

**Potential of phenolic compounds and their gut microbiota-derived metabolites to reduce 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. The most bioactive compounds tested (caffeic acid, catechin and epicatechin) reduced TMA-d<sub>9</sub> formation (compared to control) by  $57.5 \pm 1.3\%$  to  $72.5 \pm 0.4\%$  at 8 h and preserved remaining choline-d<sub>9</sub> concentrations by  $194.1 \pm 6.4\%$  to  $256.1 \pm 6.3\%$  compared to control conditions at 8 h. These inhibitory effects were achieved 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; TMA.

## 1. Introduction:

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

Quaternary amines from the diet, such as choline and L-carnitine, can be metabolized by the gut microbiota to trimethylamine (TMA) <sup>6,7</sup>, which is absorbed into circulation and further metabolized by host hepatic flavin-containing monooxygenase 3 (FMO3) into TMAO <sup>8</sup>. 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) <sup>6</sup>. 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 <sup>9</sup>. Several phytochemicals have reported promising potential to reduce TMAO formation in humans <sup>5</sup>. 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 <sup>10-13</sup>. 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 <sup>14</sup>. 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 <sup>15</sup>. 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* <sup>16,17</sup>. Microbial metabolites of flavan-3-ols include low molecular weight phenolic acids such as phenylpropionic, phenylacetic and benzoic acid derivatives <sup>11,15,18</sup>. Microbial metabolites of hydroxycinnamic acids include caffeic, ferulic and *p*-coumaric acids <sup>14</sup>.

We previously developed a high-throughput 96-well *ex vivo* anaerobic fecal fermentation assay to assess inhibition of TMA production from choline<sup>19</sup>. 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<sup>14,20,21</sup>, 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<sup>19</sup>. The aim of this study was to evaluate the potential of major hydroxycinnamic acids and flavan-3-ols in the diet and their gut-derived metabolites to inhibit microbial conversion of choline to TMA. The rationale for this study is that potent inhibitors can then be further evaluated for mechanism and dose-dependence *in vitro* and inhibitory activity *in vivo* in subsequent studies.

## 2. Materials and methods:

### 2.1. Chemicals and reagents:

Glucose, peptone water, yeast extract, KCl, NaCl, Na<sub>2</sub>HPO<sub>4</sub>, KH<sub>2</sub>PO<sub>4</sub>, MgSO<sub>4</sub>×7H<sub>2</sub>O, CaCl<sub>2</sub>×6H<sub>2</sub>O, ZnSO<sub>4</sub>×7H<sub>2</sub>O, NaHCO<sub>3</sub>, 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<sub>9</sub>, choline-1-<sup>13</sup>C-1,1,2,2-d<sub>4</sub>, TMA, TMA-d<sub>9</sub>, TMA-<sup>13</sup>C<sub>3</sub>-<sup>15</sup>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). All tested phenolic compounds presented a purity ≥98 %. 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 <sup>19</sup>, which was originally adapted from Alqurashi *et al.* <sup>22</sup>. The composition of 1 L of growth medium was 2 g peptone water, 2 g yeast extract, 0.1 g NaCl, 40 mg Na<sub>2</sub>HPO<sub>4</sub>, 40 mg KH<sub>2</sub>PO<sub>4</sub>, 10 mg MgSO<sub>4</sub> · 7H<sub>2</sub>O, 10 mg CaCl<sub>2</sub> · 6H<sub>2</sub>O, 2 g NaHCO<sub>3</sub>, 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<sub>2</sub> (g) under agitation and were then combined 1:1 in the anaerobic chamber (O<sub>2</sub> < 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<sub>2</sub> (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<sub>2</sub>, 5 % CO<sub>2</sub> and 90 % N<sub>2</sub> (Airgas, Durham, NC, USA). H<sub>2</sub> (typically 2 – 3 %) and O<sub>2</sub> (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 <sup>19</sup>, choline-d<sub>9</sub> at a final concentration of

100  $\mu\text{M}$  was added as the substrate, and the resulting TMA- $\text{d}_9$  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.*<sup>19</sup>. 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  $\mu\text{m}$ ) MicroSolv AQ syringe filter inside the anaerobic chamber. In 1.1 mL 96-well plates, 675  $\mu\text{L}$  of test compound solutions were mixed with 45  $\mu\text{L}$  of choline- $\text{d}_9$  stock solution (2 mM) in PBS 1X and 180  $\mu\text{L}$  fecal slurry (1:10 in PBS 1X,) to reach a final well volume of 900  $\mu\text{L}$  containing test compound at 2 mM (except for DMB, at 10 mM), choline- $\text{d}_9$  at 100  $\mu\text{M}$  and original fecal slurry at 2 %. These conditions were optimized previously<sup>19</sup>. 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  $\mu\text{L}$  was sampled from 0 to 12 h in 2 h intervals, analyzed for cell density (see section 2.7), then combined with 100  $\mu\text{L}$  acetonitrile and immediately frozen at – 80 °C until used for choline- $\text{d}_9$  and TMA- $\text{d}_9$  analyses. Control conditions were defined as growth media and PBS with choline- $\text{d}_9$  100  $\mu\text{M}$  in fermentations with 2 % fecal slurry (no inhibitors); vehicle conditions (VH) were defined as growth media and PBS; choline- $\text{d}_9$ -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  $\mu\text{M}$  choline- $\text{d}_9$  only. PBS 1X was used to adjust volumes to 900  $\mu\text{L}$ . 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- $\text{d}_9$ and TMA- $\text{d}_9$ :**

Externally added choline- $\text{d}_9$  and produced TMA- $\text{d}_9$  in fermentation media were extracted according to the previously reported methodology<sup>23,24</sup> with slight modifications. TMA- $\text{d}_9$  requires a derivatization process to the quaternary amine compound ethyl betaine- $\text{d}_9$  to facilitate LC-MS/MS ionization. Briefly, 25  $\mu\text{L}$  of fermentation sample were mixed with 20  $\mu\text{L}$  of TMA- $^{13}\text{C}_3$ - $^{15}\text{N}$  internal standard solution (10  $\mu\text{M}$ , for derivatization to ethyl betaine- $\text{d}_9$  or ethylbetaine- $^{13}\text{C}_3$ -

<sup>15</sup>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<sub>9</sub> 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<sub>9</sub>, 25 µL of sample were mixed with 10 µL of ZnSO<sub>4</sub> solution (5 % w/v in water), 100 µL acetonitrile and 20 µL choline-1-<sup>13</sup>C-1,1,2,2-d<sub>4</sub> (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<sub>9</sub>/choline-d<sub>9</sub> extraction and their analysis. Endogenous choline and TMA from fecal fermentation media were also extracted and analyzed as for exogenous choline-d<sub>9</sub> and TMA-d<sub>9</sub>.

## **2.6. Chromatographic analysis and quantification of choline-d<sub>9</sub> and TMA-d<sub>9</sub>:**

TMA-d<sub>9</sub> and TMA were analyzed separately from choline-d<sub>9</sub> 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. Column 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<sub>2</sub>) 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<sub>9</sub> and TMA-d<sub>9</sub> 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  $\mu$ L 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  $\mu$ L fermentation media at 600 nm in a SpectraMax iD3 plate reader (Molecular Devices, San Jose, CA, USA) at every sampling time (0 – 12 h). A cell respiration rate assay was used to estimate cell viability at 12 h. The assay in particular is usually referred to as mitochondrial toxicity test in eucaryotic cells. However, this respiration assay has been previously used to test cell respiration and viability in prokaryotic (i.e., bacteria) cells, which lack mitochondria<sup>19,25,26</sup>. Briefly, 10  $\mu$ L of fermentation mixture was mixed with 80  $\mu$ L of pre-heated (37 °C) PBS 1X with glucose 0.2% (m/v) and 10  $\mu$ L of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide at a concentration of 25 mg/mL in PBS 1X<sup>25</sup>. 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  $\mu$ L 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  $\mu$ m sterile filtering system, Corning) before their use. Results are expressed as percentages of change versus choline-free conditions  $\pm$  SEM ( $n=4$ ).

## **2.9 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<sub>9</sub>, TMA-d<sub>9</sub>, choline and TMA kinetic curves and cell density values. If a significant main effect or interaction ( $p < 0.05$ ) was reported for choline-d<sub>9</sub> and TMA-d<sub>9</sub> kinetic curves or cell density values, Sidak's *post hoc* test was used to estimate time-matched differences between control conditions (choline-d<sub>9</sub> 100  $\mu$ M) and test compounds. One-way ANOVA was used to estimate differences in choline-d<sub>9</sub>, TMA-d<sub>9</sub>, choline and TMA AUCs and cell respiration between control conditions (choline-d<sub>9</sub> 100  $\mu$ M) and test compounds (Dunnetts' *post hoc* test). In all cases, statistical significance was established *a priori* as  $p < 0.05$ .

### 3. Results:

#### 3.1. Inhibition of choline-d<sub>9</sub> use and TMA-d<sub>9</sub> production by phenolic compounds and their microbial-derived metabolites:

First, microbial dependence of TMA-d<sub>9</sub> production and choline-d<sub>9</sub> degradation were evaluated in fermentation conditions with 100  $\mu$ M exogenous choline-d<sub>9</sub> free of human fecal slurry (**Figure 1A**). Under those conditions, choline-d<sub>9</sub> levels remained fairly constant between  $90.79 \pm 4.41 \mu$ M (0 h) –  $70.28 \pm 0.08 \mu$ M (12 h), which represents a ~23 % degradation of initial choline-d<sub>9</sub> concentration over 12 h. Despite slight decrease in apparent choline-d<sub>9</sub> levels, TMA-d<sub>9</sub> was not detected during these 12 h. When fecal slurry was added (**Figure 1B**), choline-d<sub>9</sub> concentrations varied from  $94.20 \pm 2.71 \mu$ M (0h) to  $0.74 \pm 0.07 \mu$ M (12h), and TMA-d<sub>9</sub> levels increased from not detected (0 – 4 h) to  $79.00 \pm 2.34 \mu$ M (at 12 h). Choline-d<sub>9</sub> degradation and TMA-d<sub>9</sub> production mirrored each other. To evaluate if the growth media and fecal slurry contributed to choline-d<sub>9</sub> and TMA-d<sub>9</sub> signals, their levels were monitored in choline-d<sub>9</sub>-free conditions (**Figure 1C**); none of these two compounds were detected at any timepoint. Similarly, no choline-d<sub>9</sub> and TMA-d<sub>9</sub> were reported in vehicle (PBS and growth media, but no choline-d<sub>9</sub> or fecal slurry) conditions at any timepoint (**Figure 1C**). These results indicate that exogenous substrate (choline-d<sub>9</sub>) was almost completely consumed within 12 h, and roughly 84% was converted to TMA-d<sub>9</sub> within that timeframe (given the 1-to-1 stoichiometry of choline-d<sub>9</sub> bacterial fermentation to TMA-d<sub>9</sub>), consistent with our previous data<sup>19</sup>. Thus, conversion of choline-d<sub>9</sub> to TMA-d<sub>9</sub> appears to be microbially-dependent and is not affected by endogenous/background choline/TMA.

After validating that formation of TMA-d<sub>9</sub> required the presence of both fecal bacteria and choline-d<sub>9</sub> substrate, we evaluated the potential inhibition of microbial conversion of choline-d<sub>9</sub> to TMA-d<sub>9</sub> 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<sub>9</sub> and TMA-d<sub>9</sub> 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<sup>19</sup> and those of others<sup>27,28</sup>.

At the start of fermentation, most treatment conditions presented non-significantly different levels of choline-d<sub>9</sub> (**Figure 2**) against the control group (choline-d<sub>9</sub> 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<sub>9</sub> levels were slightly lower. Most screened compounds were able to significantly reduce choline-d<sub>9</sub> utilization compared to control (no inhibitors) between 6 – 10 h, the timeframe during which choline-d<sub>9</sub> is metabolized into TMA-d<sub>9</sub>, 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<sub>9</sub> levels compared to the control treatment (choline-d<sub>9</sub> 100 µM) out to the full 12 h (when choline-d<sub>9</sub> utilization and TMA-d<sub>9</sub> 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<sub>9</sub> use and TMA-d<sub>9</sub> generation<sup>19</sup>. All treatments, except for vehicle (VH) and choline-d<sub>9</sub>-free (CF) conditions, inhibited choline utilization somewhat as determined by statistically higher choline-d<sub>9</sub> AUCs vs. control conditions (**Figure 4A**) by one-way ANOVA.

In terms of TMA-d<sub>9</sub> production (**Figure 3**), several phenolic metabolites were unable to modulate TMA-d<sub>9</sub> 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<sub>9</sub> 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<sub>9</sub> than control conditions (choline-d<sub>9</sub> 100 μM) despite reducing choline-d<sub>9</sub> utilization (**Figure 2**). The rest of the compounds reported a significantly reduced TMA-d<sub>9</sub> production curve compared to control. This included significantly reduced TMA-d<sub>9</sub> levels at one or more timepoints (from 6 – 12 h) for all compounds, and all of them reported a reduction of TMA-d<sub>9</sub> levels at 8h. As for TMA-d<sub>9</sub> production, the inhibitory effect often did not persist until 12 h. The only compounds that maintained lower TMA-d<sub>9</sub> 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-dihydroxyphenylacetic acid, and phenylacetic acid. The trends reported by tested compounds in choline-d<sub>9</sub> use and TMA-d<sub>9</sub> 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<sub>9</sub> and relative reductions in TMA-d<sub>9</sub> percentages against time-matched control conditions were evaluated (**Supplementary tables 2 and 3**). For compounds that reported significant reductions in choline-d<sub>9</sub> levels at relevant metabolic timepoints (6 – 12 h), percentages of choline-d<sub>9</sub> remaining compared to choline-d<sub>9</sub> 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-hydroxyphenyl)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<sub>9</sub> levels, the percentages of TMA-d<sub>9</sub> reduction compared to choline-d<sub>9</sub> 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<sub>9</sub> use and TMA-d<sub>9</sub> production for each compound. For example, at 10 h epicatechin reported a – 24.2 ± 2.3 % decrease in TMA-d<sub>9</sub> levels and an increase of choline-d<sub>9</sub> levels of 409.7 ± 11.4 % compared to control. Nevertheless, the difference in total concentration

is quite similar: TMA-d<sub>9</sub> concentrations were reduced by ~16 μM between epicatechin (~51 μM) and control (~67 μM) groups, and choline-d<sub>9</sub> 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<sub>9</sub> use and TMA-d<sub>9</sub> 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 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<sub>9</sub> 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 respiratory units by a biologically relevant percentage (<80%). Although not statistically different from control conditions (choline-d<sub>9</sub> 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 cell respiration rate assay, we performed a cell respiration 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 cell respiration assay 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 cell respiration assay. Overall, 11 compounds presented statistically or biologically significant differences in cell density, reductions in relative cell respiration or both compared to choline-d<sub>9</sub> only control, but only two of these compounds also presented significant changes in TMA-d<sub>9</sub> 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<sub>9</sub> 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<sup>29</sup>. These methods are described in Supplementary Information. While 16s levels demonstrated significantly increased levels versus no template (water) controls that demonstrated increased Ct values as a function of dilution (**Supplementary Figure 6A**), 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 6B-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<sup>1,2</sup>. Circulating TMAO is a relatively new biomarker in terms of risk factors for cardiovascular mortality<sup>30,31</sup>. 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<sup>5</sup>. For this last application, food bioactives have been shown to hold promising TMAO-reducing properties<sup>5</sup>. Some natural compounds, such as DMB, reduce TMAO production by directly inhibiting TMA-lyase enzymes<sup>27</sup>. Other natural compounds may reduce circulating TMAO levels by modulating host gut microbiota composition and function<sup>32,33</sup> 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<sup>5</sup>. 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<sup>19</sup>. 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<sub>9</sub> into TMA-d<sub>9</sub>. Catechin and epicatechin were also selected due to their high concentrations in commonly consumed foodstuffs such as tea, cocoa and grapes<sup>10,11</sup>, as well as for their known cardioprotective functions<sup>12,13</sup>. *p*-Coumaric acid, caffeic acid and ferulic acid are found either bound or free in several commonly consumed foodstuffs, such as coffee and cereals<sup>11,34,35</sup>. 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<sup>15,36–38</sup>. 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<sup>16,17</sup>, which typically have greater bioavailability and achieve higher circulating concentrations than the native forms. Therefore, we also investigated the effects on TMA-d<sub>9</sub> formation from phenylpropionic, phenylacetic and benzoic acid derivatives, which are common microbial-derived metabolites of (epi)catechin and other phenolic compounds<sup>11,18,39</sup>. Although the primary objective of the experiments described in

this manuscript is screen for multiple compounds with the ability to reduce TMA-d<sub>9</sub> formation and not to evaluate inter-individual variability on the response to the different treatments, an aliquot of fecal slurry from two different donors were pooped together to account for some variability between individuals.

First, we tested whether TMA-d<sub>9</sub> could be formed by spontaneous degradation of choline-d<sub>9</sub> or if this was carried out primarily by the fecal slurry (presumably by the bacterial component) by incubating choline-d<sub>9</sub> with and without the presence of human fecal slurry (Figure 1). The modest decrease (~23 %) of choline-d<sub>9</sub> and the lack of detection of TMA-d<sub>9</sub> over 12 h under those conditions demonstrate that TMA-d<sub>9</sub> 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<sup>19</sup>. When compared with fermentations inoculated with fecal slurry, our results clearly indicate that TMA-d<sub>9</sub> is formed through a microbial-dependent pathway. These results are in line with our fermentation optimization study using unlabeled choline as a substrate<sup>19</sup>, and that of others<sup>28,40</sup>. Additionally, using choline-d<sub>9</sub> (as opposed to unlabeled choline) provides a cleaner assay with less background interference and a direct stoichiometric relationship between exogenous labeled choline-d<sub>9</sub> substrate and the resulting labeled TMA-d<sub>9</sub> product.

The effect of phenolic compounds and their microbial-derived metabolites on TMA-d<sub>9</sub> production were evaluated at a dose of 2 mM, which is in line with previous studies and our prior efficacy data<sup>19</sup>. Due to our statistical treatment of our kinetic data, where two-way ANOVA is applied to evaluate the effect of each phenolic compound against the control (100 μM choline-d<sub>9</sub>) 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<sub>9</sub> use and TMA-d<sub>9</sub> 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<sub>9</sub> microbial metabolism into TMA-d<sub>9</sub> 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<sub>9</sub> and TMA-d<sub>9</sub> compared to the control (inhibitor-free) group. These data suggest that most phenolic compounds and their metabolites studied in this manuscript are delaying choline-d<sub>9</sub> microbial metabolism into TMA-d<sub>9</sub>. 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 <sup>40</sup>. Of note, however, a few select compounds were able to maintain both higher choline-d<sub>9</sub> and lower TMA-d<sub>9</sub> throughout the entire experiment to the time the fermentation in the inhibitor-free 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 <sup>33,41</sup>, 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.* <sup>40</sup>, 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<sub>9</sub> use and TMA-d<sub>9</sub> production were not really marked despite being a known TMA-lyase inhibitor *in vivo* <sup>27</sup>. 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 <sup>28</sup>. Even in our previous study, DMB inhibitory effect of choline use and TMA production fluctuated <sup>19</sup>. 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 <sup>42</sup>. Antibacterial properties of catechin and epicatechin have also been reported, but at higher concentrations (i.e., > 20 mM) <sup>43,44</sup>. 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 cell respiration 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<sub>9</sub> 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 explained by an anti-bacterial (cytotoxic and/or cytostatic) effect. It is important to note that tested compounds did not interfere with the cell respiration assay (data not shown), unlike what we have previously reported for chlorogenic acid and gallic acid <sup>19</sup>. The compounds with the widest, most relevant reductions in TMA-d<sub>9</sub> levels and choline-d<sub>9</sub> 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<sub>9</sub> inhibition production by a broad-spectrum anti-bacterial mechanism, indicating other mechanisms of action and potential suitability for *in vivo* inhibition.

The present study has several limitations. First, some gut bacteria do not grow outside the gut environment regardless of the conditions employed. In vivo studies are thus necessary to validate these findings. Second, we employed pooled fecal samples from two donors, which does not fully account for potential inter-individual variability in vivo, although this is similar to other in vitro approaches using cells from one or a few donors. Third, there may be endogenous inhibitory compounds in the fecal samples employed, including dietary phenolics and their metabolites. However, the original fecal samples were diluted 50X in the final reaction mixture, and thus the contribution of these endogenous inhibitors is likely low. Additionally, all treatments were compared to control fermentation conditions, which included the same amount of fecal slurry and growth media than treatment conditions, and hence, and thus the same concentration of and endogenous or background inhibitors. Thus, these experiments controlled for the presence of inhibitory compounds in the fecal samples.

This study aimed to screen for phenolic compounds with the ability to inhibit TMA-d<sub>9</sub> production, in order to identify lead compounds to be used in future follow-up studies. We used doses of test compounds at the upper end of physiological relevance of what could be obtained in the gut lumen following dietary or supplement intake from common nutritional sources. This approach was designed to identify compounds that could be bioactive at doses obtainable from diet, supplement, or pharmaceutical delivery. The total amount of catechins found in 100 mL of green tea can be up to 220 mg, or even higher <sup>45</sup>. The consumption of two cups of green tea (~ 500 mL) would provide about 3 mmol epicatechin equivalents, which in a digestive system of 2 – 3 L would result in a concentration of 1 – 1.5 mM. Cocoa powder can have up to ~ 50 mg procyanidins/g. The consumption of 2 servings (10 g) of cocoa would provide ~ 500 mg procyanidins <sup>46</sup>, or 1.7 mM of epicatechin equivalents, which is translated into concentrations of 0.67 – 0.85 mM in a digestive system of 2 – 3 L. CocoaVia capsules provides 500 mg flavanols, 85 mg of which are epicatechin <sup>47</sup>. Following our rationale, epicatechin can be found in concentrations of 0.1 – 0.15 mM. As for microbial metabolites, a vast array of phenolic compounds can give rise to the same type of metabolites, which suggests that their concentration can be even higher than the concentration of a single native compound. Thus, overall, we believe that we are working in a range of concentrations that can be achieved by phenolic compound supplementation, or that could be even achieved through the diet

Future studies are needed to establish the dose-dependence of the effects we report in the present study, as well as to identify additional lead compounds with TMA-inhibiting activities. Additionally, *in vitro* and *in vivo* studies are warranted to assess inter-individual variability in the inhibitory effects of these compounds. Such studies could establish profiles of gut bacterial communities that predict enhanced or reduced inhibitory abilities of phenolic compounds. Mechanistic studies are also the next logical step in this line of research. Whether or not reductions in TMA-d<sub>9</sub> 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. Further work remains to be done to determine the mechanism(s) by which these phenolics inhibit TMA production. 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<sub>9</sub> into TMA-d<sub>9</sub> (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<sup>5</sup>, 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<sup>29</sup> 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<sup>48</sup>. 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.

Overall, caffeic acid, catechin, epicatechin and 3,4-dihydroxybenzoic acid were the compounds that reduced TMA-d<sub>9</sub> production and choline-d<sub>9</sub> use without interfering with cell growth and cell respiration. These compounds are led candidates to be tested in subsequent *in vitro* experiments.

547    **Abbreviations:**

548    CF: Choline-d<sub>9</sub>-free conditions.  
549    CVD: Cardiovascular disease.  
550    DMB: 3,3-Dimethyl-1-butanol.  
551    LOD: Limit of detection.  
552    LOQ: Limit of quantification.  
553    MDL: Method detection limit.  
554    MFC: Microbiota-free conditions.  
555    MQL: Method quantification limit.  
556    TMAO: Trimethylamine *N*-oxide.  
557    TMA: Trimethylamine.  
558    VH: Vehicle.

559    **Acknowledgments:**

560    The authors wish to acknowledge Lyric K. Ramsue, Gabriella P. Agnello and Kathryn C. Racine  
561    (Plants for Human Health Institute, Department of Food, Bioprocessing and Nutrition Sciences,  
562    North Carolina State University, Kannapolis, NC) for their help in preparing and aliquoting  
563    fermentation samples.

564    **Funding sources:**

565    Funding for this project was provided through startup funding from Plants for Human Health  
566    Institute North Carolina State University as well as support from the North Caroline Agricultural  
567    Research Service (NCARS) and the Hatch Program of the national Institute of Food and  
568    Agriculture (NIFA), U.S. Department of Agriculture.

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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 <sub>9</sub>	112.2	1.05	113.3>69.1	40	16
Choline-1- <sup>13</sup> C-1,1,2,2-d <sub>4</sub>	108.2	1.05	109.3>60.3	36	18
Ethyl betaine <sup>a</sup>	145.2	0.77	146.3>118.2	34	16
Ethyl betaine-d <sub>9</sub> <sup>a</sup>	154.2	0.77	155.3>127.2	34	20
Ethyl betaine- <sup>13</sup> C <sub>3</sub> - <sup>15</sup> N <sub>4</sub> <sup>a</sup>	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.

<sup>a</sup> TMA derivatives.

**Table 2:** Parameters for the quantification of choline-d<sub>9</sub> and TMA-d<sub>9</sub> (as well as non-d<sub>9</sub> isotopes) in spiked fecal fermentation samples by UPLC-MS/MS/.

Compound	Calibration curve	R <sup>2</sup>	Working linear range (μM)	LOD (nM)	LOQ (nM)	MDL (nM) <sup>b</sup>	MQL (nM) <sup>b</sup>
Choline-d <sub>9</sub>	y = 0.4401x + 0.4748	0.9974	1.0 – 300.0	63.2	210.8	126.5	421.6
Ethyl betaine-d <sub>9</sub> <sup>a</sup>	y = 0.0683x + 0.1919	0.9973	1.0 – 300.0	28.7	95.7	57.4	191.6

Abbreviations: R<sup>2</sup>, determination coefficient; LOQ, limit of detection; LOQ, limit of quantification; MDL, method detection limit; MQL, method quantification limit.

<sup>a</sup> TMA derivatives.

<sup>b</sup> MDL and MQL for 25 μL of fecal fermentation media.

**Figure captions:**

**Figure 1:** Choline-d<sub>9</sub> and TMA-d<sub>9</sub> kinetic curves in fermentations with growth media and: 100 μM choline-d<sub>9</sub> and without fecal slurry (**A**); 100 μM choline-d<sub>9</sub> and 20% fecal slurry (**B**); without 100 μM choline-d<sub>9</sub> and with 20% fecal slurry (**C**). Results are expressed as mean μM ± SEM (*n*=4). Abbreviations: ND, not detected.

**Figure 2:** Choline-d<sub>9</sub> kinetic curves in fermentations with fecal slurry 20 %, 100 μM choline-d<sub>9</sub> 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<sub>9</sub> 100 μM) and test compound (factors: treatment, time). Results are expressed as mean μM ± SEM (*n*=4). Abbreviations: VH, vehicle (choline-d<sub>9</sub> 0 μM, no fecal slurry); MFC, microbiota-free conditions (no fecal slurry); CF, choline-d<sub>9</sub>-free conditions (choline-d<sub>9</sub> 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<sub>9</sub> kinetic curves in fermentations with fecal slurry 20 %, 100 μM choline-d<sub>9</sub> 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<sub>9</sub> 100 μM) and test compound (factors: treatment and time). Results are expressed as mean μM ± SEM (*n*=4). Abbreviations: VH, vehicle (choline-d<sub>9</sub> 0 μM, no fecal slurry); MFC, microbiota-free conditions (no fecal slurry); CF, choline-d<sub>9</sub>-free conditions (choline-d<sub>9</sub> 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.

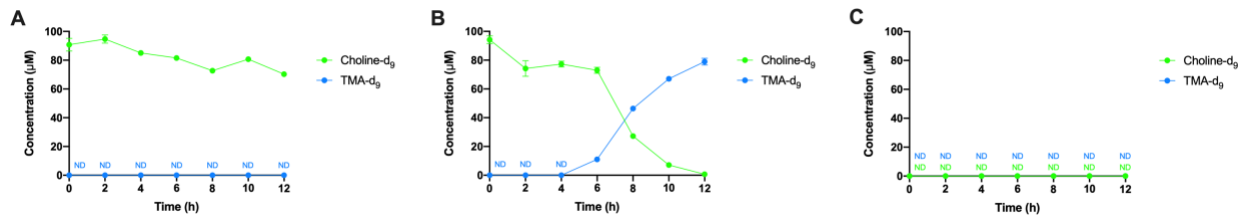
**Figure 4:** Area under the curve (AUC) of the kinetic curves of choline-d<sub>9</sub> (A), TMA-d<sub>9</sub> (B), choline (C) and TMA (D). \* Indicates statistical difference ( $p < 0.05$ ) against control conditions (choline-d<sub>9</sub> 100  $\mu$ M) by One-way ANOVA (Dunnetts' *post hoc* test). Results are expressed as mean h x  $\mu$ M  $\pm$  SEM ( $n=4$ ). Abbreviations: Control, choline-d<sub>9</sub> 100  $\mu$ M; VH, vehicle (choline-d<sub>9</sub> 0  $\mu$ M); MFC, microbiota-free conditions; CF, choline-d<sub>9</sub>-free conditions (choline-d<sub>9</sub> 0  $\mu$ M); 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  $\mu$ M choline-d<sub>9</sub> 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<sub>9</sub>-free conditions (CF). \* Indicates statistical differences ( $p < 0.05$ ) in treatment effect by Two-way ANOVA against control conditions (choline-d<sub>9</sub> 100  $\mu$ M). Results are expressed as mean arbitrary units  $\pm$  SEM ( $n=4$ ). Abbreviations: VH, vehicle (choline-d<sub>9</sub> 0  $\mu$ M, no fecal slurry); MFC, microbiota-free conditions (no fecal slurry); CF, choline-d<sub>9</sub>-free conditions (choline-d<sub>9</sub> 0  $\mu$ M); 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,

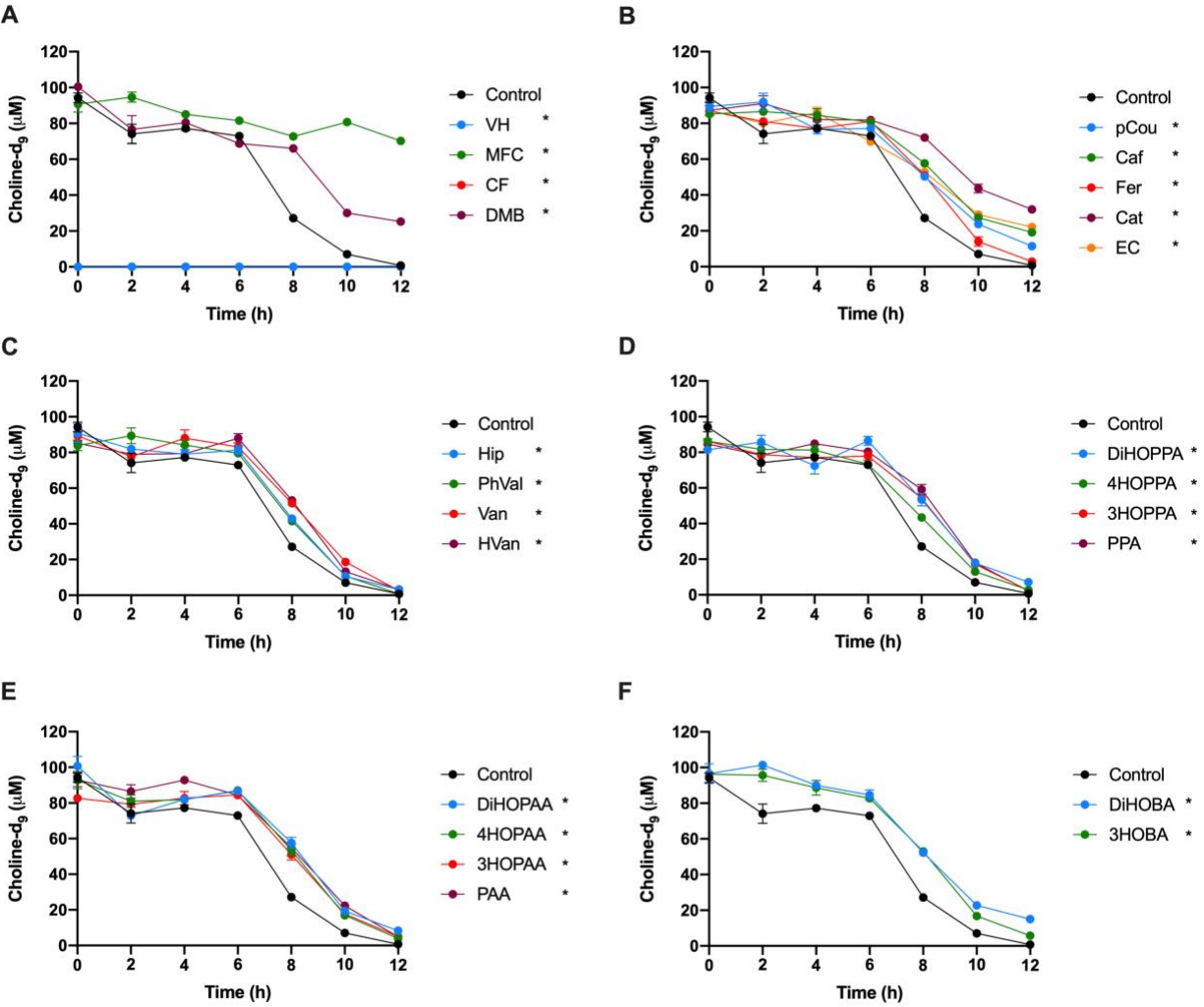
3-(4-hydroxyphenyl)propionic acid; 3HOPPA, 3-(3-hydroxyphenyl)propionic acid; PPA, 3-phenylpropionic acid; DiHOPAA, 3-(3,4-dihydroxyphenyl)acetic acid; 4HOPAA, 3-(4-hydroxyphenyl)acetic acid; 3HOPAA, 3-(3-hydroxyphenyl)acetic acid; PAA, phenylacetic acid; DiHOBA, 3,4-dihydroxybenzoic acid; and 3HOBA, 3-hydroxybenzoic acid. Note that the control treatment group is plotted on all graphs for ease of comparison.

**Figure 6:** Cell respiration relative to choline-d<sub>9</sub>-free conditions (CF) in fermentations with fecal slurry 20 %, 100 µM choline-d<sub>9</sub> and different test compounds (2 mM, except DMB 10 mM). \* Indicates statistical difference ( $p < 0.05$ ) against control conditions (choline-d<sub>9</sub> 100 µM) by One-way ANOVA (Dunnetts' *post hoc* test). Results are expressed as mean µM ± SEM ( $n=4$ ). Abbreviations: VH, vehicle (choline-d<sub>9</sub> 0 µM); MFC, microbiota-free conditions; CF, choline-d<sub>9</sub>-free conditions (choline-d<sub>9</sub> 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.

Figure 1:



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847 **Figure 3:**

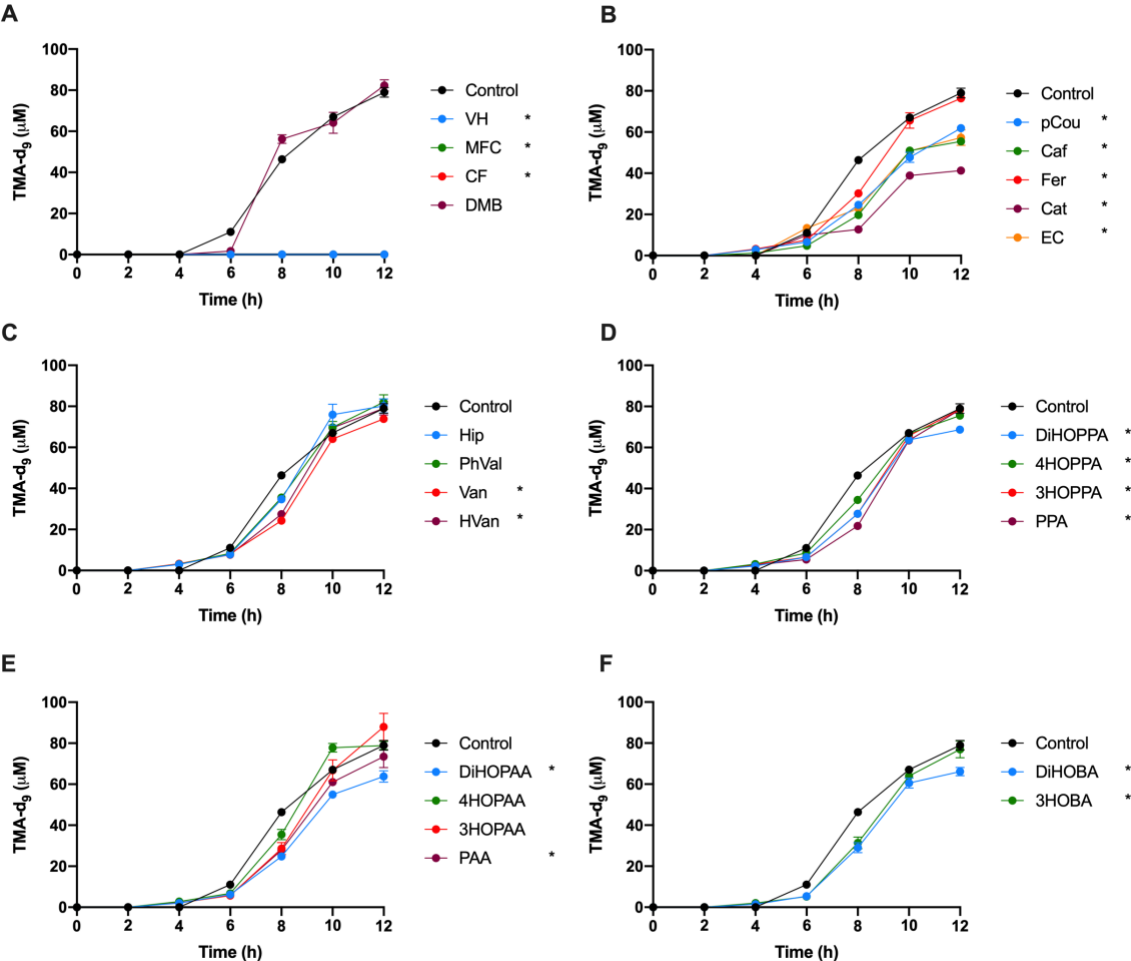


Figure 4:

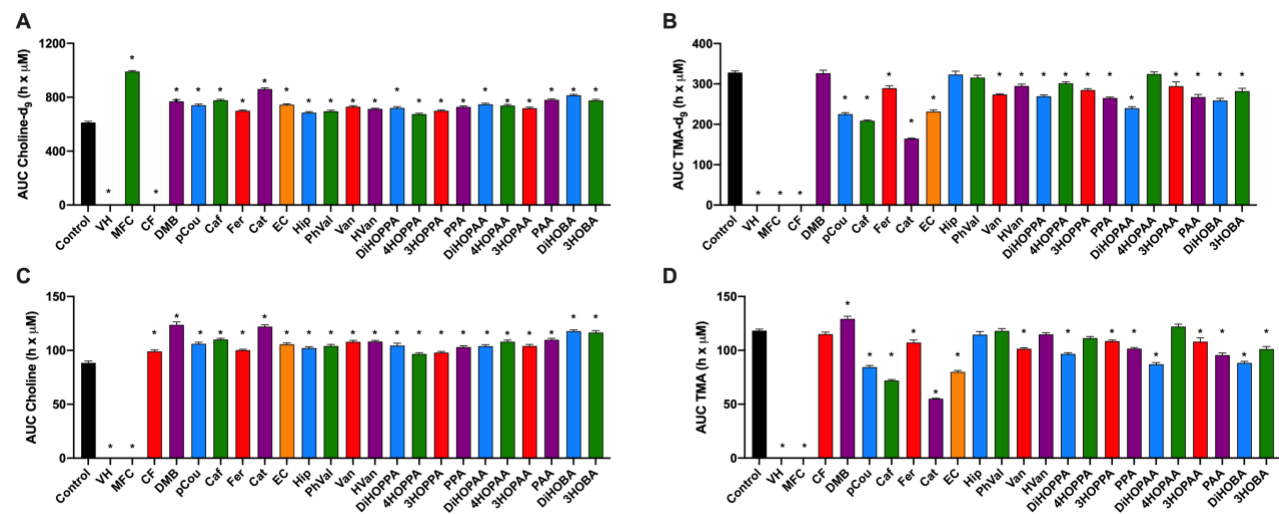




Figure 5:

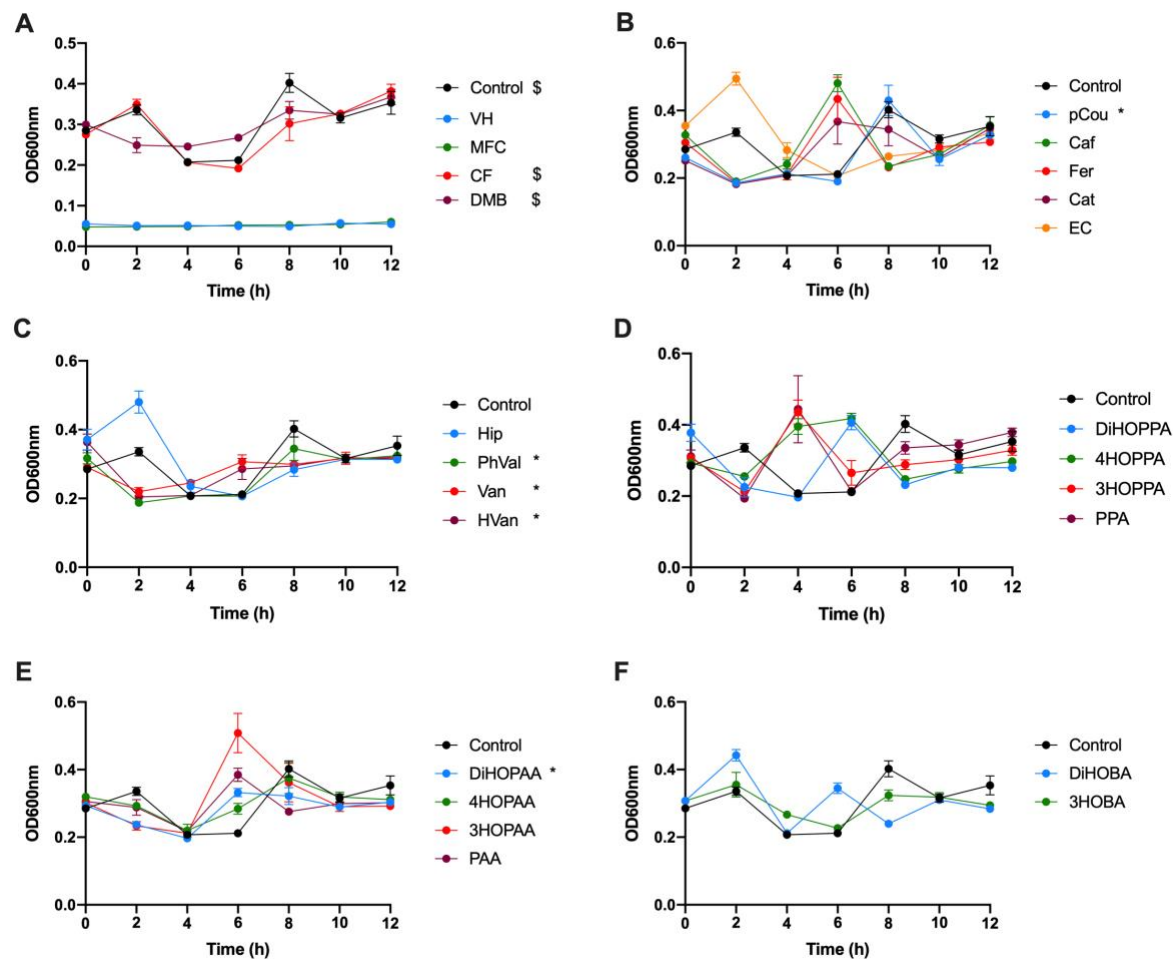
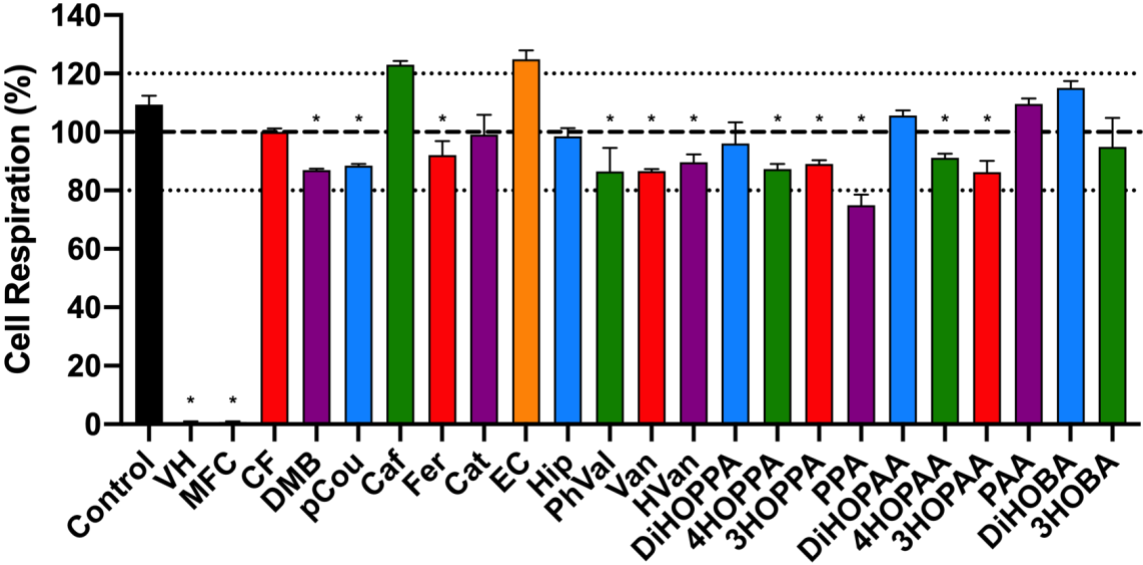
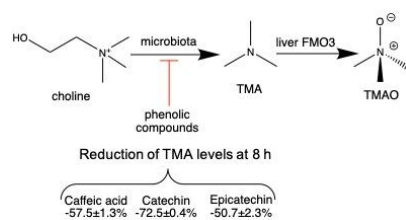


Figure 6:



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