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

4 Lisard Iglesias-Carres[a], Emily S. Krueger[b], Jacob A. Herring[b,c], Jeffery S. Tessem[b],
5 Andrew P. Neilson[a,*].

6 [a] *Plants for Human Health Institute, Department of Food, Bioprocessing and Nutrition Sciences,*
7 *North Carolina State University, Kannapolis, NC*

8 [b] *Department of Nutrition, Dietetics, and Food Science, Brigham Young University, Provo, UT*

9 [c] *Department of Microbiology and Molecular Biology, Brigham Young University, Provo, UT*

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11 * Corresponding author: Plants for Human Health Institute, Department of Food, Bioprocessing
12 and Nutrition Sciences, North Carolina State University, 600 Laureate Way, Kannapolis, NC
13 28081; 704-250-5495; aneilso@ncsu.edu

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15 # Abstract:

16 Trimethylamine *N*-oxide (TMAO) is a pro-atherosclerotic product of dietary choline metabolism
17 generated by a microbiome-host axis. The first step in this pathway is enzymatic metabolism of
18 choline to trimethylamine (TMA) by the gut microbiota. This reaction could be targeted to reduce
19 atherosclerosis risk. We aimed to evaluate potential inhibitory effects of select dietary phenolics
20 and their relevant gut microbial metabolites on TMA production via a human *ex vivo-in vitro*
21 fermentation model. Various phenolics inhibited choline use and TMA production. The most
22 bioactive compounds tested (caffeic acid, catechin and epicatechin) reduced TMA-d_9 formation
23 (compared to control) by 57.5 ± 1.3% to 72.5 ± 0.4% at 8 h and preserved remaining choline-d_9
24 concentrations by 194.1 ± 6.4% to 256.1 ± 6.3% compared to control conditions at 8 h. These
25 inhibitory effects were achieved without altering cell respiration or cell growth. However,
26 inhibitory effects decreased at late fermentation times, which suggest that these compounds delay
27 choline metabolism rather than completely inhibiting TMA formation. Overall, caffeic acid,
28 catechin and epicatechin were the most effective non-cytotoxic inhibitors of choline use and TMA
29 production. Thus, these compounds are proposed as lead bioactives to test *in vivo*.

30 **Keywords:** Atherosclerosis; Food bioactives; Gut microbiota metabolites; Phenolic compounds;
31 TMA.

32

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].

63 We previously developed a high-throughput 96-well *ex vivo* anaerobic fecal fermentation assay to
64 assess inhibition of TMA production from choline [19]. Using this assay, we are now capable of
65 screening large numbers of compounds to identify promising lead compounds for subsequent
66 focused animal studies. Due to the reported cardioprotective benefits of flavan-3-ols,
67 hydroxycinnamic acids and other phenolic compounds from foods such as cocoa [14,20,21], these
68 compounds are of particular interest as potential TMA- and TMAO-lowering agents. Indeed, we
69 found that chlorogenic acid was able to reduce TMA formation in our *ex vivo-in vitro* fermentation
70 model [19]. The aim of this study was to evaluate the potential of major hydroxycinnamic acids and
71 flavan-3-ols in the diet and their gut-derived metabolites to inhibit microbial conversion of choline
72 to TMA. The rationale for this study is that potent inhibitors can then be further evaluated for
73 mechanism and dose-dependence *in vitro* and inhibitory activity *in vivo* in subsequent studies.

74 ## 2. Materials and methods:

75 ### 2.1. Chemicals and reagents:

76 Glucose, peptone water, yeast extract, KCl, NaCl, Na_2HPO_4, KH_2PO_4, $MgSO_4 \times 7H_2O$,
77 $CaCl_2 \times 6H_2O$, $ZnSO_4 \times 7H_2O$, $NaHCO_3$, ammonium formate, hemin, bile salts, Tween 80, vitamin
78 K1, resazurin, L-cysteine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide,
79 ammonia, ethyl bromoacetate, choline, choline-d_9, choline-1-^{13}C-1,1,2,2-d_4, TMA, TMA-d_9,
80 TMA-$^{13}C_3$-^{15}N, 3,3-dimethyl-1-butanol (DMB), catechin, epicatechin, *p*-coumaric acid, caffeic
81 acid, ferulic acid, hippuric acid, 5-phenylvaleric acid, vanillic acid, homovanillic acid, 3-(3,4-
82 dihydroxyphenyl)propionic acid, 3-(4-hydroxyphenyl)propionic acid, 3-(3-
83 hydroxyphenyl)propionic acid, 3-phenylpropionic acid, 3,4-dihydroxyphenylacetic acid, 4-
84 hydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, phenylacetic acid, 3,4-dihydroxybenzoic
85 acid and 3-hydroxybenzoic acid were purchased from Sigma-Aldrich/Millipore (St. Louis, MO,
86 USA). Acetonitrile and water (HPLC grade) as well as dimethyl sulfoxide (DMSO; reagent grade)
87 were purchased from VWR International (Suwanee, GA, USA). Fecal samples from different
88 healthy donors were obtained from OpenBiome (Cambridge, MA, USA). These fecal samples
89 were from healthy donors and rigorously screened for 30 infectious diseases by OpenBiome.
90 Health histories, clinical data, pathogen screen results and 16S rDNA sequences are available at
91 OpenBiome. Samples are processed by OpenBiome in sterile 12.5% glycerol and 0.9% saline

92 buffer at 2.5 ml of buffer per gram of stool, and filtered through a 330 μm filter to remove large
93 particulate matter, and frozen at −80°C until use.
94

95 ## 2.2. Culture media preparation:

96 Fermentation media was prepared according to our optimized methodology [19], which was
97 originally adapted from Alqurashi *et al.* [22]. The composition of 1 L of growth medium was 2 g
98 peptone water, 2 g yeast extract, 0.1 g NaCl, 40 mg Na_2HPO_4, 40 mg KH_2PO_4, 10 mg $MgSO_4 \cdot$
99 $7H_2O$, 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
100 vitamin K1, 1 mg resazurin, and 0.5 g L-cysteine. Two different 500 mL solutions were prepared
101 at 2X final concentration. Solution A included all components except for resazurin and L-cysteine,
102 which were included in Solution B. The pH of both solutions was adjusted to 6.8, and solutions
103 were filter-sterilized separately through a 0.22 μm sterile filtering system (Corning, Corning, NY,
104 USA). Solution B was then boiled for 10 min. Thereafter, both solutions were sparged overnight
105 (minimum of 8 h) with N_2 (g) under agitation and were then combined 1:1 in the anaerobic
106 chamber (O_2 < 15 ppm) to the final 1X concentration. This growth media mixture was used to
107 grow bacteria in fecal slurries under different experimental conditions. The PBS 1X solution was
108 filter-sterilized (22 μm sterile filtering system, Corning, Corning, NY, USA) and sparged
109 overnight with N_2 (g) to maintain sterile and anaerobic working conditions.
110

111 ## 2.3. Anaerobic chamber conditions:

112 All fermentations were carried out inside a 4-glove 855-ACB anaerobic chamber (Plas-Labs,
113 Lansing, MI, USA). filled with a mixed gas composed of 5 % H_2, 5 % CO_2 and 90 % N_2 (Airgas,
114 Durham, NC, USA). H_2 (typically 2 – 3 %) and O_2 (typically < 15 ppm) levels were monitored
115 with a CAM-12 anaerobic monitor (Coy Lab Products, Grass Lake, MI, USA). Temperature was
116 set at 37 °C, and it was maintained constant (recorded values within 36 – 38 °C) throughout the
117 fermentation procedure. Humidity ranged between 40 and 50 % during all experiments.
118

119 ## 2.4. Fermentation experiments:

120 Due to the presence of endogenous choline and other TMA lyase substrates, and resulting
121 production of TMA in the absence of exogenous choline [19], choline-d_9 at a final concentration of

122 100 μM was added as the substrate, and the resulting TMA-d_9 was quantified in order to reduce
123 the impact of background TMA production on assay results. To screen for the potential effects of
124 our target compounds and their metabolites, fermentations were carried out in 96-well plates
125 following the optimized procedure described by Iglesias-Carres *et al.* [19]. A stock solution of growth
126 media (60 %) and PBS 1X (40 %) was prepared with filter-sterilized, overnight-sparged individual
127 solutions inside the anaerobic chamber. This solution was used to dissolve test compounds at a
128 final concentration of 2.67 mM (except DMB, 13.35 mM) and filter-sterilized again through a
129 PTFE (0.45 μm) MicroSolv AQ syringe filter inside the anaerobic chamber. In 1.1 mL 96-well
130 plates, 675 μL of test compound solutions were mixed with 45 μL of choline-d_9 stock solution (2
131 mM) in PBS 1X and 180 μL fecal slurry (1:10 in PBS 1X,) to reach a final well volume of 900 μL
132 containing test compound at 2 mM (except for DMB, at 10 mM), choline-d_9 at 100 μM and original
133 fecal slurry at 2 %. These conditions were optimized previously [19]. All solutions were pre-heated
134 at 37 °C, and fermentation was considered to start (time 0 h) when fecal slurry was inoculated into
135 the reaction mixture containing substrate. Aliquots from two different donors were used as fecal
136 slurries, with reference numbers 0105-0003-11 and 0128-0001-01. A total volume of 100 μL was
137 sampled from 0 to 12 h in 2 h intervals, analyzed for cell density (see section 2.7), then combined
138 with 100 μL acetonitrile and immediately frozen at – 80 °C until used for choline-d_9 and TMA-d_9
139 analyses. Control conditions were defined as growth media and PBS with choline-d_9 100 μM in
140 fermentations with 2 % fecal slurry (no inhibitors); vehicle conditions (VH) were defined as
141 growth media and PBS; choline-d_9-free conditions (CF) were defined as growth media and PBS
142 with fecal slurry 2 % only; and microbiota-free conditions (MFC) were defined as growth media
143 and PBS with 100 μM choline-d_9 only. PBS 1X was used to adjust volumes to 900 μL. In all cases,
144 growth media volume was 45 % of the total, and the remaining 55 % was PBS 1X.
145

146 ### 2.5. Extraction and analysis of choline-d_9 and TMA-d_9:

147 Externally added choline-d_9 and produced TMA-d_9 in fermentation media were extracted
148 according to the previously reported methodology [23,24] with slight modifications. TMA-d_9 requires
149 a derivatization process to the quaternary amine compound ethyl betaine-d_9 to facilitate LC-
150 MS/MS ionization. Briefly, 25 μL of fermentation sample were mixed with 20 μL of TMA-$^{13}C_3$-
151 ^{15}N internal standard solution (10 μM, for derivatization to ethyl betaine-d_9 or ethtylbetaine-$^{13}C_3$-

152 ^{15}N, respectively), 8 µL concentrated ammonia and 120 µL ethyl bromoacetate (20 mg/mL), and
153 let sit for 30 min. Then, 120 µL 50 % acetonitrile/0.025 % formic acid in distilled water were
154 added. TMA-d_9 samples were filtered through AcroprepAdv 0.2 µm WWPTFE 96-well filtering
155 plates (Pall Corporation, Port Washington, NY, USA) by centrifugation (10 min, 3,400 x g),
156 collected in a fresh 96-well collection plate and frozen at –80 °C until UHPLC-MS/MS analysis.
157 To extract choline-d_9, 25 µL of sample were mixed with 10 µL of $ZnSO_4$ solution (5 % w/v in
158 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
159 sonication for 5 min in a water bath, samples were filtered and stored as described above. No more
160 than 48 h passed between TMA-d_9/choline-d_9 extraction and their analysis. Endogenous choline
161 and TMA from fecal fermentation media were also extracted and analyzed as for exogenous
162 choline-d_9 and TMA-d_9.
163

164 **2.6. Chromatographic analysis and quantification of choline-d_9 and TMA-d_9:**

165 TMA-d_9 and TMA were analyzed separately from choline-d_9 and choline, but with the same
166 UHPLC-ESI-MS/MS method. Briefly, separation was achieved on a Waters Acquity UPLC
167 system (Milford, MA, USA) with an ACQUITY BHE HILIC column (1.7 µm, 2.1x100 mm)
168 coupled to an ACQUITY BHE HILIC pre-column (1.7 µm, 2.1x5 mm) (Waters). Mobile phases
169 consisted of 5 mM ammonium formate in water (pH 3.5) (A) and acetonitrile (B). The gradient
170 was isocratic at 80 % B for 3 min, with a flow rate of 0.65 mL/min. Colum temperature was set at
171 30 °C, and autosampler at 10 °C. Quantification was achieved by coupling the above system with
172 a Waters Acquity triple quadrupole mass spectrometer. Source and capillary temperatures were
173 150 and 400 °C, respectively. Capillary voltage was 0.60 kV, and desolvation and cone gas flows
174 (both N_2) were set at 800 and 20 L/h, respectively. Electrospray ionization (ESI) was operated in
175 positive mode, and data were acquired by multiple reaction monitoring (MRM) in MS/MS mode.
176 MRM fragmentation conditions of analytes and IS compounds can be found in Table 1.
177

178 For sample quantification, 45% growth media in PBS 1X was spiked with 7 different
179 concentrations each of choline-d_9 and TMA-d_9 standards to obtain external calibration curves in a
180 relevant background matrix. Samples were quantified by interpolating the analyte/IS peak
181 abundance ratio in the standard curves. Quantification of choline and TMA (inherent in samples)

182 and their d_9 isotopes (externally inoculated to samples) was performed using the calibration curves
183 constructed with the respective d_9 isotopes (choline-d_9 or TMA-d_9). Data acquisition was carried
184 out using Masslynx software (V4.1 version, Waters). Method sensitivity was determined by limit
185 of detection (LOD) and limit of quantification (LOQ), respectively defined as the concentration of
186 analyte corresponding to 3 and 10 times the signal/noise ratio. Method detection (MDL) and
187 quantification (MQL) limits were calculated for the analysis of 25 μL of non-diluted fecal
188 fermentation media samples. Method quality parameters can be found in Table 2. To determine
189 the endogenous levels of TMA and related compounds in experimental conditions, fecal slurry
190 1:10 in PBS 1X and fermentation media (45% growth media + 55% PBS 1X) with fecal slurry at
191 a final concentration of 2% were analyzed as previously stated.
192

193 **2.7. Treatment toxicity assessment:**

194 To study potential cytotoxic effects of treatments, cell count and cell respiration rate were
195 monitored as measures of viability. The number of cells present in the fermentation media was
196 evaluated by reading the optical density of 100 μL fermentation media at 600 nm in a SpectraMax
197 iD3 plate reader (Molecular Devices, San Jose, CA, USA) at every sampling time (0 – 12 h). A
198 cell respiration rate assay was used to estimate cell viability at 12 h. The assay in particular is
199 usually referred to as mitochondrial toxicity test in eucaryotic cells. However, this respiration assay
200 has been previously used to test cell respiration and viability in prokaryotic (i.e., bacteria) cells,
201 which lack mitochondria [19,25,26]. Briefly, 10 μL of fermentation mixture was mixed with 80 μL of
202 pre-heated (37 °C) PBS 1X with glucose 0.2% (m/v) and 10 μL of 3-(4,5-dimethylthiazol-2-yl)-
203 2,5-diphenyl-tetrazolium bromide at a concentration of 25 mg/mL in PBS 1X [25]. Samples were
204 allowed to react for 30 min under anaerobic conditions (37 °C and O_2 < 15 ppm), and the resulting
205 formazan crystals were resuspended to a final volume of 1 mL with DMSO by shaking for 30 min.
206 An aliquot of 100 μL was read at 560 nm in a SpectraMax iD3 plate reader (Molecular Devices,
207 San Jose, CA, USA). All reagents were prepared with overnight-sparged PBS 1X and filter-
208 sterilized 22 μm sterile filtering system, Corning) before their use. Results are expressed as
209 percentages of change versus choline-free conditions ± SEM (*n*=4).
210

211 **2.9 Data analysis and statistics**

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

221 **3. Results:**

222 **3.1. Inhibition of choline-d_9 use and TMA-d_9 production by phenolic**
223 **compounds and their microbial-derived metabolites:**

224 First, microbial dependence of TMA-d_9 production and choline-d_9 degradation were evaluated in
225 fermentation conditions with 100 μM exogenous choline-d_9 free of human fecal slurry (**Figure**
226 **1A**). Under those conditions, choline-d_9 levels remained fairly constant between 90.79 ± 4.41 μM
227 (0 h) – 70.28 ± 0.08 μM (12 h), which represents a ~23 % degradation of initial choline-d_9
228 concentration over 12 h. Despite slight decrease in apparent choline-d_9 levels, TMA-d_9 was not
229 detected during these 12 h. When fecal slurry was added (**Figure 1B**), choline-d_9 concentrations
230 varied from 94.20 ± 2.71 μM (0h) to 0.74 ± 0.07 μM (12h), and TMA-d_9 levels increased from not
231 detected (0 – 4 h) to 79.00 ± 2.34 μM (at 12 h). Choline-d_9 degradation and TMA-d_9 production
232 mirrored each other. To evaluate if the growth media and fecal slurry contributed to choline-d_9 and
233 TMA-d_9 signals, their levels were monitored in choline-d_9-free conditions (**Figure 1C**); none of
234 these two compounds were detected at any timepoint. Similarly, no choline-d_9 and TMA-d_9 were
235 reported in vehicle (PBS and growth media, but no choline-d_9 or fecal slurry) conditions at any
236 timepoint (**Figure 1C**). These results indicate that exogenous substrate (choline-d_9) was almost
237 completely consumed within 12 h, and roughly 84% was converted to TMA-d_9 within that
238 timeframe (given the 1-to-1 stoichiometry of choline-d_9 bacterial fermentation to TMA-d_9),
239 consistent with our previous data [19]. Thus, conversion of choline-d_9 to TMA-d_9 appears to be
240 microbially-dependent and is not affected by endogenous/background choline/TMA.
241

242 After validating that formation of TMA-d_9 required the presence of both fecal bacteria and choline-
243 d_9 substrate, we evaluated the potential inhibition of microbial conversion of choline-d_9 toTMA-
244 d_9 by select native phenolic compounds and their gut-derived microbial metabolites (**Figures 2
245 and 3**). Detailed information on Two-way ANOVA statistical significance for time, treatment and
246 interaction (time x treatment) effects can be found in **Supplementary Table 1**. Detailed
247 information on *post-hoc* results for choline-d_9 and TMA-d_9 levels at individual time points can be
248 found in **Supplementary Figures 1** and **2**. DMB (10 mM) was used as a positive control with
249 known TMA lyase inhibitory activity, per our previous data [19] and those of others [27,28].
250
251 At the start of fermentation, most treatment conditions presented non-significantly different levels
252 of choline-d_9 (**Figure 2**) against the control group (choline-d_9 100 μM with no added inhibitors),
253 with the exception of phenyl valeric acid, homovanillic acid, 3-(3,4-dihydroxyphenyl)propionic
254 acid, phenylacetic acid, and 3-hydroxyphenylacetic acid, where choline-d_9 levels were slightly
255 lower. Most screened compounds were able to significantly reduce choline-d_9 utilization compared
256 to control (no inhibitors) between 6 – 10 h, the timeframe during which choline-d_9 is metabolized
257 into TMA-d_9, as shown by control group kinetics. Besides the positive control (DMB 10 mM)
258 treatment, caffeic acid, catechin, epicatechin and 3,4-dihydroxybenzoic acid were the only
259 compounds able to maintain higher and statistically different residual choline-d_9 levels compared
260 to the control treatment (choline-d_9 100 μM) out to the full 12 h (when choline-d_9 utilization and
261 TMA-d_9 production was virtually over in control conditions). This is consistent with our previous
262 data showing that different inhibitory compounds (i.e., chlorogenic acid), slow but may not
263 indefinitely reduce choline-d_9 use and TMA-d_9 generation [19]. All treatments, except for vehicle
264 (VH) and choline-d_9-free (CF) conditions, inhibited choline utilization somewhat as determined
265 by statistically higher choline-d_9 AUCs vs. control conditions (**Figure 4A**) by one-way ANOVA.
266
267 In terms of TMA-d_9 production (**Figure 3**), several phenolic metabolites were unable to modulate
268 TMA-d_9 levels compared to control (non-significant two-way ANOVA for treatment effect
269 compared to control conditions), namely hippuric acid, phenylvaleric acid, 4-hydroxyphenylacetic
270 acid, and 3-hydroxyphenylacetic acid. DMB 10 mM also reported a non-significant (p>0.05) two-
271 way ANOVA treatment effect. Similarly, all these compounds except for 3-hydroxyphenylacetic
272 acid reported non-significant differences in TMA-d_9 AUCs when compared to control conditions

273 (**Figure 4B**) by one-way ANOVA. Surprisingly, positive control conditions (DMB 10 mM) did
274 not report a lower TMA-d_9 than control conditions (choline-d_9 100 μM) despite reducing choline-
275 d_9 utilization (**Figure 2**). The rest of the compounds reported a significantly reduced TMA-d_9
276 production curve compared to control. This included significantly reduced TMA-d_9 levels at one
277 or more timepoints (from 6 – 12 h) for all compounds, and all of them reported a reduction of
278 TMA-d_9 levels at 8h. As for TMA-d_9 production, the inhibitory effect often did no persist until 12
279 h. The only compounds that maintained lower TMA-d_9 levels when fermentation in the control
280 group was virtually over (at 12 h) were *p*-coumaric acid, caffeic acid, catechin, epicatechin, vanillic
281 acid, 3-(3,4-dihydroxyphenyl)propionic acid, 3,4-dihydroxyphenyacetic acid, and phenylacetic
282 acid. The trends reported by tested compounds in choline-d_9 use and TMA-d_9 production were also
283 generally maintained by background choline and TMA levels from endogenous substrates (**Figure**
284 **4** and **Supplementary figures 3 – 4**).
285
286 To further investigate the inhibitory effect of phenolic compounds and their metabolites, the
287 relative percentages of remaining choline-d_9 and relative reductions in TMA-d_9 percentages
288 against time-matched control conditions were evaluated (**Supplementary tables 2** and **3**). For
289 compounds that reported significant reductions in choline-d_9 levels at relevant metabolic
290 timepoints (6 – 12 h), percentages of choline-d_9 remaining compared to choline-d_9 alone were
291 113.8 ± 1.5 % (vanillic acid) to 120.6 ± 3.7 % (homovanillic acid) at 6 h; 152.8 ± 3.9 %
292 (phenylvaleric acid) to 265.1 ± 6.3% (catechin) at 8h; 243.1 ± 15.5 % (3-(3-
293 hydroxypheny)propionic acid) to 615.4 ± 34.3 % (catechin) at 10 h; and 2028.7 ± 67.8 % (3,4-
294 dihydroxybenzoic acid) to 4303.9 ± 69.9 % (catechin) at 12 h. For compounds that reported
295 significant reductions in TMA-d_9 levels, the percentages of TMA-d_9 reduction compared to
296 choline-d_9 alone were – 50.8 ± 1.1 % (caffeic acid) to – 56.8 ± 1.1 % (3-phenylpropionic acid) at
297 6 h; – 25.8 ± 3.9 % (3-(4-hydroxyphenyl)propionic acid) to – 72.5 ± 0.4 % (catechin) at 8 h; – 9.7
298 ± 3.7 % (3,4-dihydroxybenzoic acid) to – 42.0 ± 1.2 % (catechin) at 10 h; and – 6.5 ± 1.7 % (vanillic
299 acid) to – 47.8 ± 0.9 % (catechin) at 12 h. Overall, these data reveal a possible disconnect between
300 the inhibition of choline-d_9 use and TMA-d_9 production for each compound. For example, at 10 h
301 epicatechin reported a – 24.2 ± 2.3 % decrease in TMA-d_9 levels and an increase of choline-d_9
302 levels of 409.7 ± 11.4 % compared to control. Nevertheless, the difference in total concentration

303 is quite similar: TMA-d_9 concentrations were reduced by ~16 μM between epicatechin (~51 μM)
304 and control (~67 μM) groups, and choline-d_9 were increased by ~21 μM between epicatechin (~29
305 μM) and control (~8 μM) groups.
306

307 3.2. Cell growth and cell respiration rate:

308 To evaluate whether changes in choline-d_9 use and TMA-d_9 production were possibly due to a
309 cytotoxic or cytostatic effect of our treatments, cell density was evaluated throughout the
310 fermentation (0 – 12 h) by monitoring sample optical density at 600 nm, and cell respiration rate
311 assay was performed at the end (12 h) of the fermentation. Vehicle (VH) and microbiota-free
312 conditions (MFC), both not inoculated with human fecal slurry, presented low 600 nm optical
313 absorbance readings that did not change during 12 h incubation (Figure 5A), which was expected
314 due to the lack of cells. Although cell density kinetics fluctuated during the fermentation in all
315 other experimental conditions (Figure 5), only some compounds reported a significant (p<0.05)
316 treatment effect by Two-way ANOVA when compared to control conditions (choline-d_9 100 μM),
317 namely *p*-coumaric acid, phenylvaleric acid, vanillic acid, homovanillic acid, and 3,4-
318 dihydroxyphenylacetic acid. Detailed information on the *post hoc* results for treatment's effect on
319 cell density at individual time points can be found in Supplementary **Figure 5**. The cell respiration
320 by the end of the fermentation (12 h) was significantly reduced due to treatment with 9 out of the
321 20 different tested compounds (**Figure 6**), namely: *p*-coumaric acid, ferulic acid, phenylvaleric
322 acid, vanillic acid, 3-(4-hydroxyphenyl)propionic acid, 3-(3-hydroxyphenyl)propionic acid, 3-
323 phenylpropionic acid, 3-(4-hydroxyphenyl)acetic acid, and 3-(3-hydroxyphenyl)acetic acid.
324 However, only 3-phenylpropionic acid reduced relative cell respiratory units by a biologically
325 relevant percentage (<80%). Although not statistically different from control conditions (choline-
326 d_9 100 μM), both caffeic acid and epicatechin treatments reached relative cell respiration
327 percentages > 120 % (i.e., increased respiration, indicative of enhanced respiratory capacity and/or
328 greater cell numbers). To evaluate that tested compounds would not interfere in the cell respiration
329 rate assay, we performed a cell respiration assay with test compounds at working concentrations
330 of 2 mM (without viable cells) under anaerobic conditions in PBS 1X 0.2% glucose (data not
331 shown) as per the methodology followed for fermentation samples. Absorbances from test
332 compounds without cells ranged from 0.05 – 0.07 AU, with control conditions (PBS 1X 0.2%
333 glucose) values of 0.06 AU. Compared to typical absorbances of cell respiration assay with 10 μL

334 of sample containing viable cells that typically were >0.250 AU, it appears that tested compounds
335 do not significantly interfere with the cell respiration assay. Overall, 11 compounds presented
336 statistically or biologically significant differences in cell density, reductions in relative cell
337 respiration or both compared to choline-d_9 only control, but only two of these compounds also
338 presented significant changes in TMA-d_9 production compared to the control group (Figure 3),
339 namely *p*-coumaric acid and 3,4-dihydroxyphenylacetic acid. In no cases were significant
340 cytotoxic or cytostatic effects observed compared to control.
341

342 **3.3 TMA lyase gene abundance analysis.**

343 We hypothesized that one mechanism of action by which our tested compounds could lower
344 TMA production would be alteration of bacterial community composition to reduce TMA lyase
345 (CutC) abundance. We wished to quantify CutC gene copy number in fermentation samples in
346 order to evaluate this possible mechanism, with CntA (carnitine monooxygenase oxygenase
347 subunit, which converts carnitine to TMA) for comparison. To validate the assay, we tested our
348 method with fermentation samples from control conditions (added choline-d_9 100 µM) as we
349 postulated that these conditions would fecal select for bacterial cultures carrying the CutC gene
350 (but not CntA), thus enriching for this gene. To test this, we measured 16s (bacterial control),
351 CutC and CntA DNA levels using previously published primers. These methods are described in
352 Supplementary Information. While 16s levels demonstrated significantly increased levels versus
353 no template (water) controls that demonstrated increased Ct values as a function of dilution
354 (**Supplementary Figure 6A**), measurements of CutC or CntA demonstrated no significant
355 difference at any of the tested template DNA concentrations as compared to no template
356 controls, even after extensive primer optimization (**Supplementary Figure 6B-C**). These data
357 suggest that qPCR using these previously defined degenerative primers is not a viable tool in
358 measuring CutC and CntA bacterial DNA levels in fecal fermentation samples. Thus, we were
359 unable to evaluate the impact of our TMA-lowering phenolic compounds on CutC abundance in
360 this high-throughput *ex vivo* fecal fermentation model system.
361

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 [29,30]. 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 [27]. Other natural compounds may reduce circulating TMAO levels by modulating host gut microbiota composition and function [31,32] 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 [11,33,34]. 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 [15,35–37]. 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 [11,18,38]. Although the primary objective of the experiments described in

393 this manuscript is screen for multiple compounds with the ability to reduce TMA-d_9 formation and
394 not to evaluate inter-individual variability on the response to the different treatments, an aliquot of
395 fecal slurry from two different donors were pooped together to account for some variability
396 between individuals.
397
398 First, we tested whether TMA-d_9 could be formed by spontaneous degradation of choline-d_9 or if
399 this was caried out primarily by the fecal slurry (presumably by the bacterial component) by
400 incubating choline-d_9 with and without the presence of human fecal slurry (Figure 1). The modest
401 decrease (~23 %) of choline-d_9 and the lack of detection of TMA-d_9 over 12 h under those
402 conditions demonstrate that TMA-d_9 is not formed due to a spontaneous chemical reaction under
403 fermentation conditions in the absence of the fecal slurry. This is in line with similar dependence
404 on the presence of fecal slurry for choline transformation into TMA that we previously reported
405 [19]. When compared with fermentations inoculated with fecal slurry, our results clearly indicate that
406 TMA-d_9 is formed through a microbial-dependent pathway. These results are in line with our
407 fermentation optimization study using unlabeled choline as a substrate [19], and that of others [28,39].
408 Additionally, using choline-d_9 (as opposed to unlabeled choline) provides a cleaner assay with less
409 background interference and a direct stoichiometric relationship between exogenous labeled
410 choline-d_9 substrate and the resulting labeled TMA-d_9 product.
411
412 The effect of phenolic compounds and their microbial-derived metabolites on TMA-d_9 production
413 were evaluated at a dose of 2 mM, which is in line with previous studies and our prior efficacy
414 data [19]. Due to our statistical treatment of our kinetic data, where two-way ANOIVA is applied to
415 evaluate the effect of each phenolic compound against the control (100 µM choline-d_9) one by one,
416 without including any other tested compound, chances of type-I error are higher. In other words,
417 since not all compounds are compared to the control at the same time, there is a higher experiment-
418 wise chance of obtaining statistically significant results for any specific compound, which in this
419 case are compounds that decrease choline-d_9 use and TMA-d_9 production. However, the results
420 observed in our kinetic plots are maintained when AUCs are calculated. For AUCs statistical
421 analysis, one-way ANOVA with Dunnett's *post hoc* is applied, where although each compound is
422 compared to control conditions, the effect of other compounds is considered too by controlling the
423 experiment-wise false positive rate. Thus, chances of reporting more statistically significant results

424 decrease. Overall, our results demonstrate that phenolic compounds/metabolites inhibit choline-d_9
425 microbial metabolism into TMA-d_9 during the timeframe in which exogenous substrate was
426 consumed in the absence of inhibitors. At the end of the fermentation (12 h), most compounds
427 presented non-significantly different levels of choline-d_9 and TMA-d_9 compared to the control
428 (inhibitor-free) group. These data suggest that most phenolic compounds and their metabolites
429 studied in this manuscript are delaying choloine-d_9 microbial metabolism into TMA-d_9. The loss
430 of efficacy over time may be explained at least partly by the fact that these phenolic inhibitors
431 themselves are degraded by the microbiota. Similar trends have been reported for blood orange
432 juice phenolic compounds when choline and L-carnitine were used as substrates [39]. Of note,
433 however, a few select compounds were able to maintain both higher choline-d_9 and lower TMA-
434 d_9 throughout the entire experiment to the time the fermentation in the inhibitor-fee control group
435 was virtually over (12 h). These compounds were caffeic acid, catechin, epicatechin and 3,4-
436 dihydroxybenzoic acid. These data suggest that these four compounds possessed particularly
437 potent and lasting activities in this model. Of note, flavan-3-ols and flavan-3-ol rich foods have
438 reported mixed *in vivo* TMAO reducing properties [32,40], but our *in vitro* data suggest that
439 monomeric flavan-3-ol catechin and epicatechin are potential candidates to study in *in vivo*
440 models. It is important to acknowledge that the effects of phytochemicals could widely vary
441 depending on gut microbiota composition. For example, in the study of Bresciani *et al.* [39], coffee,
442 a rich source of caffeic acid, reported different effects depending of quaternary amine substrate
443 (i.e., choline or L-carnitine) and fecal slurry origin (i.e., vegetarian or omnivorous). We have
444 reported that caffeic acid is one of the most promising phenolic compounds to reduce TMA
445 formation in this study, along with catechin, epicatechin and 3,4-dihydroxybenzoic acid. The
446 effects of these compounds should be tested in *in vivo* models that capture the diversity of gut
447 microbiota found in humans.
448
449 Noteworthily, the inhibitory effects of DMB in choline-d_9 use and TMA-d_9 production were not
450 really marked despite being a known TMA-lyase inhibitor *in vivo* [27]. However, in a similar *ex vivo-*
451 *in vitro* fermentation set-up like ours, Day-Walsh *et al.* reported a similar behavior of DMB at 10
452 mM [28]. Even in our previous study, DMB inhibitory effect of choline use and TMA production
453 fluctuated [19]. Perhaps the insoluble water nature of DMB plays a role in such results. However, we
454 have evaluated DMB solubility in growth media, which contains agents that should help its

455 solubilization (i.e., Tween 80), and observed no such effect. Nevertheless, DMB might fall out of
456 solution over time, which might explain the disconnect between its *in vitro* and *in vivo* inhibitory
457 potential.
458
459 Whether or not the effects reported by these tested phenolic compounds and microbial-derived
460 metabolites were due to cytotoxicity or cytostatic mechanisms (undesirable *in vivo*) is a critical
461 concern. Indeed, antibacterial properties of hydroxycinnamic acids have been found at
462 concentrations between 0.9 – 9 mM in different bacteria strains [41]. Antibacterial properties of
463 catechin and epicatechin have also been reported, but at higher concentrations (i.e., > 20 mM) [42,43].
464 To shed some light into that, we monitored cell density over the fermentation course by analyzing
465 each fermentation sample's optical density at 600 nm, and evaluated cell respiration at its end (12
466 h) using the cell respiration assay. The compounds that significantly altered cell density over time
467 (significant Two-way ANOVA treatment effect) when compared to the inhibitor-free control
468 group were *p*-coumaric acid, phenylvaleric acid, vanillic acid, homovanillic acid, and 3,4-
469 dihydroxyphenylacetic acid. The relative cell respiration was reduced by *p*-coumaric acid, ferulic
470 acid, phenylvaleric acid, vanillic acid, 3-(4-hydroxyphenyl)propionic acid, 3-(3-
471 hydroxyphenyl)propionic acid, 3-phenylpropionic acid, 4-hydroxyphenylacetic acid, and 3-
472 hydroxyphenylacetic acid when compared to the control group. Out of the 11 compounds that
473 presented either changes in cell density or reductions in relative cell respiration only *p*-coumaric
474 acid and 3,4-dihydroxyphenylacetic acid presented relevant changes in TMA-d_9 production
475 compared to the control group (**Figure 3**). Thus, it is possible that the results reported by only *p*-
476 coumaric acid and 3,4-dihydroxyphenylacetic acid could be partially optioned by an anti-bacterial
477 (cytotoxic and/or cytostatic) effect. It is important to note that tested compounds did not interfere
478 with the cell respiration assay (data not shown), unlike what we have previously reported for
479 chlorogenic acid and gallic acid [19]. The compounds with the widest, most relevant reductions in
480 TMA-d_9 levels and choline-d_9 use, namely caffeic acid, catechin, epicatechin and 3,4-
481 dihydroxybenzoic acid, did not report significant changes in cell density or relative cell respiration.
482 This suggests that none of these compounds achieved TMA-d_9 inhibition production by a broad-
483 spectrum anti-bacterial mechanism, indicating other mechanisms of action and potential suitability
484 for *in vivo* inhibition.
485

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

498

499 This study aimed to screen for phenolic compounds with the ability to inhibit TMA-d_9 production,
500 in order to identify lead compounds to be used in future follow-up studies. We used doses of test
501 compounds at the upper end of physiological relevance of what could be obtained in the gut lumen
502 following dietary or supplement intake from common nutritional sources. This approach was
503 designed to identify compounds that could be bioactive at doses obtainable from diet, supplement,
504 or pharmaceutical delivery. The total amount of catechins found in 100 mL of green tea can be up
505 to 220 mg, or even higher [44]. The consumption of two cups of green tea (~ 500 mL) would provide
506 about 3 mmol epicatechin equivalents, which in a digestive system of 2 – 3 L would result in a
507 concentration of 1 – 1.5 mM. Cocoa powder can have up to ~ 50 mg procyanidins/g. The
508 consumption of 2 servings (10 g) of cocoa would provide ~ 500 mg procyanidins [45], or 1.7 mM of
509 epicatechin equivalents, which is translated into concentrations of 0.67 – 0.85 mM in a digestive
510 system of 2 – 3 L. CocoaVia capsules provides 500 mg flavanols, 85 mg of which are epicatechin
511 [46]. Following our rationale, epicatechin can be found in concentrations of 0.1 – 0.15 mM. As for
512 microbial metabolites, a vast array of phenolic compounds can give rise to the same type of
513 metabolites, which suggests that their concentration can be even higher that the concentration of a
514 single native compound. Thus, overall, we believe that we are working in a range of concentrations
515 that can be achieved by phenolic compound supplementation, or that could be even achieved
516 through the diet

517

518 Future studies are needed to establish the dose-dependence of the effects we report in the present
519 study, as well as to identify additional lead compounds with TMA-inhibiting activities.
520 Additionally, *in vitro* and *in vivo* studies are warranted to assess inter-individual variability in the
521 inhibitory effects of these compounds. Such studies could establish profiles of gut bacterial
522 communities that predict enhanced or reduced inhibitory abilities of phenolic compounds.
523 Mechanistic studies are also the next logical step in this line of research. Whether or not reductions
524 in TMA-d_9 production are achieved by selectively killing and/or inhibiting certain bacteria strains
525 that carry TMA lyase genes, and/or by promoting the growth of others is unknown. Further work
526 remains to be done to determine the mechanism(s) by which these phenolics inhibit TMA
527 production. Evaluation of probiotic (microbiome-shifting) effects of tested compound should be
528 performed. As a matter of fact, although changes in cell respiration rate and cell density give an
529 overview of potential cytotoxic effects, an evaluation of changes in bacteria function, understood
530 as changes in the levels of bacteria with the capacity to metabolize choline-d_9 into TMA-d_9 (CutC
531 gene abundance), should be performed. This would provide relevant information on gut microbiota
532 function modulation as a potential mechanism of action. As described above, we attempted
533 multiple times to quantify the levels of CutC in our samples via qPCR using previously reported
534 degenerative primers, but were unable to amplify this gene. The low abundance of bacteria
535 containing TMA-lyase gene CutC [5], the use of diluted human fecal slurries with a wide diversity
536 of bacteria with different CutC isoforms, and the use of degenerative primers to capture the
537 diversity in CutC gene sequences [47] might have been some obstacles not overcome in our attempts.
538 Additionally, studies have suggested that capacity for TMA production may not correlate well with
539 gut levels of CutC/CutD gene expression [48]. Utilization of other primers may be able to achieve
540 this goal of characterizing the impact of phenolic compounds on TMA lyase abundance in
541 subsequent projects.

542

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

546 **Abbreviations:**

547 CF: Choline-d_9-free conditions.

548 CVD: Cardiovascular disease.

549 DMB: 3,3-Dimethil-1-butanol.

550 LOD: Limit of detection.

551 LOQ: Limit of quantification.

552 MDL: Method detection limit.

553 MFC: Microbiota-free conditions.

554 MQL: Method quantification limit.

555 TMAO: Trimethylamine *N*-oxide.

556 TMA: Trimethylamine.

557 VH: Vehicle.

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751 # Tables:

752

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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-$^{13}C_3$-$^{15}N_4$ [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.

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755

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.

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758

759 **Figure captions:**

760 **Figure 1:** Choline-d_9 and TMA-d_9 kinetic curves in fermentations with growth media and: 100
761 μM choline-d_9 and without fecal slurry (**A**); 100 μM choline-d_9 and 20% fecal slurry (**B**); without
762 100 μM choline-d_9 and with 20% fecal slurry (**C**). Results are expressed as mean μM ± SEM
763 (*n*=4). Abbreviations: ND, not detected.

764

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

779

780 **Figure 3:** TMA-d_9 kinetic curves in fermentations with fecal slurry 20 %, 100 μM choline-d_9 and
781 different test compounds (2 mM, except DMB 10 mM). * Indicates statistical differences ($p<0.05$)
782 in treatment effect by Two-way ANOVA between control conditions (no inhibitors, choline-d_9
783 100 μM) and test compound (factors: treatment and time). Results are expressed as mean μM ±
784 SEM (*n*=4). Abbreviations: VH, vehicle (choline-d_9 0 μM, no fecal slurry); MFC, microbiota-free
785 conditions (no fecal slurry); CF, choline-d_9-free conditions (choline-d_9 0 μM); DMB, 3,3-
786 dimethyl-1-butanol; pCou, *p*-coumaric acid; Caf, caffeic acid; Fer, ferulic acid; Cat, catechin; EC,
787 epicatechin; Hip, hippuric acid; PhVal, phenylvaleric acid; Van, vanillic; HVan, homovanillic
788 acid; DiHOPPA, 3-(3,4-dihydroxyphenyl)propionic acid; 4HOPPA, 3-(4-

789 hydroxyphenyl)propionic acid; 3HOPPA, 3-(3-hydroxyphenyl)propionic acid; PPA, 3-
790 phenylpropionic acid; DiHOPAA, 3-(3,4-dihydroxyphenyl)acetic acid; 4HOPAA, 3-(4-
791 hydroxyphenyl)acetic acid; 3HOPAA, 3-(3-hydroxyphenyl)acetic acid; PAA, phenylacetic acid;
792 DiHOBA, 3,4-dihydroxybenzoic acid; and 3HOBA, 3-hydroxybenzoic acid. Note that the control
793 treatment group is plotted on all graphs for ease of comparison.
794

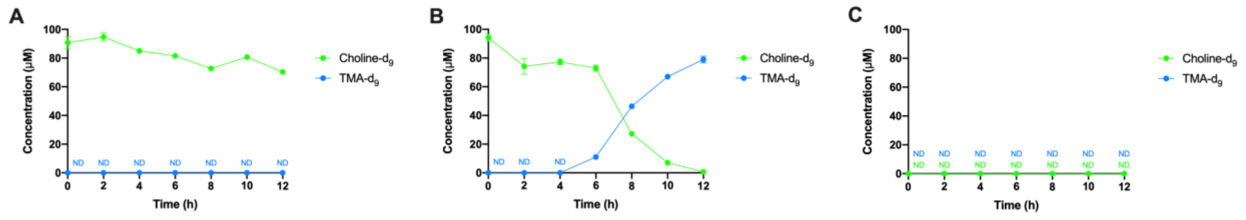
795 **Figure 4:** Area under the curve (AUC) of the kinetic curves of choline-d_9 (**A**), TMA-d_9 (**B**), choline
796 (**C**) and TMA (**D**). * Indicates statistical difference (p<0.05) against control conditions (choline-
797 d_9 100 μM) by One-way ANOVA (Dunnetts' *post hoc* test). Results are expressed as mean h x μM
798 ± SEM (n=4). Abbreviations: Control, choline-d_9 100 μM; VH, vehicle (choline-d_9 0 μM); MFC,
799 microbiota-free conditions; CF, choline-d_9-free conditions (choline-d_9 0 μM); DMB, 3,3-
800 dimethyl-1-butanol; pCou, *p*-coumaric acid; Caf, caffeic acid; Fer, ferulic acid; Cat, catechin; EC,
801 epicatechin; Hip, hippuric acid; PhVal, phenylvaleric acid; Van, vanillic; HVan, homovanillic
802 acid; DiHOPPA, 3-(3,4-dihydroxyphenyl)propionic acid; 4HOPPA, 3-(4-
803 hydroxyphenyl)propionic acid; 3HOPPA, 3-(3-hydroxyphenyl)propionic acid; PPA, 3-
804 phenylpropionic acid; DiHOPAA, 3-(3,4-dihydroxyphenyl)acetic acid; 4HOPAA, 3-(4-
805 hydroxyphenyl)acetic acid; 3HOPAA, 3-(3-hydroxyphenyl)acetic acid; PAA, phenylacetic acid;
806 DiHOBA, 3,4-dihydroxybenzoic acid; and 3HOBA, 3-hydroxybenzoic acid. Note that the control
807 treatment group is plotted on all graphs for ease of comparison.
808

809 **Figure 5:** Cell density measured as optical density at 600 nm (OD 600nm) in fermentations with
810 fecal slurry 20 %, 100 μM choline-d_9 and different test compounds (2 mM, except DMB 10 mM).
811 $ Indicates statistical differences (p<0.05) in treatment effect by Two-way ANOVA against
812 vehicle (VH); # Indicates statistical differences (p<0.05) in treatment effect by Two-way ANOVA
813 against choline-d_9-free conditions (CF). * Indicates statistical differences (p<0.05) in treatment
814 effect by Two-way ANOVA against control conditions (choline-d_9 100 μM). Results are expressed
815 as mean arbitrary units ± SEM (n=4). Abbreviations: VH, vehicle (choline-d_9 0 μM, no fecal
816 slurry); MFC, microbiota-free conditions (no fecal slurry); CF, choline-d_9-free conditions
817 (choline-d_9 0 μM); DMB, 3,3-dimethyl-1-butanol; pCou, *p*-coumaric acid; Caf, caffeic acid; Fer,
818 ferulic acid; Cat, catechin; EC, epicatechin; Hip, hippuric acid; PhVal, phenylvaleric acid; Van,
819 vanillic; HVan, homovanillic acid; DiHOPPA, 3-(3,4-dihydroxyphenyl)propionic acid; 4HOPPA,

820 3-(4-hydroxyphenyl)propionic acid; 3HOPPA, 3-(3-hydroxyphenyl)propionic acid; PPA, 3-
821 phenylpropionic acid; DiHOPAA, 3-(3,4-dihydroxyphenyl)acetic acid; 4HOPAA, 3-(4-
822 hydroxyphenyl)acetic acid; 3HOPAA, 3-(3-hydroxyphenyl)acetic acid; PAA, phenylacetic acid;
823 DiHOBA, 3,4-dihydroxybenzoic acid; and 3HOBA, 3-hydroxybenzoic acid. Note that the control
824 treatment group is plotted on all graphs for ease of comparison.
825
826 **Figure 6:** Cell respiration relative to choline-d_9-free conditions (CF) in fermentations with fecal
827 slurry 20 %, 100 μM choline-d_9 and different test compounds (2 mM, except DMB 10 mM). *
828 Indicates statistical difference ($p<0.05$) against control conditions (choline-d_9 100 μM) by One-
829 way ANOVA (Dunnetts' *post hoc* test). Results are expressed as mean μM ± SEM (n=4).
830 Abbreviations: VH, vehicle (choline-d_9 0 μM); MFC, microbiota-free conditions; CF, choline-d_9-
831 free conditions (choline-d_9 0 μM); DMB, 3,3-dimethyl-1-butanol; pCou, *p*-coumaric acid; Caf,
832 caffeic acid; Fer, ferulic acid; Cat, catechin; EC, epicatechin; Hip, hippuric acid; PhVal,
833 phenylvaleric acid; Van, vanillic; HVan, homovanillic acid; DiHOPPA, 3-(3,4-
834 dihydroxyphenyl)propionic acid; 4HOPPA, 3-(4-hydroxyphenyl)propionic acid; 3HOPPA, 3-(3-
835 hydroxyphenyl)propionic acid; PPA, 3-phenylpropionic acid; DiHOPAA, 3-(3,4-
836 dihydroxyphenyl)acetic acid; 4HOPAA, 3-(4-hydroxyphenyl)acetic acid; 3HOPAA, 3-(3-
837 hydroxyphenyl)acetic acid; PAA, phenylacetic acid; DiHOBA, 3,4-dihydroxybenzoic acid; and
838 3HOBA, 3-hydroxybenzoic acid.

839

840 **Figure 1:**



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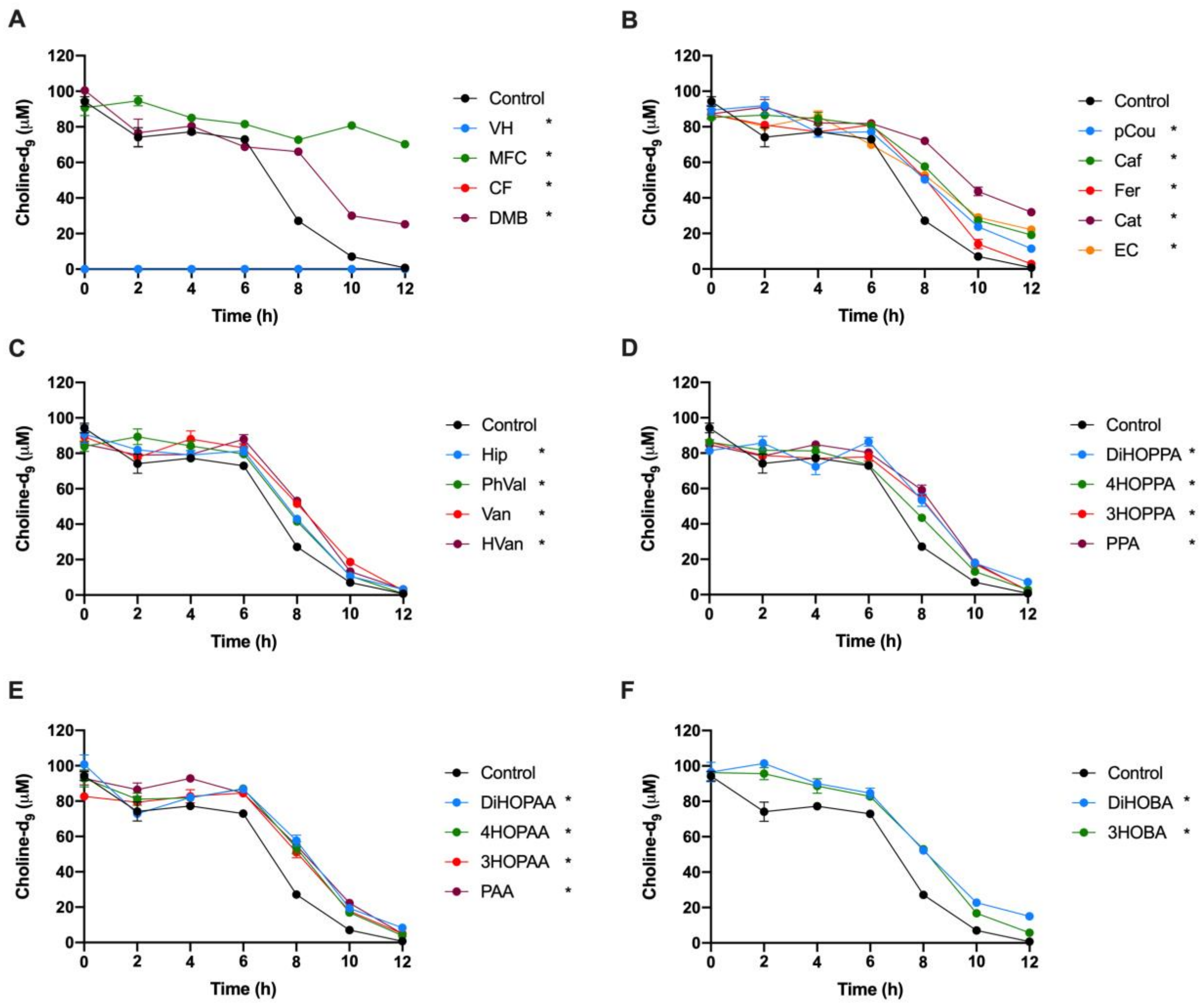

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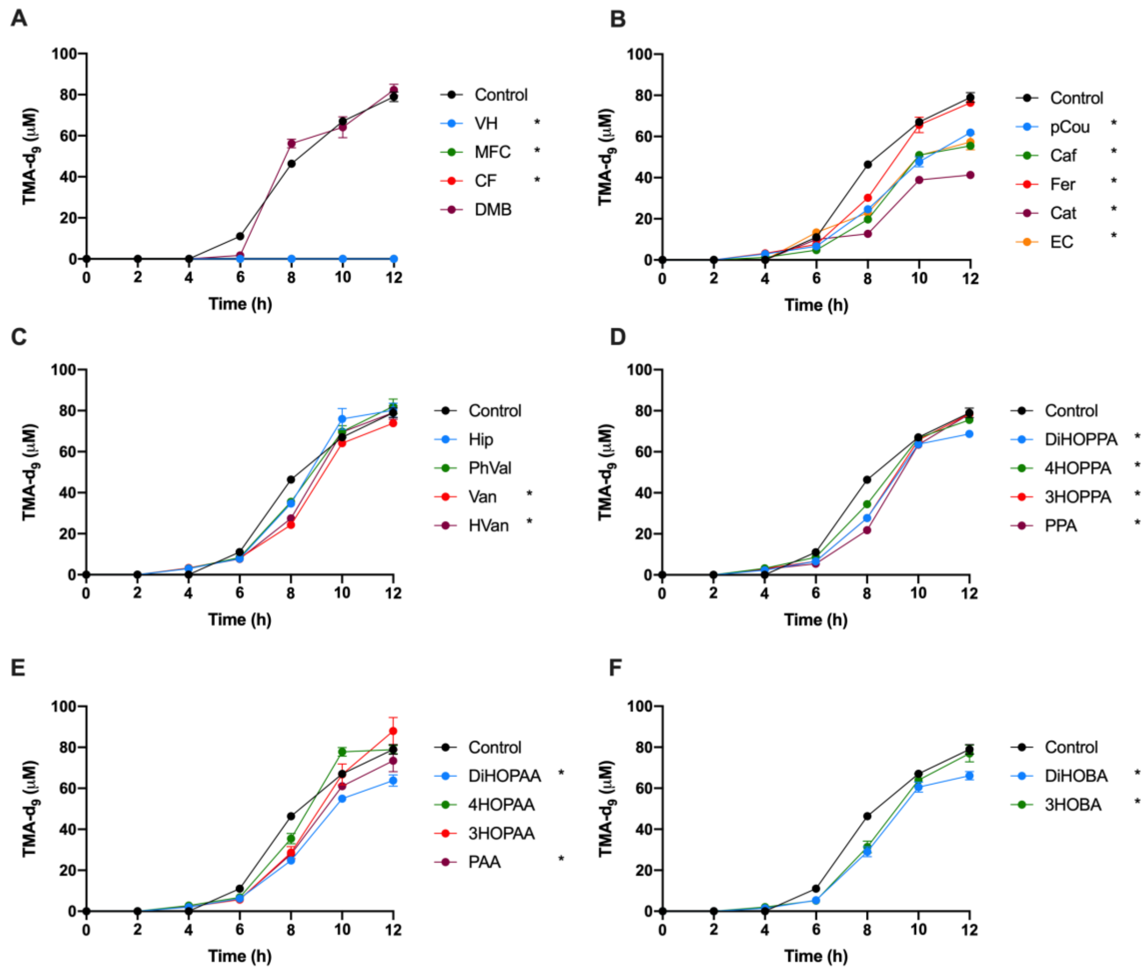

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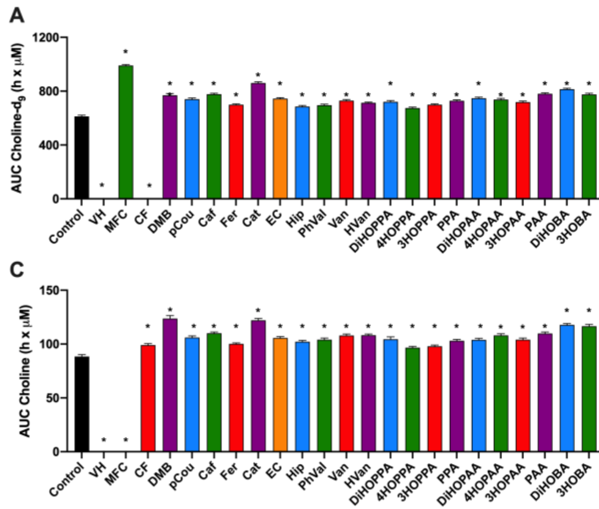
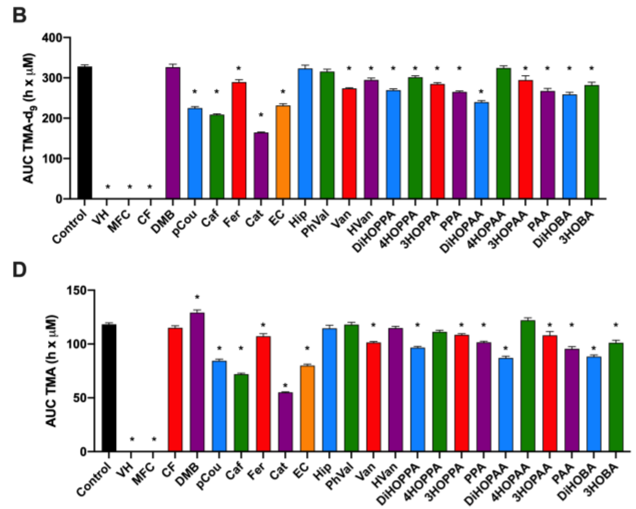
850 **Figure 4:**





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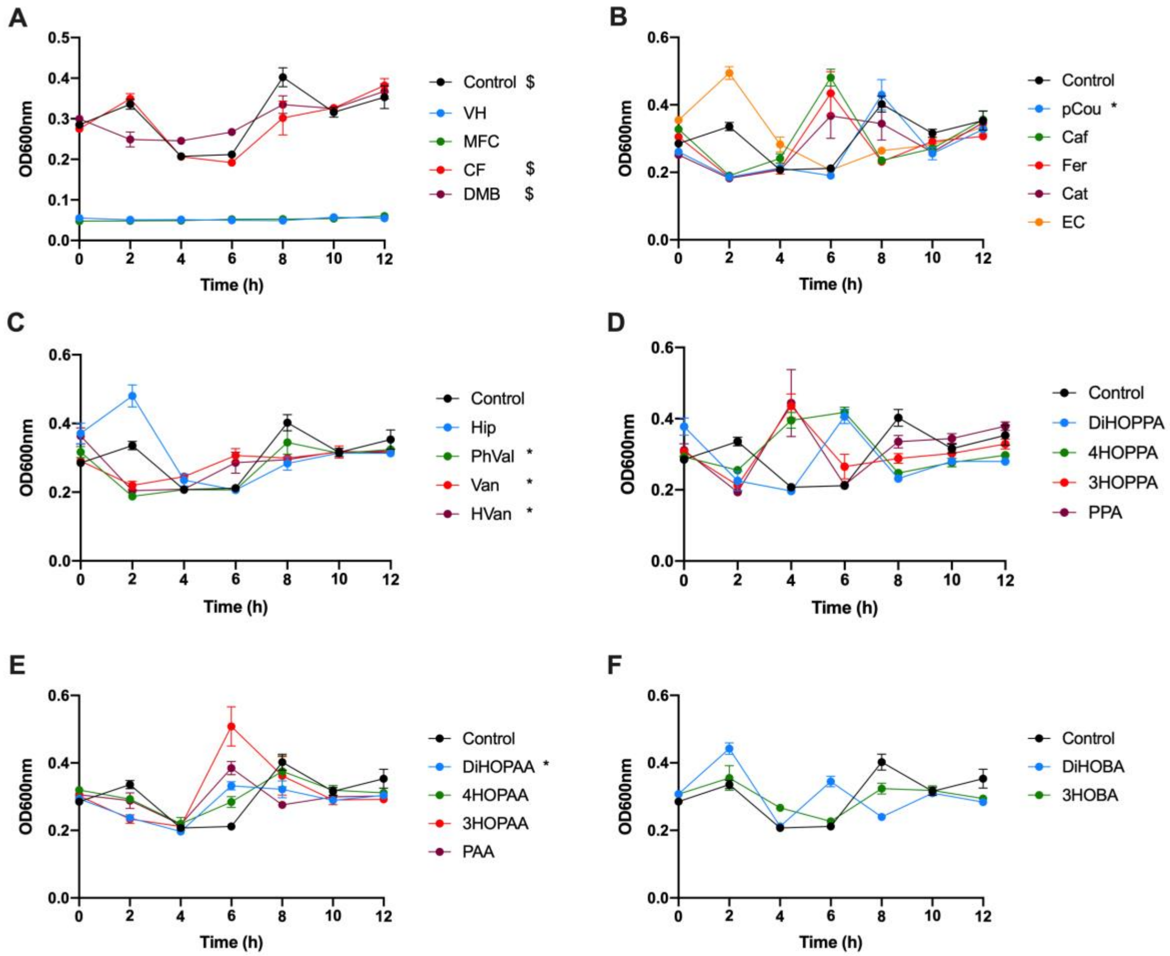

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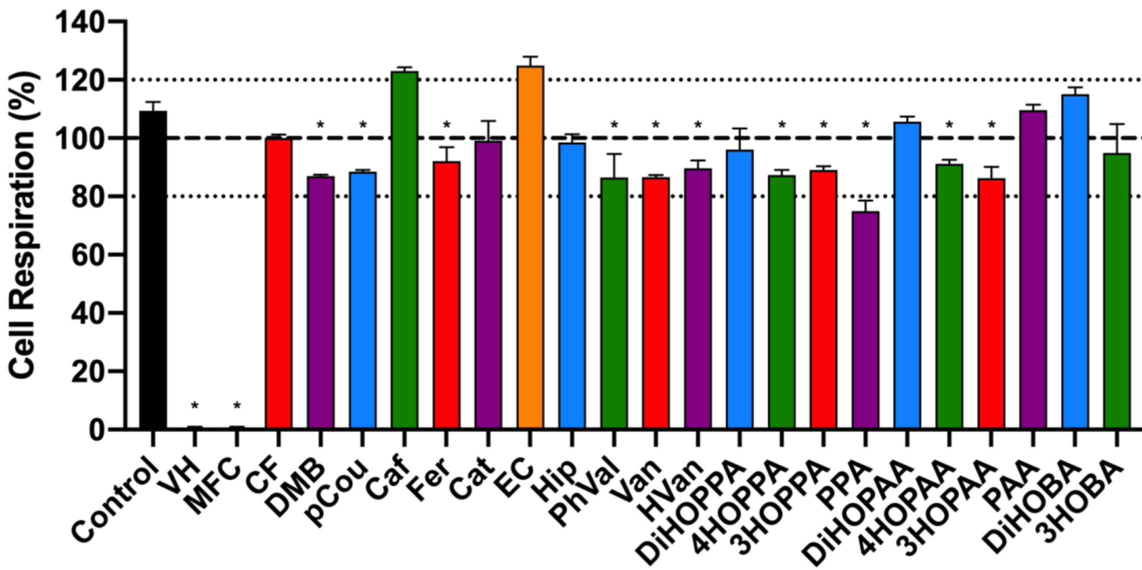

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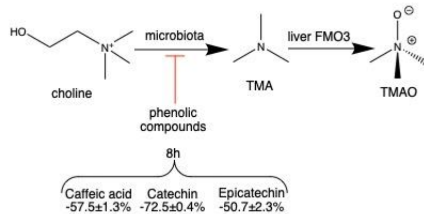

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