 100 μL acetonitrile and immediately frozen at – 80 °C until used for choline-d_9 and TMA-d_9 analyses. Control conditions were defined as growth media and PBS with choline-d_9 100 μM in fermentations with 2 % fecal slurry (no inhibitors); vehicle conditions (VH) were defined as growth media and PBS; choline-d_9-free conditions (CF) were defined as growth media and PBS with fecal slurry 2 % only; and microbiota-free conditions (MFC) were defined as growth media and PBS with 100 μM choline-d_9 only. PBS 1X was used to adjust volumes to 900 μL. In all cases, growth media volume was 45 % of the total, and the remaining 55 % was PBS 1X.

2.5. Extraction and analysis of choline-d_9 and TMA-d_9:

Externally added choline-d_9 and produced TMA-d_9 in fermentation media were extracted according to the previously reported methodology [23,24] with slight modifications. TMA-d_9 requires a derivatization process to the quaternary amine compound ethyl betaine-d_9 to facilitate LC-MS/MS ionization. Briefly, 25 μL of fermentation sample were mixed with 20 μL of TMA-$^{13}C_3$-^{15}N internal standard solution (10 μM, for derivatization to ethyl betaine-d_9 or ethtylbetaine-$^{13}C_3$-^{15}N, respectively), 8 μL concentrated ammonia and 120 μL ethyl bromoacetate (20 mg/mL), and let sit for 30 min. Then, 120 μL 50 % acetonitrile/0.025 % formic acid in distilled water were added. TMA-d_9 samples were filtered through AcroprepAdv 0.2 μm WWPTFE 96-well filtering plates (Pall Corporation, Port Washington, NY, USA) by centrifugation (10 min, 3,400 x g), collected in a fresh 96-well collection plate and frozen at –80 °C until UHPLC-MS/MS analysis. To extract choline-d_9, 25 μL of sample were mixed with 10 μL of $ZnSO_4$ solution (5 % w/v in water), 100 μL acetonitrile and 20 μL choline-1-^{13}C-1,1,2,2-d_4 (IS; 10 μM) in 96-well plates. After sonication for 5 min in a water bath, samples were filtered and stored as described above. No more than 48 h passed between TMA-d_9/choline-d_9 extraction and their analysis. Endogenous choline and TMA from fecal fermentation media were also extracted and analyzed as for exogenous choline-d_9 and TMA-d_9.

2.6. Chromatographic analysis and quantification of choline-d_9 and TMA-d_9:

TMA-d_9 and TMA were analyzed separately from choline-d_9 and choline, but with the same UHPLC-ESI-MS/MS method. Briefly, separation was achieved on a Waters Acquity UPLC system (Milford, MA, USA) with an ACQUITY BHE HILIC column (1.7 μm, 2.1x100 mm) coupled to an ACQUITY BHE HILIC pre-column (1.7 μm, 2.1x5 mm) (Waters). Mobile phases consisted of 5 mM ammonium formate in water (pH 3.5) (A) and acetonitrile (B). The gradient was isocratic at 80 % B for 3 min, with a flow rate of 0.65 mL/min. Colum temperature was set at 30 °C, and autosampler at 10 °C. Quantification was achieved by coupling the above system with a Waters Acquity triple quadrupole mass spectrometer. Source and capillary temperatures were 150 and 400 °C, respectively. Capillary voltage was 0.60 kV, and desolvation and cone gas flows (both N_2) were set at 800 and 20 L/h, respectively. Electrospray ionization (ESI) was operated in

positive mode, and data were acquired by multiple reaction monitoring (MRM) in MS/MS mode. MRM fragmentation conditions of analytes and IS compounds can be found in Table 1.

For sample quantification, 45% growth media in PBS 1X was spiked with 7 different concentrations each of choline-d_9 and TMA-d_9 standards to obtain external calibration curves in a relevant background matrix. Samples were quantified by interpolating the analyte/IS peak abundance ratio in the standard curves. Quantification of choline and TMA (inherent in samples) and their d_9 isotopes (externally inoculated to samples) was performed using the calibration curves constructed with the respective d_9 isotopes (choline-d_9 or TMA-d_9). Data acquisition was carried out using Masslynx software (V4.1 version, Waters). Method sensitivity was determined by limit of detection (LOD) and limit of quantification (LOQ), respectively defined as the concentration of analyte corresponding to 3 and 10 times the signal/noise ratio. Method detection (MDL) and quantification (MQL) limits were calculated for the analysis of 25 μL of non-diluted fecal fermentation media samples. Method quality parameters can be found in Table 2. To determine the endogenous levels of TMA and related compounds in experimental conditions, fecal slurry 1:10 in PBS 1X and fermentation media (45% growth media + 55% PBS 1X) with fecal slurry at a final concentration of 2% were analyzed as previously stated.

2.7. Treatment toxicity assessment:

To study potential cytotoxic effects of treatments, cell count and cell respiration rate were monitored as measures of viability. The number of cells present in the fermentation media was evaluated by reading the optical density of 100 μL fermentation media at 600 nm in a SpectraMax iD3 plate reader (Molecular Devices, San Jose, CA, USA) at every sampling time (0 – 12 h). The MTT cell respiration assay was used to estimate cell viability at 12 h. While typically used to measure mitochondrial respiration in eucaryotic cells, the assay has been previously used to test cell respiration and viability in prokaryotic (i.e., bacteria) cells, which lack mitochondria but do have the NAD(P)H oxidoreductases that carry out the MTT reaction [19,25,26]. Briefly, 10 μL of fermentation mixture was mixed with 80 μL of pre-heated (37 °C) PBS 1X with glucose 0.2% (m/v) and 10 μL of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] at a concentration of 25 mg/mL in PBS 1X [25]. Samples were allowed to react for 30 min under anaerobic conditions (37 °C and O_2 < 15 ppm), and the resulting formazan crystals were

resuspended to a final volume of 1 mL with DMSO by shaking for 30 min. An aliquot of 100 µL was read at 560 nm in a SpectraMax iD3 plate reader (Molecular Devices, San Jose, CA, USA). All reagents were prepared with overnight-sparged PBS 1X and filter-sterilized 22 µm sterile filtering system, Corning) before their use. Results are expressed as percentages of change versus choline-free conditions ± SEM (*n*=4).

2.9 TMA lyase gene abundance analysis.

To optimize the assay, DNA from fermented fecal slurry 2% with 100 uM choline-d_9 was harvested per manufacturer's procedures (MP Biomedical #116570200). Briefly, fecal slurry samples were prepared in sodium phosphate buffer and PLS solution, mixed, and centrifuged. Pellet was homogenized and proteins precipitate out using PPS solution. DNA was bound to a SPIN filter tube, washed, dried, and eluted into TES elution solution. Real-time PCRs were performed using the QuantStudio 6 Flex Real-Time PCR Sytem (ThermoFisher). SYBR green-based degenerative primers were used to detect 16s (internal control), CntA (carnitine monooxygenase oxygenase subunit, which converts carnitine to TMA) and CutC. Averages of three independent CntA or CutC reactions were performed and ΔCt values were calculated. All sequences for the degenerate primers were previously described [27]. The optimal concentrations for the forward and reverse primer sets were identified by an optimization matrix protocol [28] comparing Ct results between reactions with water and reactions with a sample of isolated DNA not part of the study. The optimal concentrations were chosen based on reduced background fluorescence and used to investigate all study DNA samples.

2.10 Data analysis and statistics

Prism 8.0 (GraphPad, La Jolla, CA, USA) was used for statistical analyses and graph creation purposes. Two-way ANOVA (main effect of Treatment) was used to estimate differences in choline-d_9, TMA-d_9, choline and TMA kinetic curves and cell density values. If a significant main effect or interaction ($p<0.05$) was reported for choline-d_9 and TMA-d_9 kinetic curves or cell density values, Sidak's *post hoc* test was used to estimate time-matched differences between control conditions (choline-d_9 100 µM) and test compounds. One-way ANOVA was used to estimate differences in choline-d_9, TMA-d_9, choline and TMA AUCs and cell respiration between control

conditions (choline-d_9 100 μM) and test compounds (Dunnetts' *post hoc* test). In all cases, statistical significance was stablished *a priori* as $p < 0.05$.

3. Results:

3.1. Inhibition of choline-d_9 use and TMA-d_9 production by phenolic compounds and their microbial-derived metabolites:

First, microbial dependence of TMA-d_9 production and choline-d_9 degradation were evaluated in fermentation conditions with 100 μM exogenous choline-d_9 free of human fecal slurry (**Figure 1A**). Under those conditions, choline-d_9 levels remained fairly constant between 90.79 ± 4.41 μM (0 h) – 70.28 ± 0.08 μM (12 h), which represents a ~23 % degradation of initial choline-d_9 concentration over 12 h. Despite slight decrease in apparent choline-d_9 levels, TMA-d_9 was not detected during these 12 h. When fecal slurry was added (**Figure 1B**), choline-d_9 concentrations varied from 94.20 ± 2.71 μM (0h) to 0.74 ± 0.07 μM (12h), and TMA-d_9 levels increased from not detected (0 – 4 h) to 79.00 ± 2.34 μM (at 12 h). Choline-d_9 degradation and TMA-d_9 production mirrored each other. To evaluate if the growth media and fecal slurry contributed to choline-d_9 and TMA-d_9 signals, their levels were monitored in choline-d_9-free conditions (**Figure 1C**); none of these two compounds were detected at any timepoint. Similarly, no choline-d_9 and TMA-d_9 were reported in vehicle (PBS and growth media, but no choline-d_9 or fecal slurry) conditions at any timepoint (**Figure 1C**). These results indicate that exogenous substrate (choline-d_9) was almost completely consumed within 12 h, and roughly 84% was converted to TMA-d_9 within that timeframe (given the 1-to-1 stoichiometry of choline-d_9 bacterial fermentation to TMA-d_9), consistent with our previous data [19]. Thus, conversion of choline-d_9 to TMA-d_9 appears to be microbially-dependent and is not affected by endogenous/background choline/TMA.

After validating that formation of TMA-d_9 required the presence of both fecal bacteria and choline-d_9 substrate, we evaluated the potential inhibition of microbial conversion of choline-d_9 toTMA-d_9 by select native phenolic compounds and their gut-derived microbial metabolites (**Figures 2 and 3**). Detailed information on Two-way ANOVA statistical significance for time, treatment and interaction (time x treatment) effects can be found in **Supplementary Table 1**. Detailed information on *post-hoc* results for choline-d_9 and TMA-d_9 levels at individual time points can be

found in **Supplementary Figures 1** and **2**. DMB (10 mM) was used as a positive control with known TMA lyase inhibitory activity, per our previous data [19] and those of others [29,30].

At the start of fermentation, most treatment conditions presented non-significantly different levels of choline-d_9 (**Figure 2**) against the control group (choline-d_9 100 μM with no added inhibitors), with the exception of phenyl valeric acid, homovanillic acid, 3-(3,4-dihydroxyphenyl)propionic acid, phenylacetic acid, and 3-hydroxyphenylacetic acid, where choline-d_9 levels were slightly lower. Most screened compounds were able to significantly reduce choline-d_9 utilization compared to control (no inhibitors) between 6 – 10 h, the timeframe during which choline-d_9 is metabolized into TMA-d_9, as shown by control group kinetics. Besides the positive control (DMB 10 mM) treatment, caffeic acid, catechin, epicatechin and 3,4-dihydroxybenzoic acid were the only compounds able to maintain higher and statistically different residual choline-d_9 levels compared to the control treatment (choline-d_9 100 μM) out to the full 12 h (when choline-d_9 utilization and TMA-d_9 production was virtually over in control conditions). This is consistent with our previous data showing that different inhibitory compounds (i.e., chlorogenic acid), slow but may not indefinitely reduce choline-d_9 use and TMA-d_9 generation [19]. All treatments, except for vehicle (VH) and choline-d_9-free (CF) conditions, inhibited choline utilization somewhat as determined by statistically higher choline-d_9 AUCs vs. control conditions (**Figure 4A**) by one-way ANOVA.

In terms of TMA-d_9 production (**Figure 3**), several phenolic metabolites were unable to modulate TMA-d_9 levels compared to control (non-significant two-way ANOVA for treatment effect compared to control conditions), namely hippuric acid, phenylvaleric acid, 4-hydroxyphenylacetic acid, and 3-hydroxyphenylacetic acid. DMB 10 mM also reported a non-significant (p>0.05) two-way ANOVA treatment effect. Similarly, all these compounds except for 3-hydroxyphenylacetic acid reported non-significant differences in TMA-d_9 AUCs when compared to control conditions (**Figure 4B**) by one-way ANOVA. Surprisingly, positive control conditions (DMB 10 mM) did not report a lower TMA-d_9 than control conditions (choline-d_9 100 μM) despite reducing choline-d_9 utilization (**Figure 2**). The rest of the compounds reported a significantly reduced TMA-d_9 production curve compared to control. This included significantly reduced TMA-d_9 levels at one or more timepoints (from 6 – 12 h) for all compounds, and all of them reported a reduction of TMA-d_9 levels at 8h. As for TMA-d_9 production, the inhibitory effect often did no persist until 12

h. The only compounds that maintained lower TMA-d_9 levels when fermentation in the control group was virtually over (at 12 h) were *p*-coumaric acid, caffeic acid, catechin, epicatechin, vanillic acid, 3-(3,4-dihydroxyphenyl)propionic acid, 3,4-dihydroxyphenyacetic acid, and phenylacetic acid. The trends reported by tested compounds in choline-d_9 use and TMA-d_9 production were also generally maintained by background choline and TMA levels from endogenous substrates (**Figure 4** and **Supplementary figures 3 – 4**).

To further investigate the inhibitory effect of phenolic compounds and their metabolites, the relative percentages of remaining choline-d_9 and relative reductions in TMA-d_9 percentages against time-matched control conditions were evaluated (**Supplementary Tables 2** and **3**). For compounds that reported significant reductions in choline-d_9 levels at relevant metabolic timepoints (6 – 12 h), percentages of choline-d_9 remaining compared to choline-d_9 alone were 113.8 ± 1.5 % (vanillic acid) to 120.6 ± 3.7 % (homovanillic acid) at 6 h; 152.8 ± 3.9 % (phenylvaleric acid) to 265.1 ± 6.3% (catechin) at 8h; 243.1 ± 15.5 % (3-(3-hydroxypheny)propionic acid) to 615.4 ± 34.3 % (catechin) at 10 h; and 2028.7 ± 67.8 % (3,4-dihydroxybenzoic acid) to 4303.9 ± 69.9 % (catechin) at 12 h. For compounds that reported significant reductions in TMA-d_9 levels, the percentages of TMA-d_9 reduction compared to choline-d_9 alone were – 50.8 ± 1.1 % (caffeic acid) to – 56.8 ± 1.1 % (3-phenylpropionic acid) at 6 h; – 25.8 ± 3.9 % (3-(4-hydroxyphenyl)propionic acid) to – 72.5 ± 0.4 % (catechin) at 8 h; – 9.7 ± 3.7 % (3,4-dihydroxybenzoic acid) to – 42.0 ± 1.2 %(catechin) at 10 h; and – 6.5 ± 1.7 % (vanillic acid) to – 47.8 ± 0.9 % (catechin) at 12 h. Overall, these data reveal a possible disconnect between the inhibition of choline-d_9 use and TMA-d_9 production for each compound. For example, at 10 h epicatechin reported a – 24.2 ± 2.3 % decrease in TMA-d_9 levels and an increase of choline-d_9 levels of 409.7 ± 11.4 % compared to control. Nevertheless, the difference in total concentration is quite similar: TMA-d_9 concentrations were reduced by ~16 μM between epicatechin (~51 μM) and control (~67 μM) groups, and choline-d_9 were increased by ~21 μM between epicatechin (~29 μM) and control (~8 μM) groups.

3.2. Cell growth and cell respiration rate:

To evaluate whether changes in choline-d_9 use and TMA-d_9 production were possibly due to a cytotoxic or cytostatic effect of our treatments, cell density was evaluated throughout the fermentation (0 – 12 h) by monitoring sample optical density at 600 nm, and the MTT cell respiration rate assay was performed at the end (12 h) of the fermentation. Vehicle (VH) and microbiota-free conditions (MFC), both not inoculated with human fecal slurry, presented low 600 nm optical absorbance readings that did not change during 12 h incubation (Figure 5A), which was expected due to the lack of cells. Although cell density kinetics fluctuated during the fermentation in all other experimental conditions (Figure 5), only some compounds reported a significant (p<0.05) treatment effect by Two-way ANOVA when compared to control conditions (choline-d_9 100 μM), namely *p*-coumaric acid, phenylvaleric acid, vanillic acid, homovanillic acid, and 3,4-dihydroxyphenylacetic acid. Detailed information on the *post hoc* results for treatment's effect on cell density at individual time points can be found in Supplementary **Figure 5**. The cell respiration by the end of the fermentation (12 h) was significantly reduced due to treatment with 9 out of the 20 different tested compounds (**Figure 6**), namely: *p*-coumaric acid, ferulic acid, phenylvaleric acid, vanillic acid, 3-(4-hydroxyphenyl)propionic acid, 3-(3-hydroxyphenyl)propionic acid, 3-phenylpropionic acid, 3-(4-hydroxyphenyl)acetic acid, and 3-(3-hydroxyphenyl)acetic acid. However, only 3-phenylpropionic acid reduced relative cell respiration by a biologically relevant percentage (<80%). Although not statistically different from control conditions (choline-d_9 100 μM), both caffeic acid and epicatechin treatments reached relative cell respiration percentages > 120 % (i.e., increased respiration, indicative of enhanced respiratory capacity and/or greater cell numbers). To evaluate that tested compounds would not interfere in the MTT assay, we performed the assay with test compounds at working concentrations of 2 mM (without viable cells) under anaerobic conditions in PBS 1X 0.2% glucose (data not shown) as per the methodology followed for fermentation samples. Absorbances from test compounds without cells ranged from 0.05 – 0.07 AU, with control conditions (PBS 1X 0.2% glucose) values of 0.06 AU. Compared to typical absorbances of the MTT assay performed with with 10 μL of sample containing viable cells that typically were >0.250 AU, it appears that tested compounds do not significantly interfere with the MTT assay. Overall, 11 compounds presented statistically or biologically significant differences in cell density, reductions in relative cell respiration or both compared to choline-d_9 only control, but only two of these compounds also presented significant changes in TMA-d_9 production

compared to the control group (Figure 3), namely *p*-coumaric acid and 3,4-dihydroxyphenylacetic acid. In no cases were significant cytotoxic or cytostatic effects observed compared to control.

3.3 TMA lyase gene abundance analysis.

We hypothesized that one mechanism of action by which our tested compounds could lower TMA production would be alteration of bacterial community composition to reduce TMA lyase (CutC) abundance. We wished to quantify CutC gene copy number in fermentation samples in order to evaluate this possible mechanism, with CntA (carnitine monooxygenase oxygenase subunit, which converts carnitine to TMA) for comparison. To validate the assay, we tested our method with fermentation samples from control conditions (added choline-d_9 100 μM) as we postulated that these conditions would fecal select for bacterial cultures carrying the CutC gene (but not CntA), thus enriching for this gene. To test this, we measured 16s (bacterial control), CutC and CntA DNA levels using previously published primers. While 16s levels demonstrated significantly increased levels versus no template (water) controls that demonstrated increased Ct values as a function of dilution (**Supplementary Figure 7A**), measurements of CutC or CntA demonstrated no significant difference at any of the tested template DNA concentrations as compared to no template controls, even after extensive primer optimization (**Supplementary Figure 7B-C**). These data suggest that qPCR using these previously defined degenerative primers is not a viable tool in measuring CutC and CntA bacterial DNA levels in fecal fermentation samples. Thus, we were unable to evaluate the impact of our TMA-lowering phenolic compounds on CutC abundance in this high-throughput *ex vivo* fecal fermentation model system.

4. Discussion:

CVD is the leading cause of death worldwide, and atherosclerosis plays an important part in its pathogenesis [1,2]. Circulating TMAO is a relatively new biomarker in terms of risk factors for cardiovascular mortality [31,32]. So far, there is not an approved drug to reduce TMAO formation, but several strategies to reduce its formation have been proposed. These include reducing substrate intake, pre- and probiotics to alter gut microbiome composition and function, and direct TMA lyase inhibition [5]. For this last application, food bioactives have been shown to hold promising

TMAO-reducing properties [5]. Some natural compounds, such as DMB, reduce TMAO production by directly inhibiting TMA-lyase enzymes [29]. Other natural compounds may reduce circulating TMAO levels by modulating host gut microbiota composition and function [33,34] or other processes. Most natural compounds achieve TMAO reductions, at least in part, by targeting the gut microbiota and inhibiting the conversion of quaternary amines (i.e., choline) to TMA through various mechanisms [5]. We recently developed a high-throughput method to screen for natural compounds with TMA production inhibitory bioactivities. Through this methodology, we reported that two common dietary phenolic compounds (gallic acid and chlorogenic acid) significantly inhibit microbial choline metabolism to TMA in a non-toxic fashion [19]. Our current aim in this study was to further investigate the TMA production inhibitory properties of other common dietary phenolic compounds and their gut microbiota-derived metabolites. We evaluated the potential of 5 additional common phenolic compounds to inhibit microbial transformation of choline-d_9 into TMA-d_9. Catechin and epicatechin were also selected due to their high concentrations in commonly consumed foodstuffs such as tea, cocoa and grapes [10,11], as well as for their known cardioprotective functions [12,13]. *p*-Coumaric acid, caffeic acid and ferulic acid are found either bound or free in several commonly consumed foodstuffs, such as coffee and cereals [11,35,36]. Moreover, in addition to occurring in the diet, cinnamic acids *p*-coumaric, caffeic and ferulic acids can also be formed due to the microbial metabolism of certain phenolic compounds [15,37–39]. Of note, it has been shown that some of the bioactivities presumably attributed to native compounds may in fact belong to their microbial-derived metabolites [16,17], which typically have greater bioavailability and achieve higher circulating concentrations than the native forms. Therefore, we also investigated the effects on TMA-d_9 formation from phenylpropionic, phenylacetic and benzoic acid derivates, which are common microbial-derived metabolites of (epi)catechin and other phenolic compounds [11,18,40].

First, we tested whether TMA-d_9 could be formed by spontaneous degradation of choline-d_9 or if this was caried out primarily by the fecal slurry (presumably by the bacterial component) by incubating choline-d_9 with and without the presence of human fecal slurry (Figure 1). The modest decrease (~23 %) of choline-d_9 and the lack of detection of TMA-d_9 over 12 h under those conditions demonstrate that TMA-d_9 is not formed due to a spontaneous chemical reaction under fermentation conditions in the absence of the fecal slurry. This is in line with similar dependence

on the presence of fecal slurry for choline transformation into TMA that we previously reported [19]. When compared with fermentations inoculated with fecal slurry, where TMA-d_9 ranges from non-detected levels initially to 79.00 ± 2.34 μM at 12 h and choline-d_9 from 94.20 ± 2.71 initially to 0.74 ± 0.07 μM at 12 h, these results clearly indicate that TMA-d_9 is formed through a microbial-dependent pathway. These results are in line with our fermentation optimization study using unlabeled choline as a substrate [19], and that of others [30,41]. Additionally, using choline-d_9 (as opposed to unlabeled choline) provides a cleaner assay with less background interference and a direct stoichiometric relationship between exogenous labeled choline-d_9 substrate and the resulting labeled TMA-d_9 product.

The effect of phenolic compounds and their microbial-derived metabolites on TMA-d_9 production were evaluated at a dose of 2 mM, which is in line with previous studies and our prior efficacy data [19]. All tested phenolic compounds and metabolites reported higher choline-d_9 levels than control (inhibitor-free) conditions for at least one timepoint over the 12 h course of fermentation (**Figure 2, Supplementary Figure** 1). All of them were able to maintain higher choline-d_9 levels compared to control (inhibitor-free) conditions at one or more timepoints between 6 – 10 h, the time when most choline-d_9 is metabolized into TMA-d_9 in the control group (**Supplementary Figure 1**). These effects were reflected in a lower TMA-d_9 formation on that time interval (**Figure 3, Supplementary Figure 2**), although not reaching statistical significance in all cases. Due to our statistical treatment of our kinetic data, where two-way ANOIVA is applied to evaluate the effect of each phenolic compound against the control (100 μM choline-d_9) one by one, without including any other tested compound, chances of type-I error are higher. In other words, since not all compounds are compared to the control at the same time, there is a higher experiment-wise chance of obtaining statistically significant results for any specific compound, which in this case are compounds that decrease choline-d_9 use and TMA-d_9 production. However, the results observed in our kinetic plots are maintained when AUCs are calculated. For AUCs statistical analysis, one-way ANOVA with Dunnett's *post hoc* is applied, where although each compound is compared to control conditions, the effect of other compounds is considered too by controlling the experiment-wise false positive rate. Thus, chances of reporting more statistically significant results decrease. Overall, our results demonstrate that phenolic compounds/metabolites inhibit choline-d_9 microbial metabolism into TMA-d_9 during the timeframe in which exogenous substrate was consumed in the

absence of inhibitors. At the end of the fermentation (12 h), most compounds presented non-significantly different levels of choline-d_9 and TMA-d_9 compared to the control (inhibitor-free) group. These data suggest that most phenolic compounds and their metabolites studied in this manuscript are delaying choloine-d_9 microbial metabolism into TMA-d_9. The loss of efficacy over time may be explained at least partly by the fact that these phenolic inhibitors themselves are degraded by the microbiota. Similar trends have been reported for blood orange juice phenolic compounds when choline and L-carnitine were used as substrates [41]. Of note, however, a few select compounds were able to maintain both higher choline-d_9 and lower TMA-d_9 throughout the entire experiment to the time the fermentation in the inhibitor-fee control group was virtually over (12 h). These compounds were caffeic acid, catechin, epicatechin and 3,4-dihydroxybenzoic acid. These data suggest that these four compounds possessed particularly potent and lasting activities in this model. Of note, flavan-3-ols and flavan-3-ol rich foods have reported mixed *in vivo* TMAO reducing properties [34,42], but our *in vitro* data suggest that monomeric flavan-3-ol catechin and epicatechin are potential candidates to study in *in vivo* models. It is important to acknowledge that the effects of phytochemicals could widely vary depending on gut microbiota composition. For example, in the study of Bresciani *et al.* [41], coffee, a rich source of caffeic acid, reported different effects depending of quaternary amine substrate (i.e., choline or L-carnitine) and fecal slurry origin (i.e., vegetarian or omnivorous). We have reported that caffeic acid is one of the most promising phenolic compounds to reduce TMA formation in this study, along with catechin, epicatechin and 3,4-dihydroxybenzoic acid. The effects of these compounds should be tested in *in vivo* models that capture the diversity of gut microbiota found in humans.

Noteworthy, the inhibitory effects of DMB in choline-d_9 use and TMA-d_9 production were not really marked despite being a known TMA-lyase inhibitor *in vivo* [29]. However, in a similar *ex vivo-in vitro* fermentation set-up like ours, Day-Walsh *et al.* reported a similar behavior of DMB at 10 mM [30]. Even in our previous study, DMB inhibitory effect of choline use and TMA production fluctuated [19]. Perhaps the insoluble water nature of DMB plays a role in such results. However, we have evaluated DMB solubility in growth media, which contains agents that should help its solubilization (i.e., Tween 80), and observed no such effect. Nevertheless, DMB might fall out of solution over time, which might explain the disconnect between its *in vitro* and *in vivo* inhibitory potential.

Whether or not the effects reported by these tested phenolic compounds and microbial-derived metabolites were due to cytotoxicity or cytostatic mechanisms (undesirable *in vivo*) is a critical concern. Indeed, antibacterial properties of hydroxycinnamic acids have been found at concentrations between 0.9 – 9 mM in different bacteria strains [43]. Antibacterial properties of catechin and epicatechin have also been reported, but at higher concentrations (i.e., > 20 mM) [44,45]. To shed some light into that, we monitored cell density over the fermentation course by analyzing each fermentation sample's optical density at 600 nm, and evaluated cell respiration at its end (12 h) using the MTT assay. The compounds that significantly altered cell density over time (significant Two-way ANOVA treatment effect) when compared to the inhibitor-free control group were *p*-coumaric acid, phenylvaleric acid, vanillic acid, homovanillic acid, and 3,4-dihydroxyphenylacetic acid. The relative cell respiration was reduced by *p*-coumaric acid, ferulic acid, phenylvaleric acid, vanillic acid , 3-(4-hydroxyphenyl)propionic acid, 3-(3-hydroxyphenyl)propionic acid, 3-phenylpropionic acid, 4-hydroxyphenylacetic acid, and 3-hydroxyphenylacetic acid when compared to the control group. Out of the 11 compounds that presented either changes in cell density or reductions in relative cell respiration only *p*-coumaric acid and 3,4-dihydroxyphenylacetic acid presented relevant changes in TMA-d_9 production compared to the control group (**Figure 3**). Thus, it is possible that the results reported by only *p*-coumaric acid and 3,4-dihydroxyphenylacetic acid could be partially optioned by an anti-bacterial (cytotoxic and/or cytostatic) effect. It is important to note that tested compounds did not interfere with the MTT assay (data not shown), unlike what we have previously reported for chlorogenic acid and gallic acid [19]. The compounds with the widest, most relevant reductions in TMA-d_9 levels and choline-d_9 use, namely caffeic acid, catechin, epicatechin and 3,4-dihydroxybenzoic acid, did not report significant changes in cell density or relative cell respiration. This suggests that none of these compounds achieved TMA-d_9 inhibition production by a broad-spectrum anti-bacterial mechanism, indicating other mechanisms of action and potential suitability for *in vivo* inhibition.

Whether or not reductions in TMA-d_9 production are achieved by selectively killing and/or inhibiting certain bacteria strains that carry TMA lyase genes, and/or by promoting the growth of others is unknown. For example, catechin and caffeic acid supplementation has been shown to change gut microbiota composition in different models [46,47]. Thus, the reduction in TMA-d_9

production by these compounds could be achieved through a prebiotic effect, which in this context would be promoting the growth of non-TMA producing bacteria over TMA-producing ones [48,49]. Indeed, it could also be possible that the reduction in TMA-d_9 production shown by caffeic acid, catechin, epicatechin and 3,4-dihydroxybenzoic acid was achieved because bacteria preferred those compounds as sources of energy than choline-d_9 itself. As a matter of fact, Bresciani *et al.* [41] found that the blood orange juice components with the most potent inhibition of TMA production from choline and L-carnitine were sugars (i.e., glucose, fructose and sucrose), and not phenolic compounds (i.e., ferulic acid, hesperidin and cyanidin-3-glucoside). In this sense, caffeic acid, catechin and epicatechin are compounds that can be metabolized by gut microbiota into smaller phenolic acids [50,51], thus serving as sources of energy. Overall, the phenolic microbial metabolites were not as efficient at reducing TMA-d_9 production or choline-d_9 use as the native phenolic compounds. Thus, our data seem to indicate that larger phenolic compounds are better TMA-d_9 production inhibitors, which supports the hypothesis that their bioactivity may be due to their use by the bacteria as energy-production substrates. The only exception to this are 3-(3,4-dihydroxyphenyl)propionic acid and 3,4-dihydroxyphenylacetic acid, which are phenolic acid metabolites typically formed after native compounds' microbial catabolic pathways [15,52,53]. Although the results reported by 3-(3,4-dihydroxyphenyl)propionic acid could be partially explained by a toxic effect, this suggests that those compounds might achieve TMA-d_9 production inhibition by directly inhibiting TMA-lyase.

Further work remains to be done to determine the mechanism(s) by which these phenolics inhibit TMA production. First, inhibition experiments should be performed with cell lysates or recombinant TMA lyase isoforms to determine whether direct inhibition is occurring, and the nature of the inhibitory mechanism (competitive, non-competitive, etc.). In the present study, we repeatedly but unsuccessfully tried to use lysates of fecal slurry bacteria to release TMA-lyase enzymes with which to perform enzyme inhibition studies. To our knowledge, only Wand *et al.* [29] have conducted similar studies, but only with pure bacteria cell lines. Of note, only 0.16 ± 0.15 % of all bacteria in human fecal samples present specific TMA-lyases for choline [5], which may explain our unsuccessful results to produce cell lysates with relevant TMA-lyase activity. Second, evaluation of probiotic (microbiome-shifting) effects of tested compound should be performed. As a matter of fact, although changes in cell respiration rate and cell density give an overview of

potential cytotoxic effects, an evaluation of changes in bacteria function, understood as changes in the levels of bacteria with the capacity to metabolize choline-d_9 into TMA-d_9 (CutC gene abundance), should be performed. This would provide relevant information on gut microbiota function modulation as a potential mechanism of action. As described above, we attempted multiple times to quantify the levels of CutC in our samples via qPCR using previously reported degenerative primers, but were unable to amplify this gene. The low abundance of bacteria containing TMA-lyase gene CutC [5], the use of diluted human fecal slurries with a wide diversity of bacteria with different CutC isoforms, and the use of degenerative primers to capture the diversity in CutC gene sequences [27] might have been some obstacles not overcome in our attempts. Additionally, studies have suggested that capacity for TMA production may not correlate well with gut levels of CutC/CutD gene expression [54]. Utilization of other primers may be able to achieve this goal of characterizing the impact of phenolic compounds on TMA lyase abundance in subsequent projects. Third, work must be done to determine whether the mechanism of action is unique to specific TMA lyase forms from distinct bacteria, and how broadly applicable these inhibitory activity activities are to the unique microbial communities in individual subjects. Finally, there are thousands of additional bioactive plant secondary metabolites (phenolics, terpenoids and alkaloids) that could be screened for TMA reducing activities. We believe that these encouraging findings, coupled with the utility of an exogenous labeled substrate assay in a 96-well (or potentially 384-well) assay [19], represent a framework for accomplishing the needed advances in this area.

Overall, caffeic acid, catechin, epicatechin and 3,4-dihydroxybenzoic acid were the compounds that reduced TMA-d_9 production and choline-d_9 use without interfering with cell growth and cell respiration. These compounds are led candidates to be tested in subsequent *in vitro* experiments.

Abbreviations used:

CF: Choline-d_9-free conditions.
CVD: Cardiovascular disease.
DMB: 3,3-Dimethil-1-butanol.
LOD: Limit of detection.
LOQ: Limit of quantification.

MDL: Method detection limit.

MFC: Microbiota-free conditions.

MQL: Method quantification limit.

TMAO: Trimethylamine *N*-oxide.

TMA: Trimethylamine.

VH: Vehicle.

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Supporting Information:

Individual choline-d_9 and TMA-d_9 kinetic curves, kinetic curves for endogenous substrate (unlabeled choline) and product (unlabeled TMA), cell density time course data, MTT assay interference data, qPCR data, two-way ANOVA main effects and interactions significance summary table, % inhibition of choline-d_9 utilization and TMA-d_9 production at individual time points.

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Tables:

Table 1: Optimized multi-reaction monitoring conditions for detection of choline, TMA and related compounds.

Compound	MW	RT (min)	MS/MS transition	CV (V)	CE (eV)
Choline	103.2	1.05	104.2>60.0	38	16
Choline-d_9	112.2	1.05	113.3>69.1	40	16
Choline-1-^{13}C-1,1,2,2-d_4	108.2	1.05	109.3>60.3	36	18
Ethyl betaine [a]	145.2	0.77	146.3>118.2	34	16
Ethyl betaine-d_9 [a]	154.2	0.77	155.3>127.2	34	20
Ethyl betaine-$^{13}C_3$-d_4 [a]	149.2	0.77	150.3>122.2	34	18

Abbreviations: TMA, trimethylamine; MW, molecular weight; RT, retention time; CV, cone voltage; and CE, collision energy.
[a] TMA derivatives

Table 2: Parameters for the quantification of choline-d_9 and TMA-d_9 (as well as non-d_9 isotopes) in spiked fecal fermentation samples by UPLC-MS/MS/.

Compound	Calibration curve	R^2	Working linear range (µM)	LOD (nM)	LOQ (nM)	MDL (nM) [b]	MQL (nM) [b]
Choline-d_9	y = 0.4401x + 0.4748	0.9974	1.0 – 300.0	63.2	210.8	126.5	421.6
Ethyl betaine-d_9 [a]	y = 0.0683x + 0.1919	0.9973	1.0 – 300.0	28.7	95.7	57.4	191.6

Abbreviations: R^2, determination coefficient; LOQ, limit of detection; LOQ, limit of quantification; MDL, method detection limit; MQL, method quantification limit.
[a] TMA derivatives.
[b] MDL and MQL for 25 µL of fecal fermentation media.

Figures:

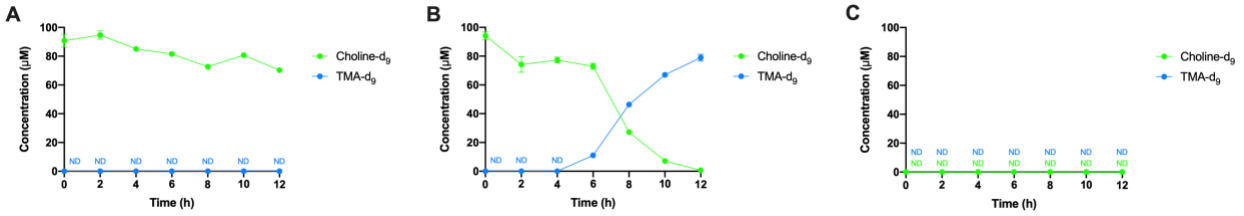


Figure 1: Choline-d_9 and TMA-d_9 kinetic curves in fermentations with growth media and: 100 µM choline-d_9 and without fecal slurry (**A**); 100 µM choline-d_9 and 20% fecal slurry (**B**); without 100 µM choline-d_9 and with 20% fecal slurry (**C**). Results are expressed as mean µM ± SEM (*n*=4). Abbreviations: ND, not detected.

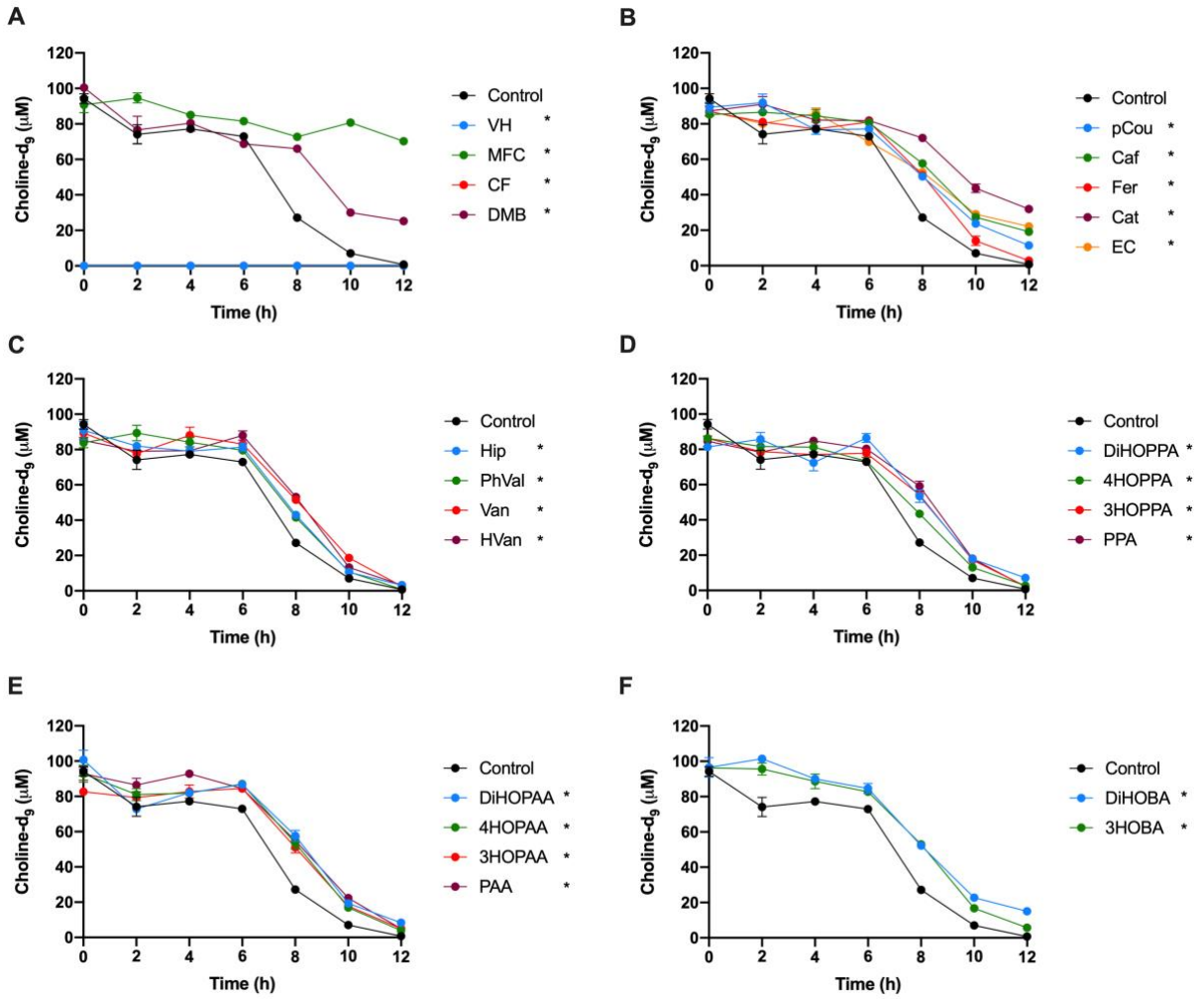


Figure 2: Choline-d_9 kinetic curves in fermentations with fecal slurry 20 %, 100 μM choline-d_9 and different test compounds (2 mM, except DMB 10 mM). * Indicates statistical differences (p<0.05) in treatment main effect by Two-way ANOVA between control conditions (no inhibitors, choline-d_9 100 μM) and test compound (factors: treatment, time). Results are expressed as mean μM ± SEM (n=4). Abbreviations: VH, vehicle (choline-d_9 0 μM, no fecal slurry); MFC, microbiota-free conditions (no fecal slurry); CF, choline-d_9-free conditions (choline-d_9 0 μM); DMB, 3,3-dimethyl-1-butanol; pCou, *p*-coumaric acid; Caf, caffeic acid; Fer, ferulic acid; Cat, catechin; EC, epicatechin; Hip, hippuric acid; PhVal, phenylvaleric acid; Van, vanillic; HVan, homovanillic acid; DiHOPPA, 3-(3,4-dihydroxyphenyl)propionic acid; 4HOPPA, 3-(4-hydroxyphenyl)propionic acid; 3HOPPA, 3-(3-hydroxyphenyl)propionic acid; PPA, 3-phenylpropionic acid; DiHOPAA, 3-(3,4-dihydroxyphenyl)acetic acid; 4HOPAA, 3-(4-hydroxyphenyl)acetic acid; 3HOPAA, 3-(3-hydroxyphenyl)acetic acid; PAA, phenylacetic acid; DiHOBA, 3,4-dihydroxybenzoic acid; and 3HOBA, 3-hydroxybenzoic acid. Note that the control treatment group is plotted on all graphs for ease of comparison.

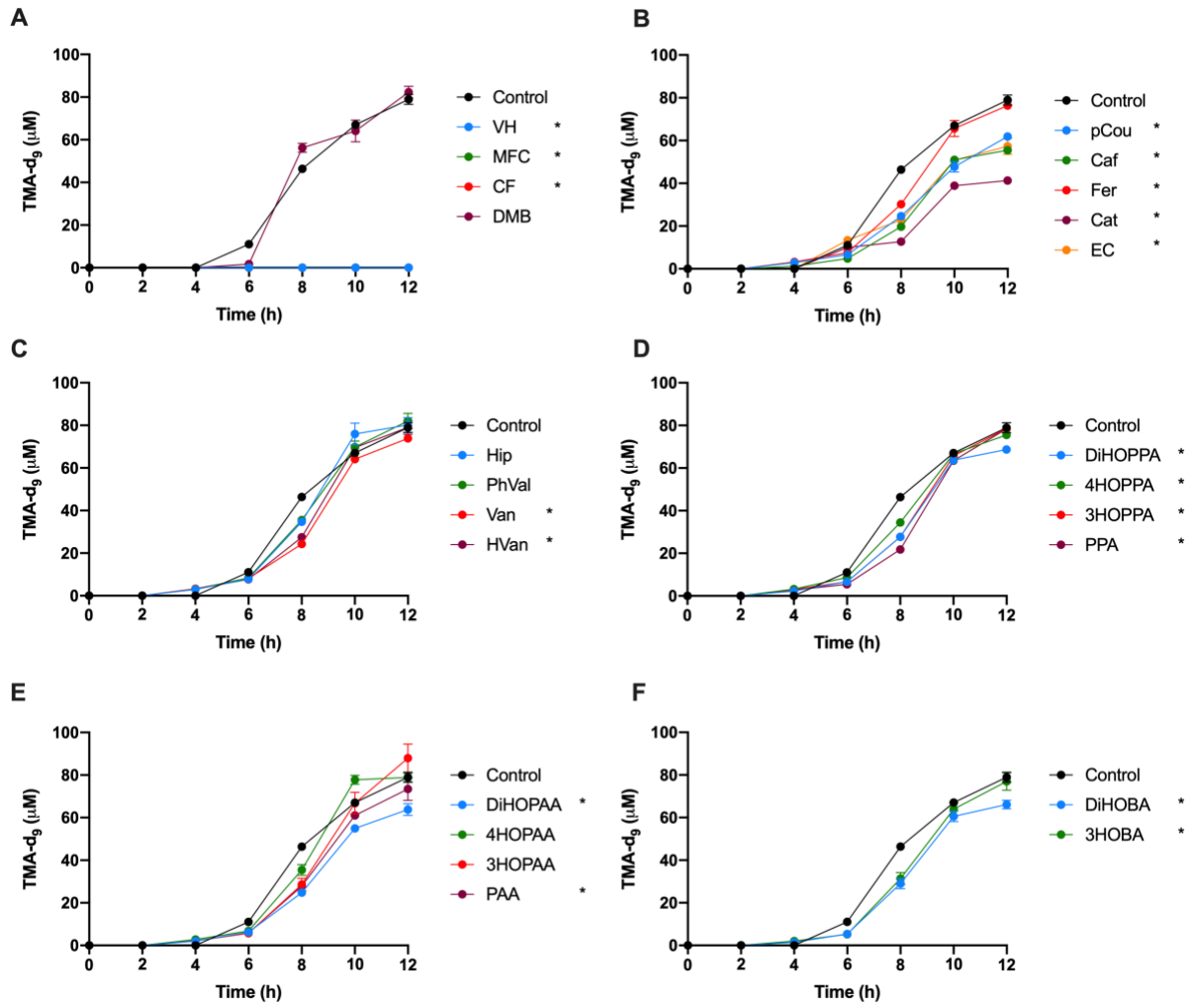


Figure 3: TMA-d_9 kinetic curves in fermentations with fecal slurry 20 %, 100 μM choline-d_9 and different test compounds (2 mM, except DMB 10 mM). * Indicates statistical differences (p<0.05) in treatment effect by Two-way ANOVA between control conditions (no inhibitors, choline-d_9 100 μM) and test compound (factors: treatment and time). Results are expressed as mean μM ± SEM (n=4). Abbreviations: VH, vehicle (choline-d_9 0 μM, no fecal slurry); MFC, microbiota-free conditions (no fecal slurry); CF, choline-d_9-free conditions (choline-d_9 0 μM); DMB, 3,3-dimethyl-1-butanol; pCou, *p*-coumaric acid; Caf, caffeic acid; Fer, ferulic acid; Cat, catechin; EC, epicatechin; Hip, hippuric acid; PhVal, phenylvaleric acid; Van, vanillic; HVan, homovanillic acid; DiHOPPA, 3-(3,4-dihydroxyphenyl)propionic acid; 4HOPPA, 3-(4-hydroxyphenyl)propionic acid; 3HOPPA, 3-(3-hydroxyphenyl)propionic acid; PPA, 3-phenylpropionic acid; DiHOPAA, 3-(3,4-dihydroxyphenyl)acetic acid; 4HOPAA, 3-(4-hydroxyphenyl)acetic acid; 3HOPAA, 3-(3-hydroxyphenyl)acetic acid; PAA, phenylacetic acid; DiHOBA, 3,4-dihydroxybenzoic acid; and 3HOBA, 3-hydroxybenzoic acid. Note that the control treatment group is plotted on all graphs for ease of comparison.

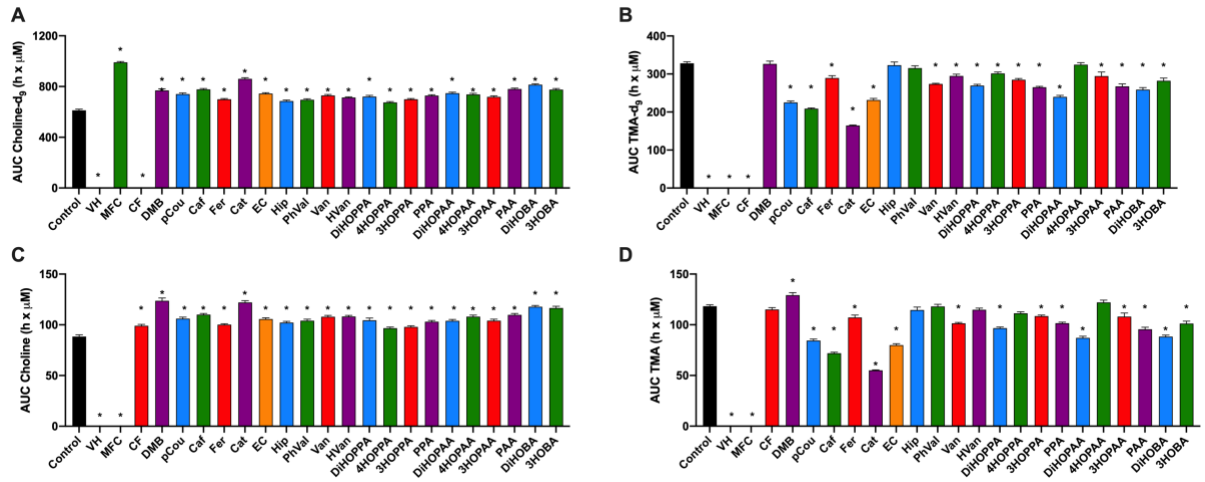


Figure 4: Area under the curve (AUC) of the kinetic curves of choline-d_9 (**A**), TMA-d_9 (**B**), choline (**C**) and TMA (**D**). * Indicates statistical difference (p<0.05) against control conditions (choline-d_9 100 μM) by One-way ANOVA (Dunnetts' *post hoc* test). Results are expressed as mean h x μM ± SEM (n=4). Abbreviations: Control, choline-d_9 100 μM; VH, vehicle (choline-d_9 0 μM); MFC, microbiota-free conditions; CF, choline-d_9-free conditions (choline-d_9 0 μM); DMB, 3,3-dimethyl-1-butanol; pCou, *p*-coumaric acid; Caf, caffeic acid; Fer, ferulic acid; Cat, catechin; EC, epicatechin; Hip, hippuric acid; PhVal, phenylvaleric acid; Van, vanillic; HVan, homovanillic acid; DiHOPPA, 3-(3,4-dihydroxyphenyl)propionic acid; 4HOPPA, 3-(4-hydroxyphenyl)propionic acid; 3HOPPA, 3-(3-hydroxyphenyl)propionic acid; PPA, 3-phenylpropionic acid; DiHOPAA, 3-(3,4-dihydroxyphenyl)acetic acid; 4HOPAA, 3-(4-hydroxyphenyl)acetic acid; 3HOPAA, 3-(3-hydroxyphenyl)acetic acid; PAA, phenylacetic acid; DiHOBA, 3,4-dihydroxybenzoic acid; and 3HOBA, 3-hydroxybenzoic acid. Note that the control treatment group is plotted on all graphs for ease of comparison.

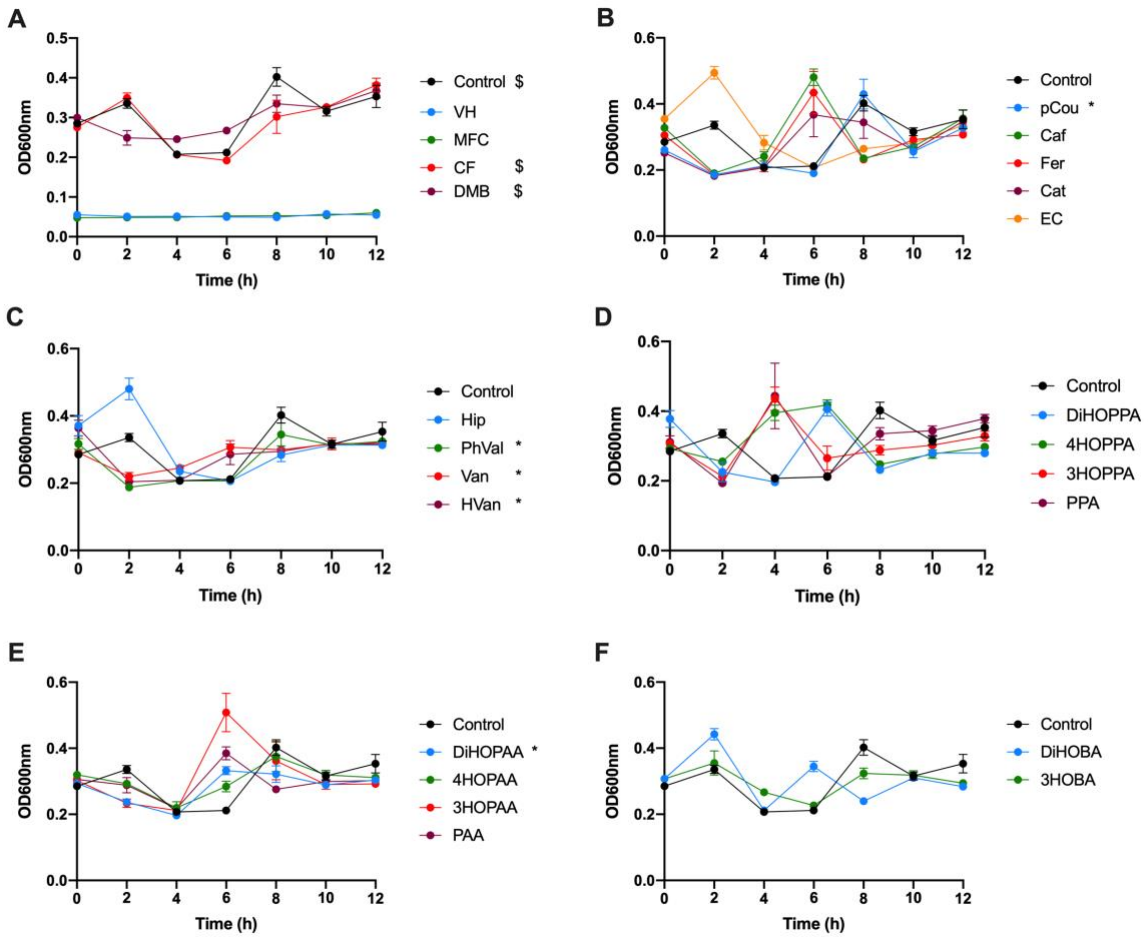
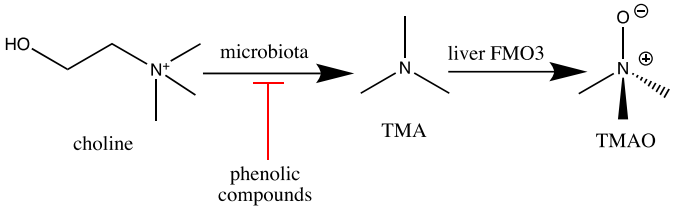


Figure 5: Cell density measured as optical density at 600 nm (OD 600nm) in fermentations with fecal slurry 20 %, 100 μM choline-d_9 and different test compounds (2 mM, except DMB 10 mM). $ Indicates statistical differences (p<0.05) in treatment effect by Two-way ANOVA against vehicle (VH); # Indicates statistical differences (p<0.05) in treatment effect by Two-way ANOVA against choline-d_9-free conditions (CF). * Indicates statistical differences (p<0.05) in treatment effect by Two-way ANOVA against control conditions (choline-d_9 100 μM). Results are expressed as mean arbitrary units ± SEM (n=4). Abbreviations: VH, vehicle (choline-d_9 0 μM, no fecal slurry); MFC, microbiota-free conditions (no fecal slurry); CF, choline-d_9-free conditions (choline-d_9 0 μM); DMB, 3,3-dimethyl-1-butanol; pCou, *p*-coumaric acid; Caf, caffeic acid; Fer, ferulic acid; Cat, catechin; EC, epicatechin; Hip, hippuric acid; PhVal, phenylvaleric acid; Van, vanillic; HVan, homovanillic acid; DiHOPPA, 3-(3,4-dihydroxyphenyl)propionic acid; 4HOPPA, 3-(4-hydroxyphenyl)propionic acid; 3HOPPA, 3-(3-hydroxyphenyl)propionic acid; PPA, 3-phenylpropionic acid; DiHOPAA, 3-(3,4-dihydroxyphenyl)acetic acid; 4HOPAA, 3-(4-hydroxyphenyl)acetic acid; 3HOPAA, 3-(3-hydroxyphenyl)acetic acid; PAA, phenylacetic acid; DiHOBA, 3,4-dihydroxybenzoic acid; and 3HOBA, 3-hydroxybenzoic acid. Note that the control treatment group is plotted on all graphs for ease of comparison.

865 **For Table of Contents Only**



866