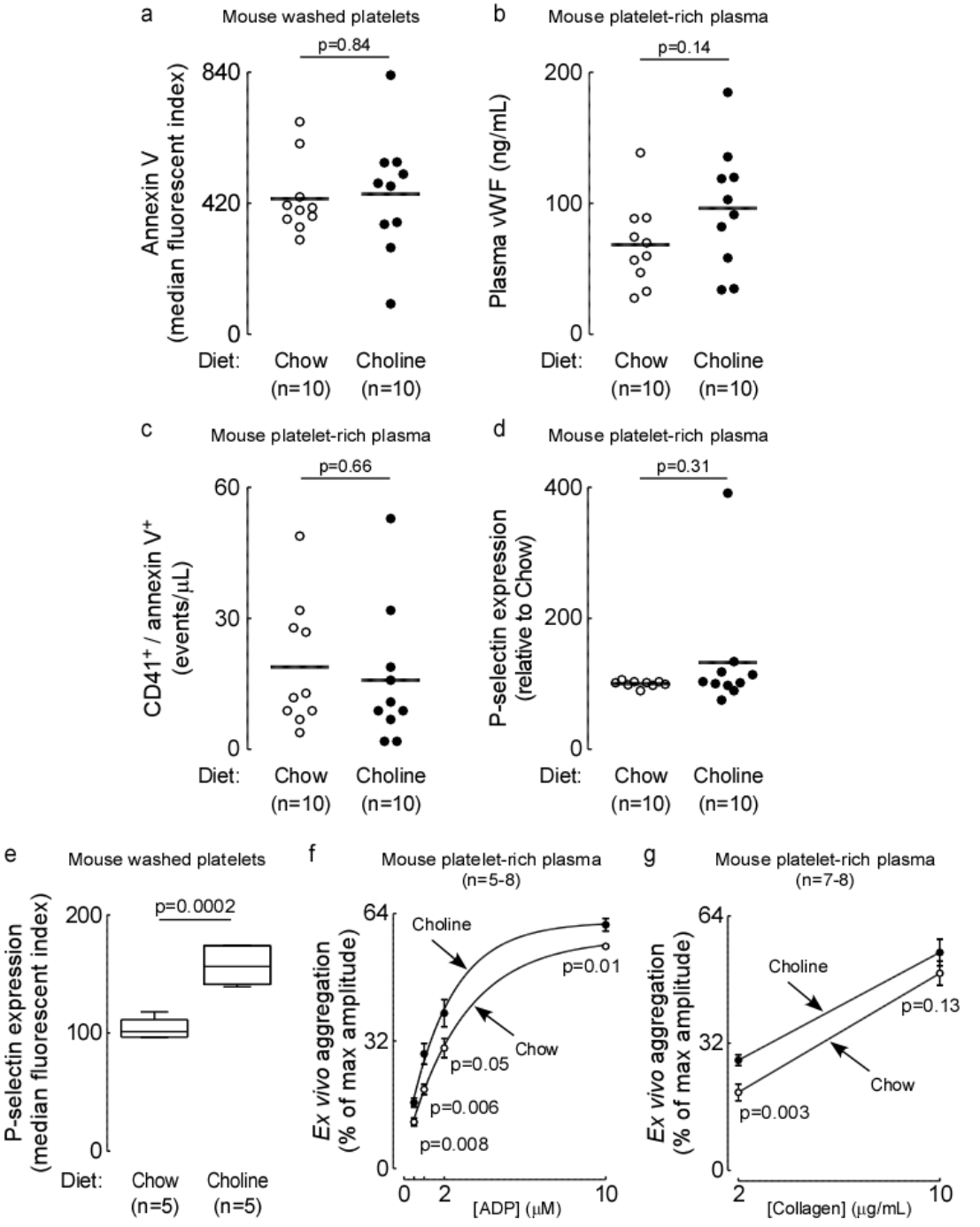
Supplementary Figure 1

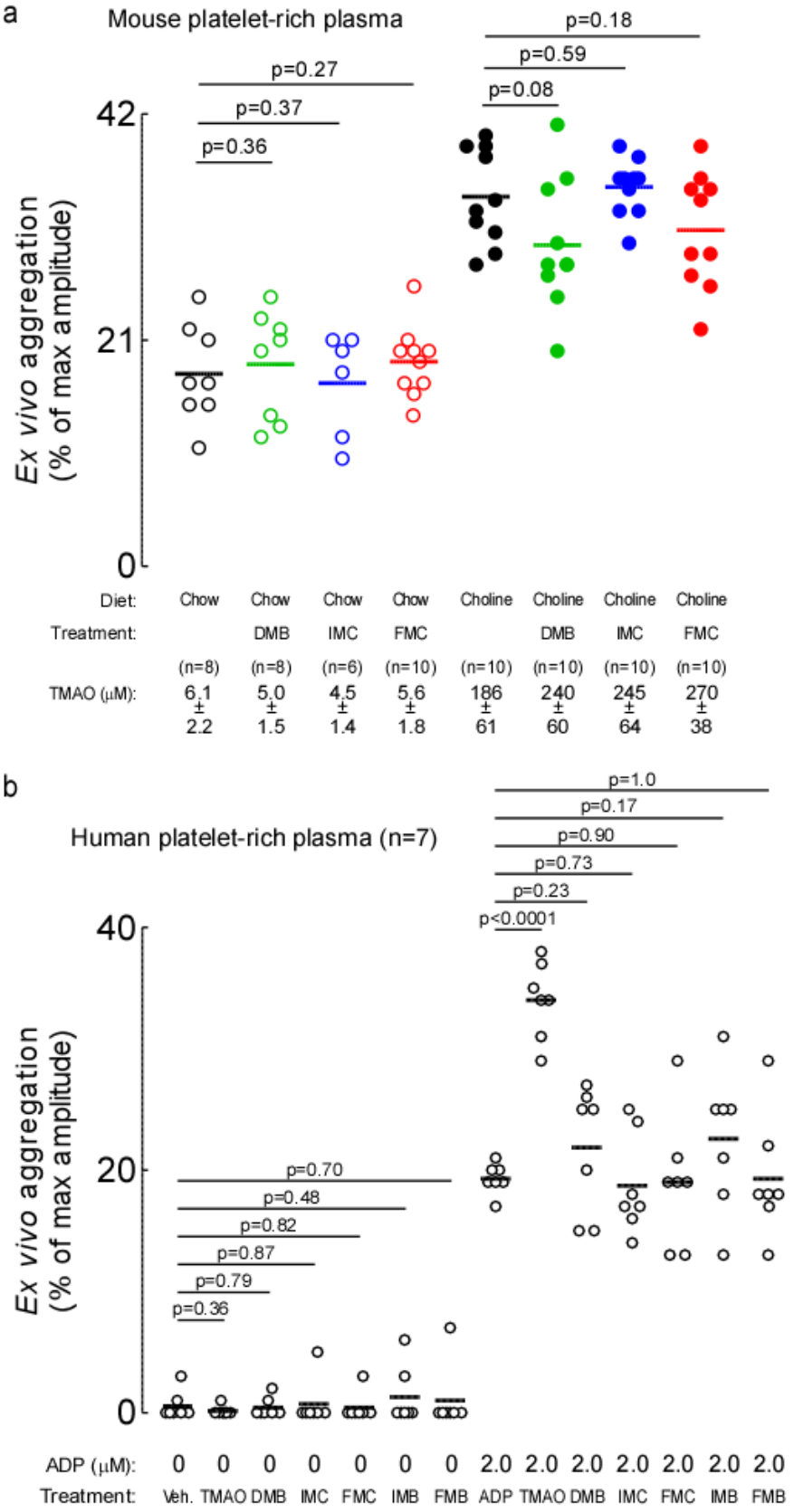


Supplementary Figure 1) Choline diet-induced effects on platelet aggregation and prothrombotic markers

a) C57BL/6J female mice (10-13 weeks of age) were placed on the indicated diets supplemented with or without 1% choline for ≥1 week. Washed platelets were stimulated with ADP (2 μM) then stained with FITC annexin V conjugate (1:50 dilution). Data are presented as median fluorescence intensity for each mouse (bars represent the mean for each group; n=10 mice per dietary group). P-values shown are for comparisons between the indicated groups using a two-tailed Student's t-test.

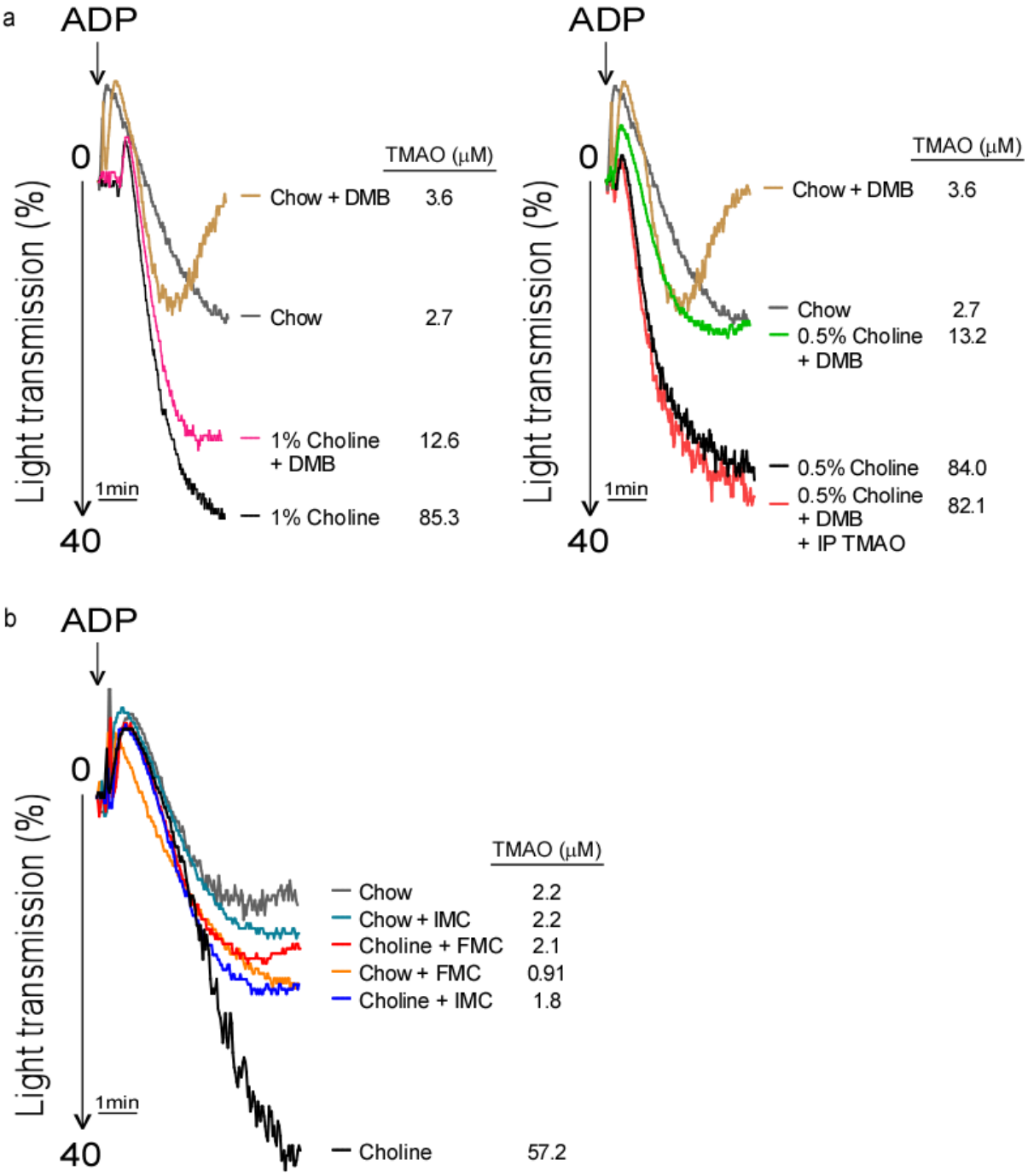
b) C57BL/6J female mice (10-13 weeks of age) were placed on the indicated diets supplemented with or without 1% choline for ≥1 week. Endpoint plasma was used to quantify the levels of circulating von Willebrand Factor (vWF) using a sandwich ELISA assay. Data points represent plasma vWF levels for each mouse (bars represent the mean for each group; n=10 mice per dietary group). P-values shown are for comparisons between the indicated groups using a two-tailed Student's test.
c) C57BL/6J female mice (10-13 weeks of age) were placed on the indicated diets supplemented with or without 1% choline for ≥1 week. Microvesicles were isolated by differential centrifugation. FITC-PE conjugated CD41 antibody and FITC annexin V conjugate were used to assess the quantities of CD41+/annexin V+ microvesicles. Data points represent microvesicle levels for each mouse (bars represent the mean for each group; n=10 mice per dietary group). P-values shown are for comparisons between the indicated groups using a two-tailed Student's test.
d) C57BL/6J female mice (10-13 weeks of age) were placed on the indicated diets supplemented with or without 1% choline for ≥1 week. Platelet-rich plasma was isolated and alpha granule release in response to diet was evaluated by p-selectin cell-surface expression. P-selectin was quantified using FITC-conjugated CD62p antibody. Data points represent median fluorescent intensity for each mouse relative to chow-fed mice (bars represent the mean for each group; n=10 mice per dietary group). P-values shown are for comparisons between the indicated groups using a two-tailed Student's t-test.
e) C57BL/6J female mice (10-13 weeks old) were fed the indicated diets supplemented with or without 1% choline for ≥1 week. Platelets were isolated, washed, and then stimulated with ADP (2 μM) and p-selectin cell-surface expression was determined using FITC-conjugated CD62p antibody. Data are presented as a box-whisker plot where the box represents the first and third quartiles, the center line is the median, and the whiskers represent the 5 and 95 percentiles (n=5 mice per dietary group). P-values shown are for comparisons between the indicated groups using a two-tailed Student's t-test.
f-g) C57BL/6J female mice (10-13 weeks old) were fed the indicated diets supplemented with or without 1% choline for ≥1 week. Platelet aggregation was monitored in platelet-rich plasma in response to the indicated levels of **(f)** ADP (0.5 – 10 μM) or **(g)** collagen (2 or 3 μg/mL). Data are represented as % of maximum amplitude (mean +/- SEM) for each group. At least 5 animals were used to generate each data point. Exact numbers of mice used for each data point can be found in the Source Data (n=5-8). P-values shown are for comparisons between the indicated groups using a two-tailed Student's t-test.

Supplementary Figure 2



Supplementary Figure 2) Control Studies: TMA lyase inhibitors alone do not induce platelet aggregation, nor do they alter ADP-induced platelet aggregation.
a) C57BL/6J female mice (10-13 weeks old) were fed either the chemically-defined chow diet or choline-supplemented (1%) diet (all without inhibitor present) for ≥1 week. Platelet-rich plasma was isolated and incubated with the indicated "Treatment" (i.e. 20 μM of IMC, FMC, DMB or vehicle) at room temperature for 20 minutes prior to platelet aggregation in response to ADP (1 μM). Data points represent aggregation as % of maximum amplitude in platelet-rich plasma recovered from each mouse, and bars represent mean levels for each group. Sample sizes shown represent indicated numbers of mice in each group. Plasma TMAO levels are also shown and represent mean ± SEM for each group. All p-values were measured by two-tailed Student's t-tests.
b) Human plasma was obtained from healthy consenting volunteers. Platelet aggregation was monitored in platelet-rich plasma in the absence of ADP (left) or presence of ADP (2 μM, right) following incubation with either vehicle or the indicated inhibitors (20 μM IMC, FMC, DMB), or the primary metabolites (20 μM IMB, FMB), or TMAO (100 μM). Data points (n=7) represent the % of maximum amplitude levels and bars represent group means from the indicated numbers of mice. All p-values were measured by two-tailed Student's t-tests.

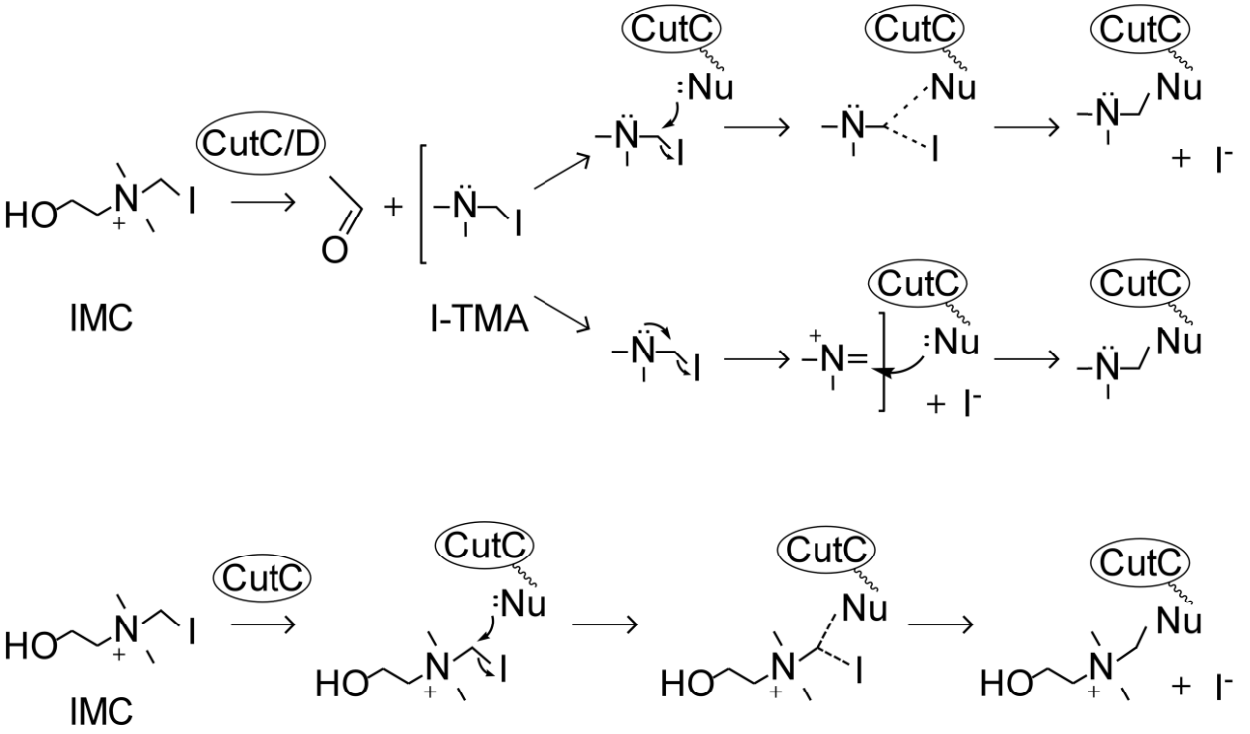
Supplementary Figure 3



Supplementary Figure 3) Representative platelet aggregation tracings from mice fed a low- or high-choline diet with or without inhibitor

C57BL/6J female mice (12 weeks old) were fed **(a)** the indicated diets ± DMB (1.3% v/v) or **(b)** the indicated diets ± FMC (0.006% w/w) or IMC (0.06% w/w) for >2 weeks. Shown are representative aggregation tracings in platelet-rich plasma from a mouse in each of the indicated groups in response to submaximal (1 μM) ADP, along with that mouse's corresponding plasma TMAO level. Complete study results including replications are shown for **(a)** in Figure 1a and for **(b)** in Figure 4a.

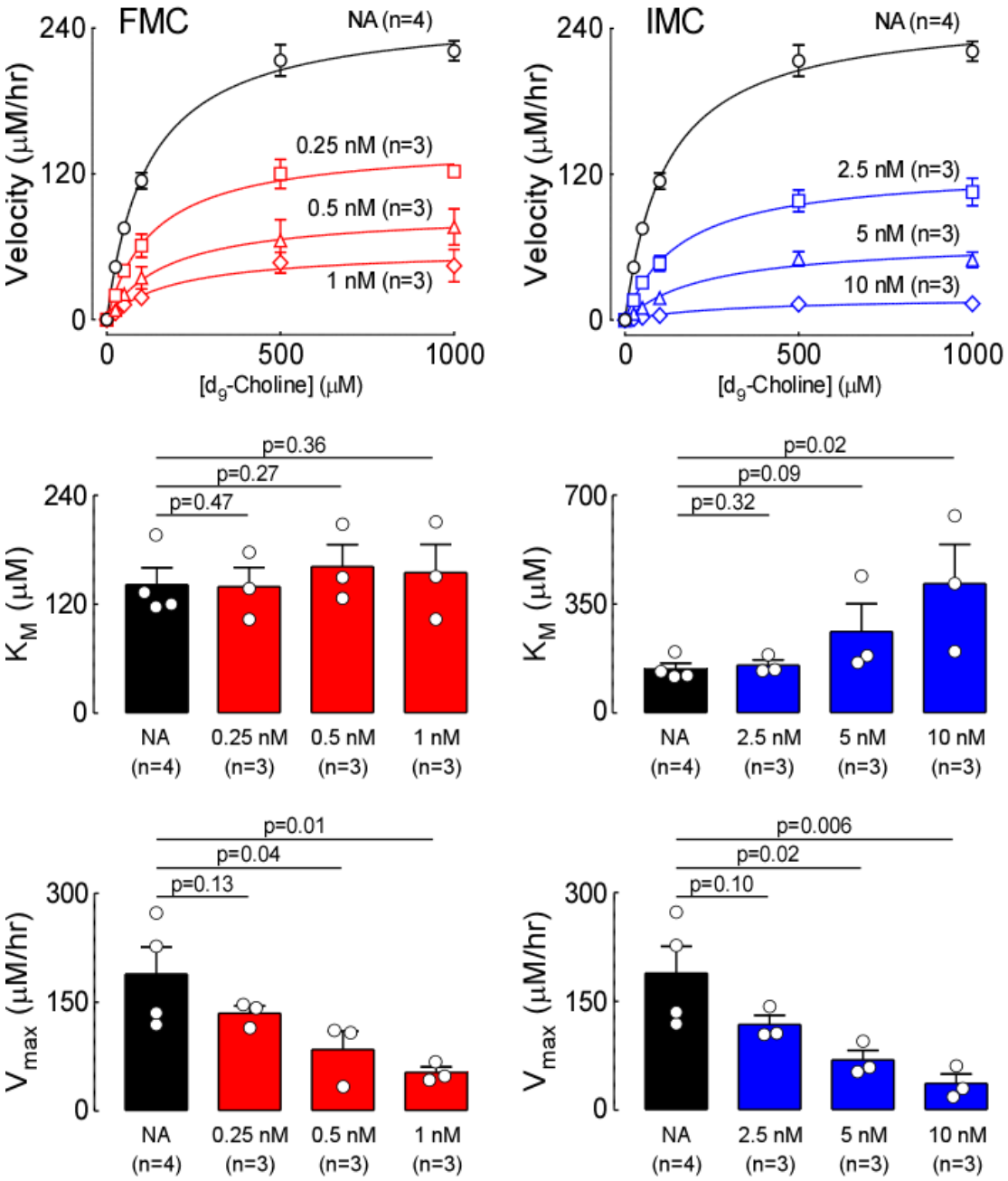
Supplementary Figure 4



Supplementary Figure 4) Hypothetical reaction mechanism for IMC inhibition of the microbial CutC/D enzyme complex

(Top) Schematic reaction mechanism for IMC lysis by CutC/D. In this hypothetical scenario, the suicide substrate inhibitor IMC undergoes C-N bond cleavage catalyzed by CutC/D, producing acetaldehyde and the halo-TMA analogue iodotrimethylamine (I-TMA). I-TMA is predicted to be quite labile, and may either undergo a backside nucleophilic attack by any suitably positioned nucleophilic residue (also outlined in Figure 1d), or it may undergo an elimination reaction, losing its reactive leaving group (I^-) generating a reactive imine, before generating a covalent adduct with a CutC nucleophilic residue ("Nu"). In both of these latter mechanisms, time dependent non-competitive irreversible inhibition is anticipated. The brackets represent the concerted formation of the imine directly from IMC with I-TMA present only as a transition state, based on quantum mechanical calculations that predict I-TMA is too labile and may not be generated. **(Bottom)** In another hypothetical pathway for CutC inhibition by IMC, the nucleophilic CutC residue ("Nu") attack on IMC precedes completion of the C-N bond cleavage, resulting in an adduct comprised of an entire choline molecule.

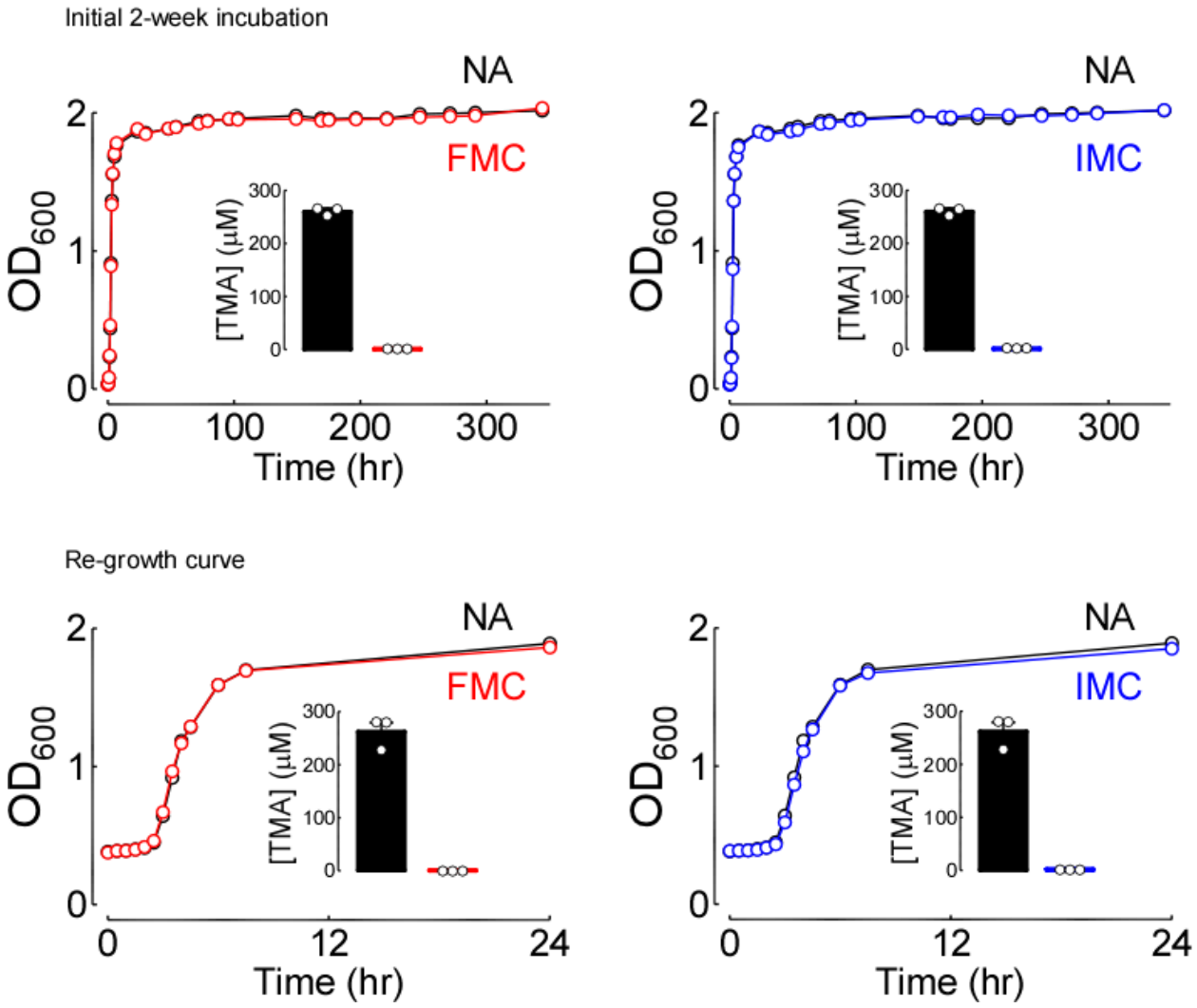
Supplementary Figure 5



Supplementary Figure 5) Michaelis-Menten kinetic analyses of FMC and IMC dependent inhibition of TMA formation from d_9-choline substrate in recombinant *P. mirabilis* CutC/D

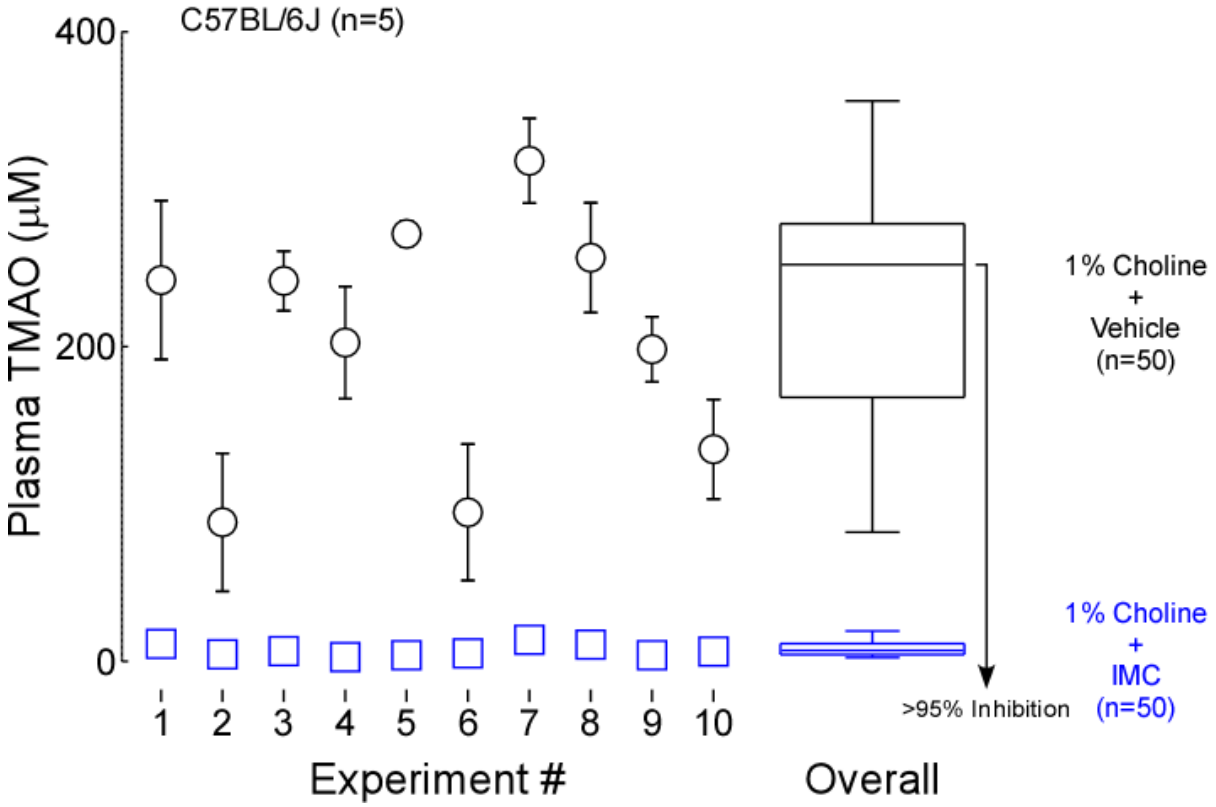
In the presence of FMC (left) and IMC (right), compared to "no addition" (NA) controls, the initial rate of d_9-TMA formation was quantified by LC/MS/MS analyses of samples as a function of changes in substrate concentration across a range of inhibitor concentrations. Data points represent the mean ± SEM for the indicated number of experiments, each performed in triplicate. The effect of FMC or IMC on kinetic parameters K_M (middle) and V_{max} (bottom) are represented by bar graphs (mean ± SEM). Significance determined by one-tailed Student's t-test.

Supplementary Figure 6



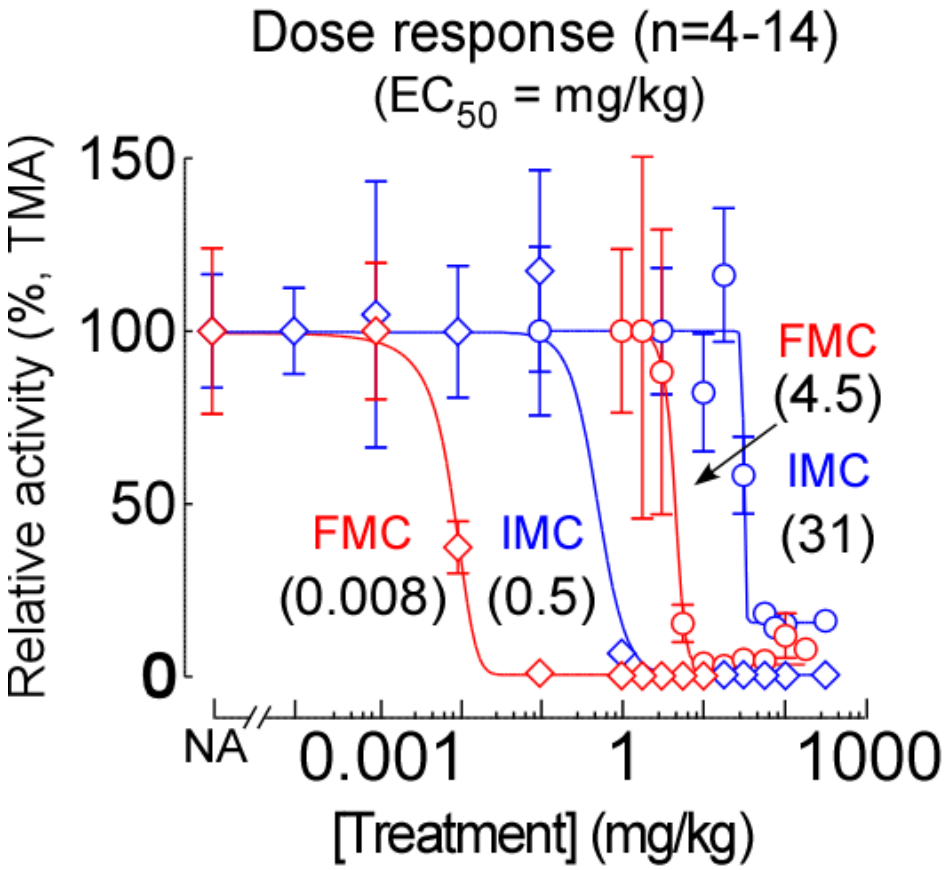
Supplementary Figure 6) There is no observable effect on long-term growth or fitness of a TMA-producing human gut commensal with high concentration (1 mM) exposure to IMC or FMC.
Wild-type *P. mirabilis* was grown in LB media with either vehicle (water) or the addition of 1 mM of either FMC or IMC. During the log-growth phase, aliquots were taken and OD_{600} measurements were taken every ~30 minutes. Additional aliquots were removed when OD_{600}=0.5 to quantify enzymatic activity with the addition of d_9-labeled choline as the substrate (in triplicate). After the first day, OD_{600} measurements were taken every ~12 or ~24 hours during the stationary phase. After ~2 weeks, aliquots of each group of cells were recovered and diluted 1:100 into fresh LB media. The log-growth phase was again recorded, and similarly, additional aliquots were removed when OD_{600}=0.6 to measure enzymatic activity. Growth curves represent a single experiment with the activity test performed in triplicate (mean +/- SEM).

Supplementary Figure 7



Supplementary Figure 7) IMC consistently suppresses TMAO levels in mice on a high-choline diet. C57BL/6J female mice (≥8 weeks of age) were placed on a 1% choline diet and treated with either vehicle or 100 mg/kg IMC via gastric gavage once/daily. Blood was collected 24 hours post-gavage and plasma TMAO levels were quantified using stable isotope dilution LC/MS/MS. Data shown are from 10 separate experiments conducted across a 2 year period (n=5 mice per group). Results shown are reported as mean +/- SEM for each group. The "Overall" box-plot represents the first and third quartiles, the center line is the median, and the whiskers represent the 5 and 95 percentiles for results averaged over all studies over a 2 year period (n=50 per group). We routinely perform these studies "identically" with respect to age of animals, timing of day inhibitor is initiated, gavage of drug administered, and then 24h later, timing of day blood is collected, yet still note substantial biological variation in the TMAO level observed on a high choline diet. Importantly, use of the inhibitor IMC always results in >95% inhibition in TMAO levels. Moreover, the choline diet-induced elevation in plasma TMAO is always >10-fold higher than the chow.

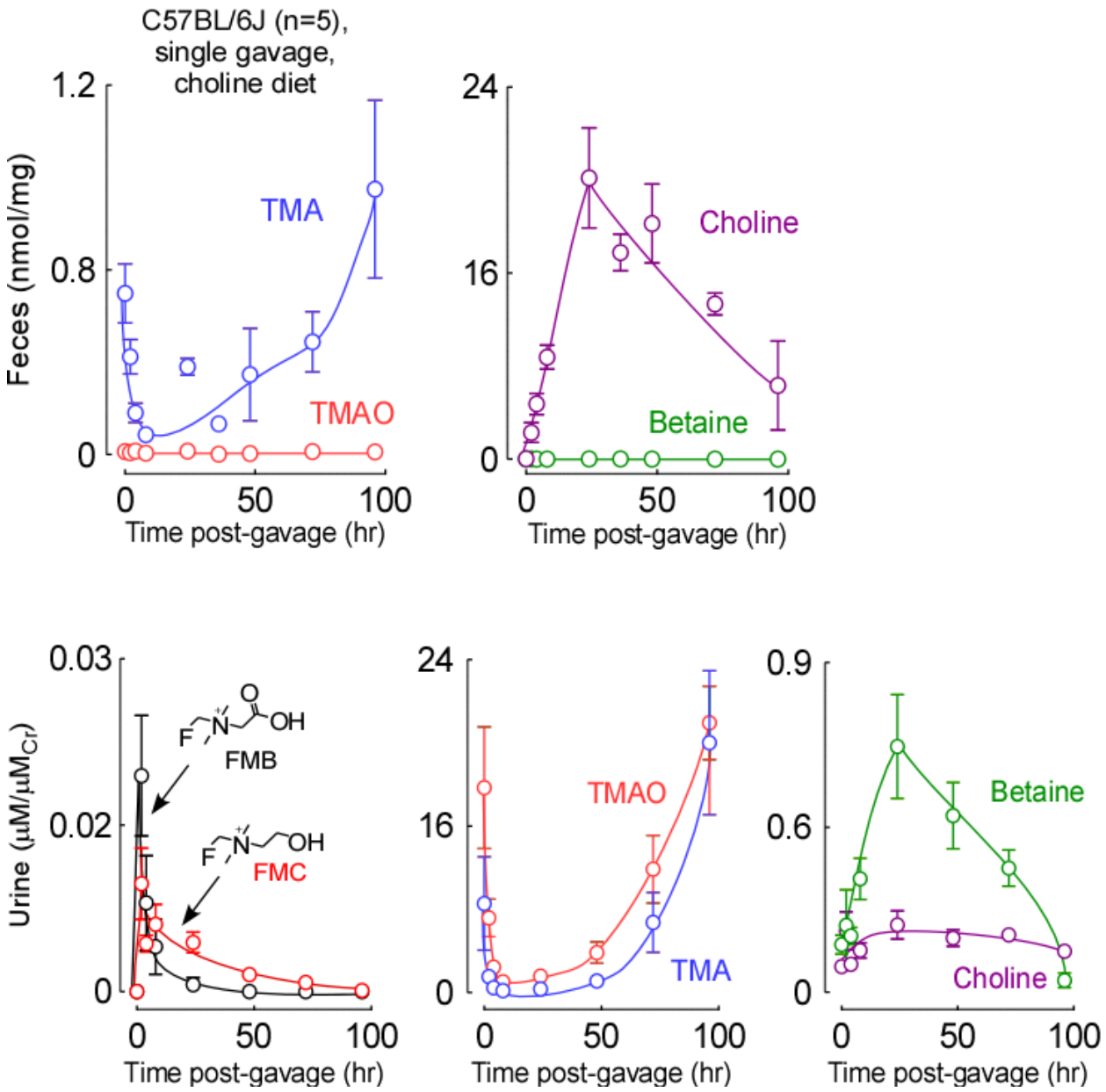
Supplementary Figure 8



Supplementary Figure 8) Dose response curves examining impact of FMC and IMC on either systemic TMA/TMAO levels in chronic choline diet-supplemented mice, or d_9-TMA/ d_9-TMAO production from oral d_9-choline challenged mice

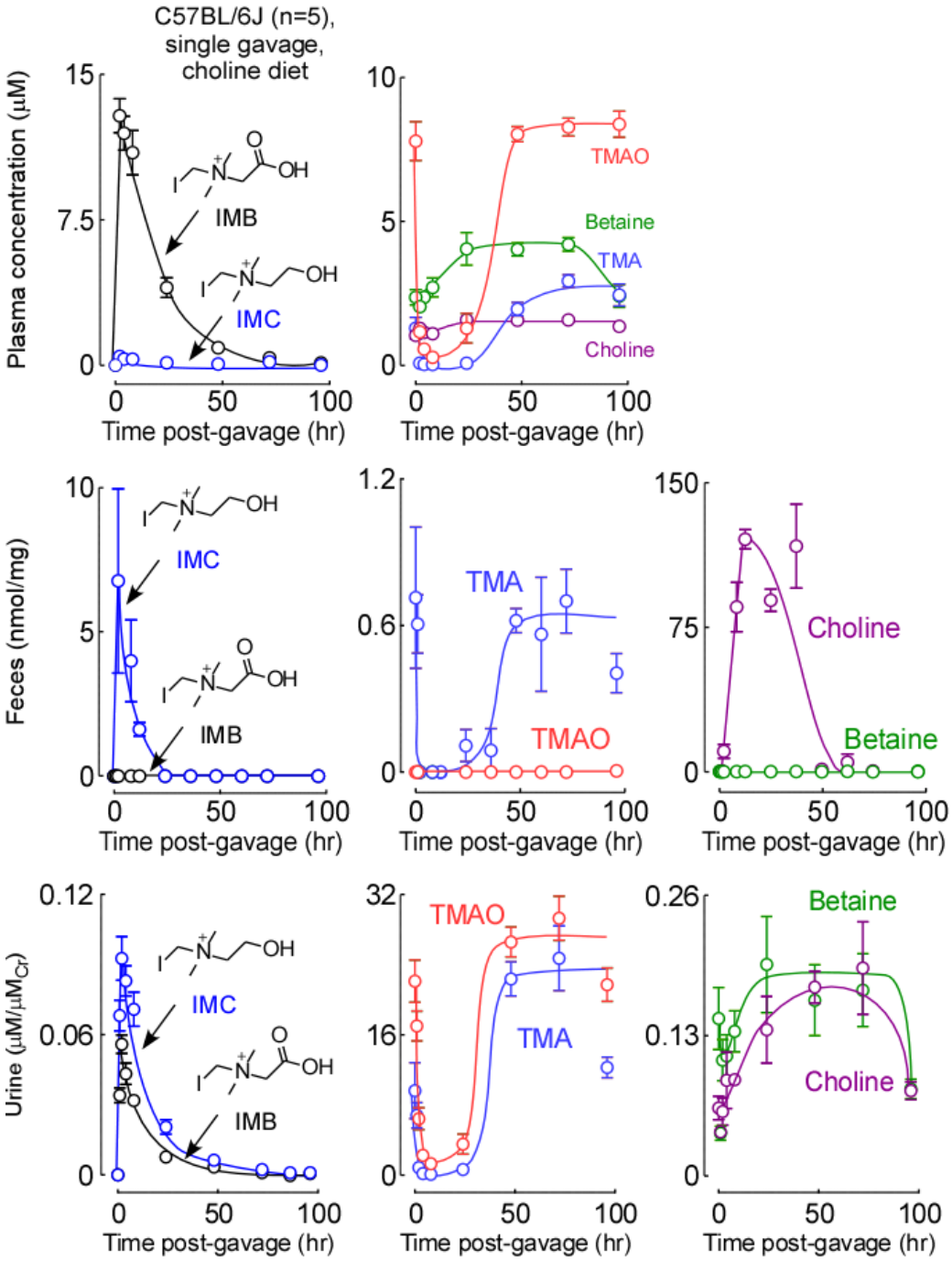
(Top) C57BL/6J female mice (18-20 g body weight) were treated by oral gavage with either vehicle ("No Addition", NA) or a range of doses (0.0001 – 310 mg/kg) of either FMC or IMC. In the "d_9-choline challenge" (◊), groups of mice maintained on a chemically-defined diet comparable in composition to a normal chow were simultaneously gavaged with 10 mg/mL d_9-choline plus the indicated dose of either FMC or IMC. Blood was collected 3 hours post-gavage and relative activity was measured as the amount of d_9-TMA produced relative to vehicle controls. In the "q24h post-gavage" model (O), groups of mice were maintained on the chemically-defined diet supplemented with choline (1% w/w) and given once-daily oral gavage of the indicated dose of either FMC or IMC for 4 successive days. On day 5, blood was collected 24 hours after the last oral gavage. Relative activity represents plasma TMA levels relative to vehicle controls. Data points represent the mean ± SEM. Numbers in parentheses represent the calculated EC_{50} dose for the indicated inhibitor using data either from the (i) d_9-choline challenge model, which gives estimate of oral dose needed to inhibit 50% of microbial d_9-choline → d_9-TMA *in vivo*; or (ii) the q24h post-gavage model, which gives estimate of the oral dose needed to inhibit 50% of plasma level of TMA at trough time in a chronic once daily oral gavage dosing regimen in mice maintained on a high choline diet. Dose-response curves were performed multiple times with results shown representing the cumulative results from all drug concentrations tested. Each data point represents at least four mice at the indicated drug concentration. Exact numbers of mice used for each data point can be found in the Source Data (n=4-14).

Supplementary Figure 9



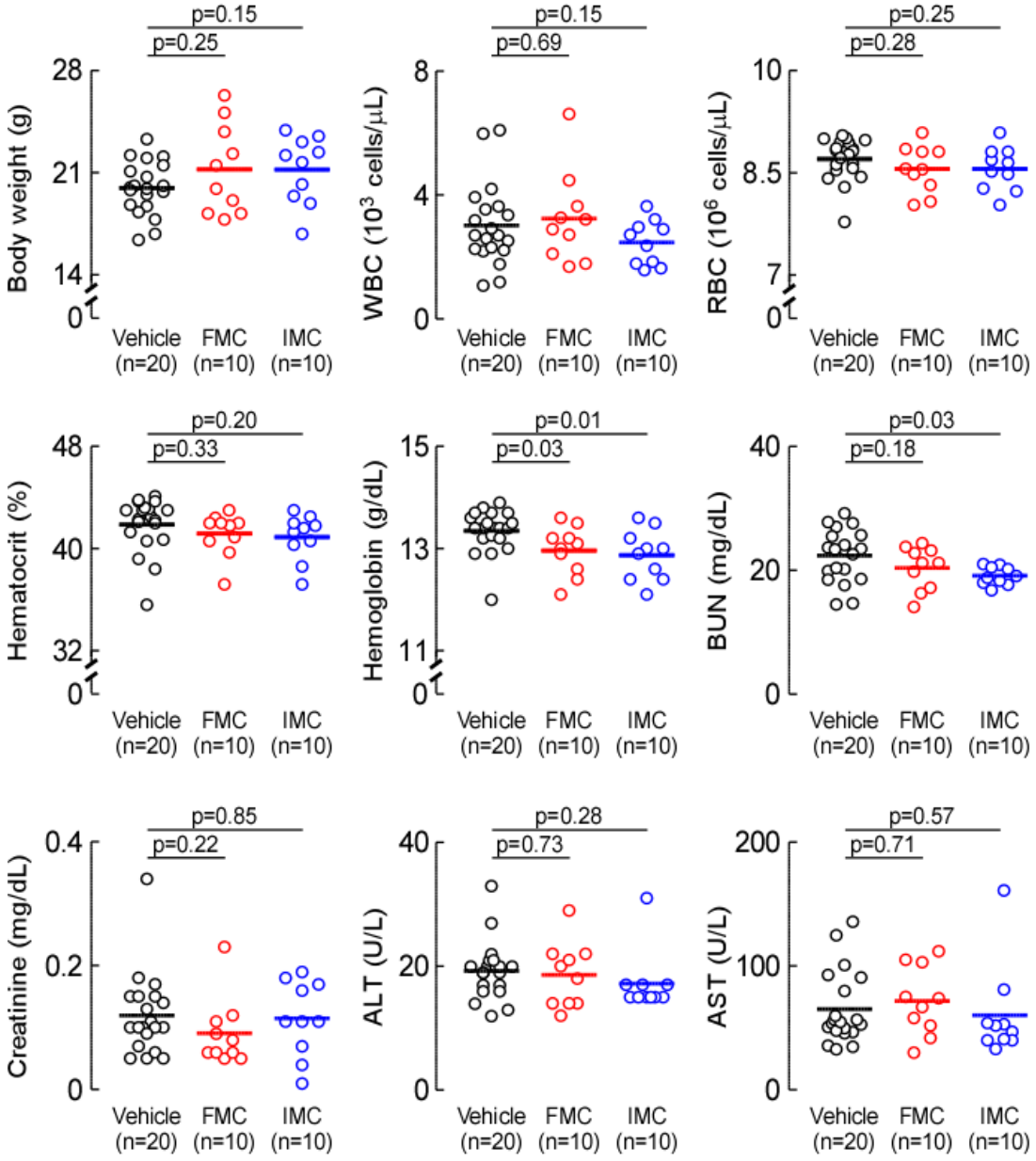
Supplementary Figure 9) The *in vivo* urinary and fecal pharmacokinetic properties of FMC
Groups of C57BL/6J female mice (12 weeks old) maintained on a choline-supplemented diet (1% w/w) for 3 weeks were given a single oral gavage of 100 mg/kg FMC. At specified time points, fresh feces were collected and levels of choline, betaine, TMA, and TMAO were measured by LC/MS/MS (top). Fecal metabolites are reported per mg of dried feces. At specified time points, urine was collected and levels of FMC, FMB, choline, betaine, TMA, and TMAO were measured by LC/MS/MS (bottom). Urinary levels of metabolites are reported as a ratio with simultaneously quantified creatinine, to account for alterations in urinary dilution. Data points represent the mean ± SEM for the indicated number of animals.

Supplementary Figure 10



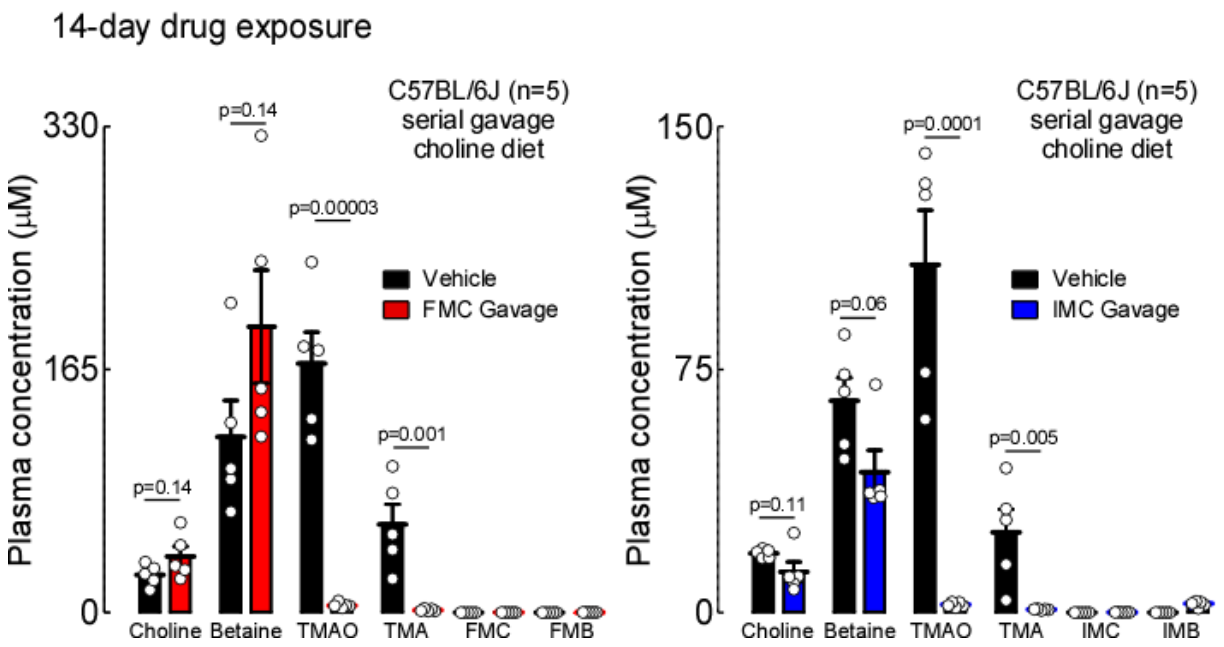
Supplementary Figure 10) The *in vivo* plasma, fecal, and urinary pharmacokinetic properties of IMC
Groups of C57BL/6J female mice (12 weeks old) maintained on a choline-supplemented diet (1% w/w) for 3 weeks were given a single oral gavage of 100 mg/kg IMC. At the indicated time points post-gavage, blood was drawn and plasma levels of IMC, IMB, choline, betaine, TMA, and TMAO were measured by LC/MS/MS (top). Fresh fecal samples were also collected and levels of IMC, IMB, choline, betaine, TMA, and TMAO were quantified by LC/MS/MS and normalized to the dry weight of samples (center). At the indicated time points, urine was collected and levels of IMC, IMB, choline, betaine, TMA, and TMAO were measured by LC/MS/MS (bottom). Data points represent the mean ± SEM for the indicated number of animals.

Supplementary Figure 11



Supplementary Figure 11) Toxicity studies for FMC and IMC
Groups of C57BL/6J female mice (8-12 weeks old) were maintained on a choline-supplemented diet (1% w/w) and given a single, daily oral gavage of either vehicle, 10 mg/kg FMC, or 100 mg/kg IMC. Mice were also weighed daily. After 14 days, the mice were weighed, and plasma was collected and analyzed for signs of toxicity using the indicated assays. Data points represent individual values for each mouse (bars represent the mean for each group for the indicated numbers of mice). Significance determined by two-tailed Student's t-test.

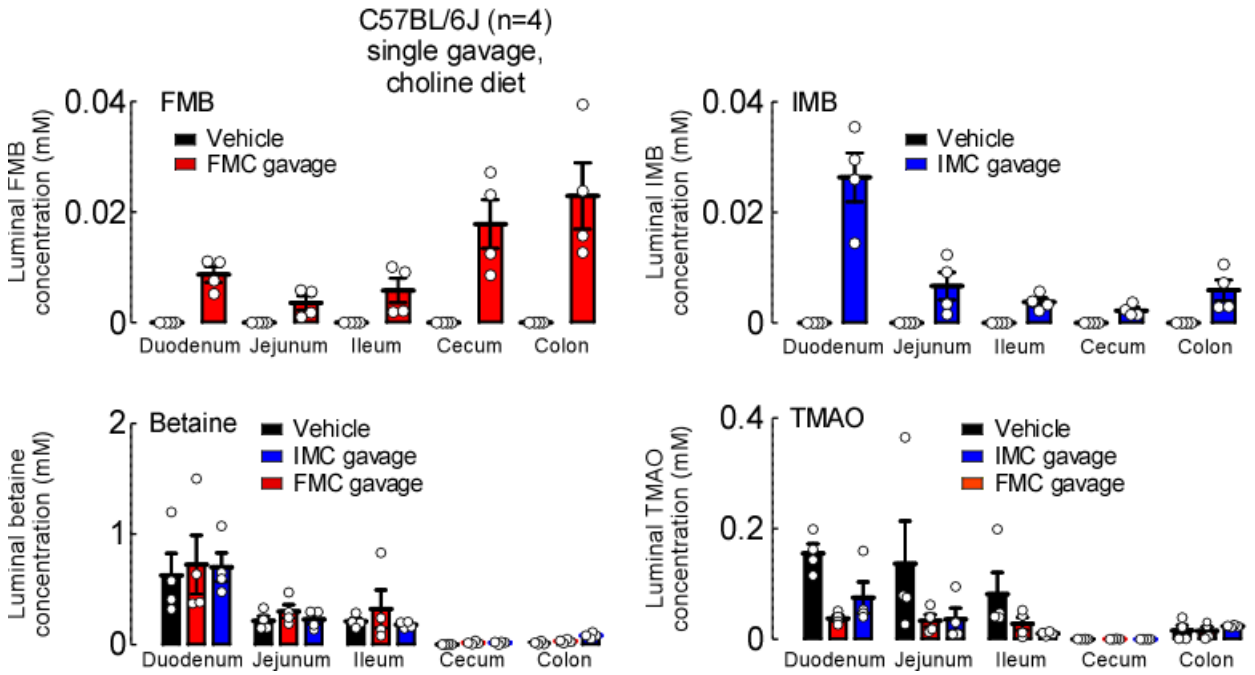
Supplementary Figure 12



Supplementary Figure 12) Metabolism of halomethylcholines and choline related pathway during chronic exposure

C57BL/6J female mice (8-12 weeks old) were maintained on a choline-supplemented diet (1% w/w) and given a single, daily oral gavage of either vehicle, 10 mg/kg FMC (left), or 100 mg/kg IMC (right). After 14 days, plasma was collected and analyzed by LC/MS/MS for choline, betaine, TMA, TMAO, and either FMC or FMB (left) or IMC or IMB (right). Bars represent the mean ± SEM for the indicated numbers of mice. Significance determined by two-tailed Student's t-test.

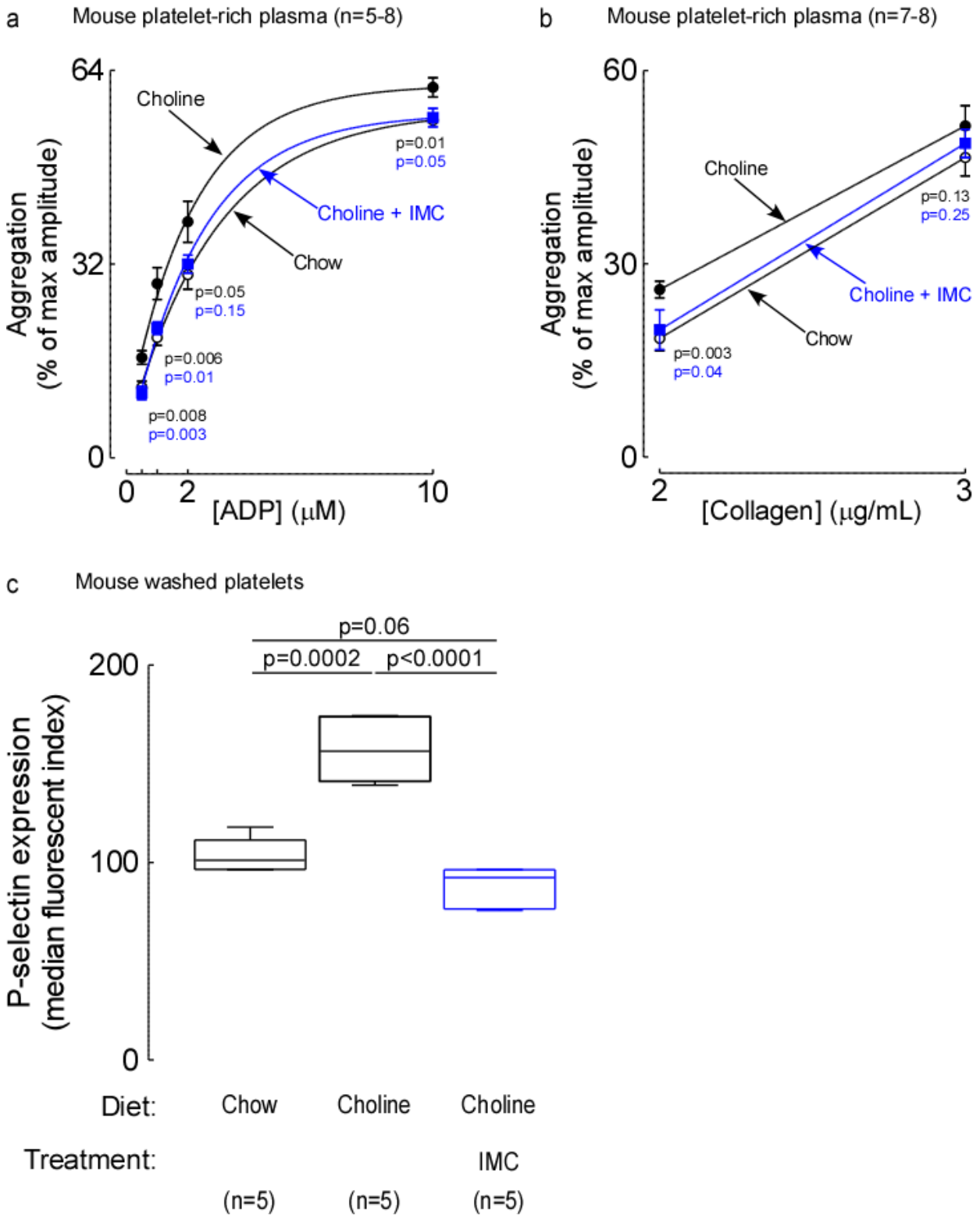
Supplementary Figure 13



Supplementary Figure 13) Intestinal microbial accumulation of halomethylcholines, choline, and metabolites after oral gavage

C57BL/6J female mice (10 weeks old) maintained on a high-choline diet (1% w/w) for 3 weeks were given a single oral gavage of either vehicle, 100 mg/kg FMC, or 100 mg/kg IMC. Intestinal luminal accumulation of halomethylcholines, FMB, IMB, betaine, and TMAO were measured as described in Methods. Groups of mice (n=4 per group) were sacrificed at 4 hours post-gavage. The intestines were extracted and divided into 5 sections: duodenum, jejunum, ileum, cecum and colon. The luminal contents were recovered, homogenized in buffer, and the clarified supernatant was analyzed by LC/MS/MS. Bars represent the mean ± SEM.

Supplementary Figure 14



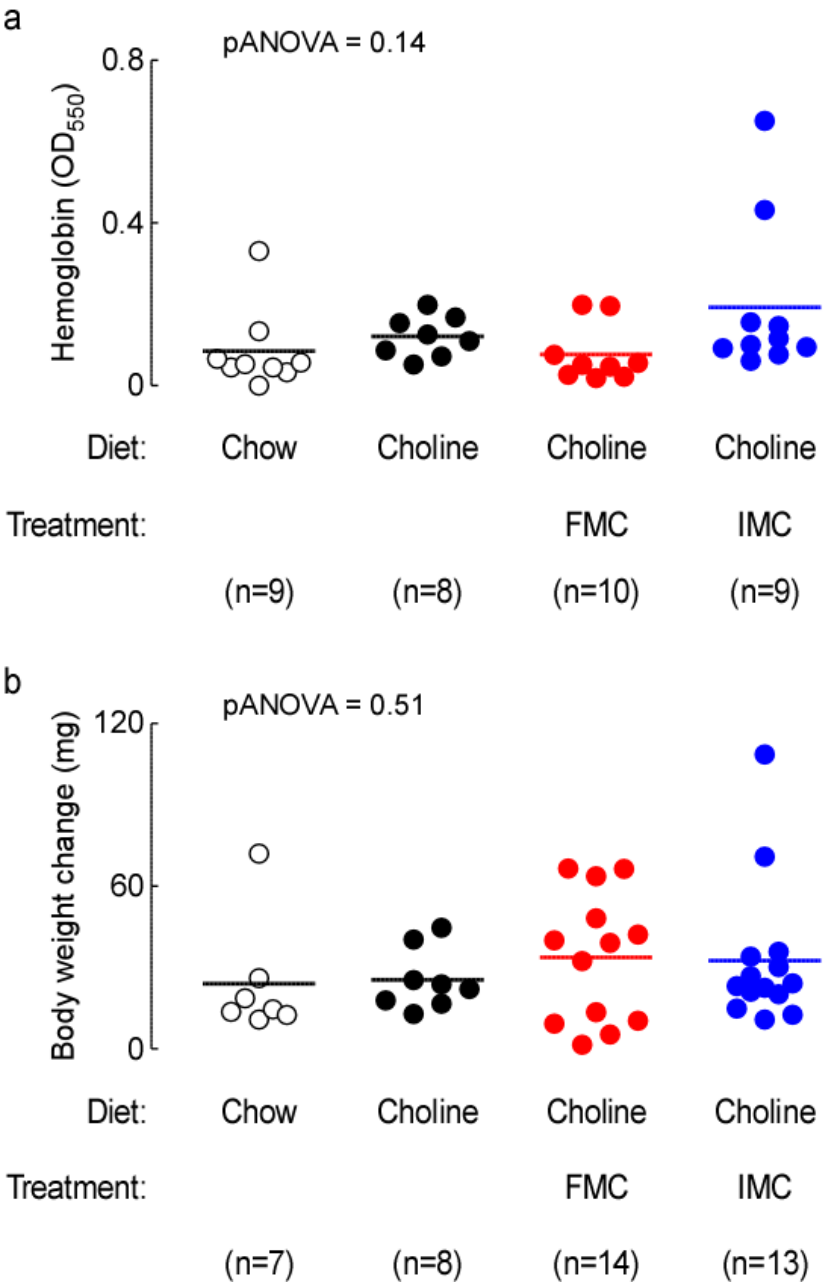
Supplementary Figure 14) Impact of IMC on choline diet-enhanced platelet activation with various levels of stimulus and in washed platelets

The impact of IMC treatment on the choline diet-induced phenotype shown in Supplementary Figure 1 is illustrated here.

a-b) C57BL/6J female mice (10-13 weeks old) were fed either a chemically-defined chow diet, choline-supplemented (1%) diet, or choline with the addition of IMC (0.06%) for ≥1 week. Platelet aggregation was monitored in platelet-rich plasma in response to the indicated levels of **(a)** ADP (0.5 – 10 µM) or **(b)** collagen (2 or 3 µg/mL). Data are represented as % of maximum amplitude (mean +/- SEM) for each group with at least 5 different mice in each group. P-values were determined by two-tailed Student's t-test between "Choline" and "Chow" (p-values in black) and "Choline" and "Choline + IMC" (p-values in blue). Exact numbers of mice used for each data point can be found in the Source Data (n=5-8).

c) C57BL/6J female mice (10-13 weeks old) were fed either a chemically-defined chow diet, choline-supplemented (1%) diet, or choline with the addition of IMC (0.06%) for ≥1 week. Platelets were isolated, washed, and then stimulated with ADP (2 µM) and p-selectin cell-surface expression was determined using FITC-conjugated CD62p antibody and quantified using FlowJo software. Data are presented as a box-whisker plot where the box represents the first and third quartiles, the center line is the median, and the whiskers represent the 5 and 95 percentiles (n=5 mice per group). P-values shown are for comparisons between the indicated groups using a two-tailed Student's t-test.

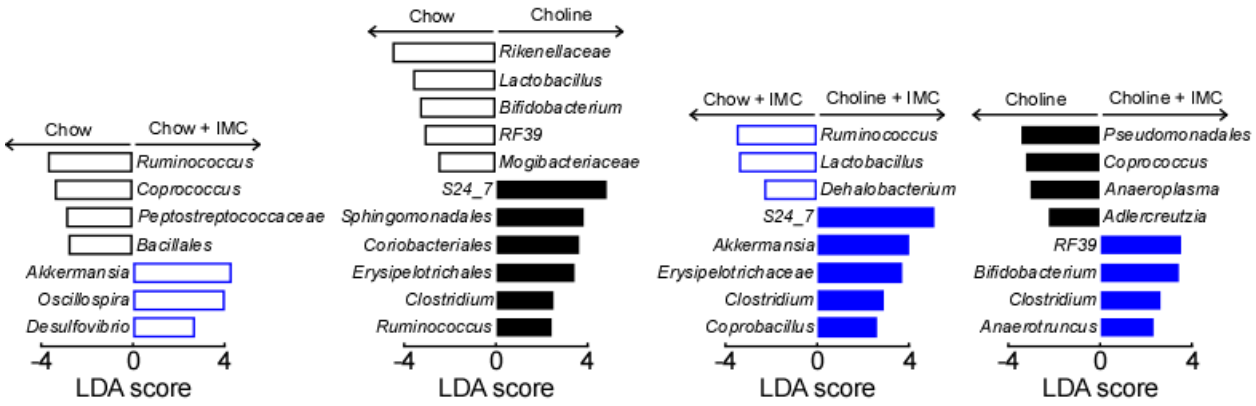
Supplementary Figure 15



Supplementary Figure 15) Impact of FMC and IMC on bleeding parameters following tail tip amputation.

a-b) C57BL/6J female mice (8-12 weeks old) were placed on the indicated diets in the presence of FMC (0.006% w/w) or IMC (0.06% w/w) for 1 week. Mice were anesthetized and a consistent excision injury was made 3 mm from the tip of the tail. The tail was immersed in 37 °C saline and the cumulative time of bleeding for a total of 10 minutes, hemoglobin recovered in the warm saline, and change in weight of the mice were recorded. Data shown are for **(a)** hemoglobin loss measured as OD_{550} and **(b)** change in body weight. Data points represent the indicated levels for each mouse (bars represent the mean for each group for the indicated number of mice per group). Significance was determined using two-way ANOVA.

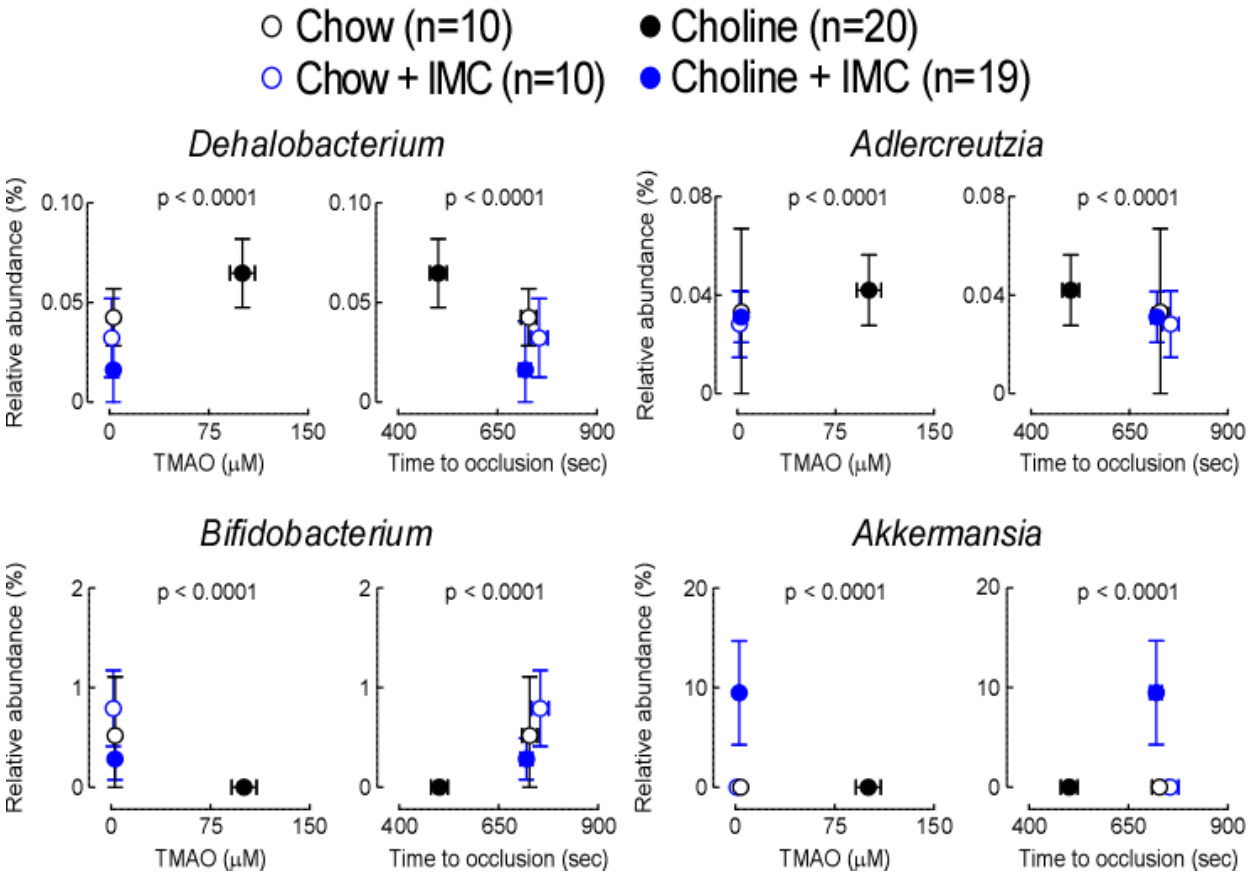
Supplementary Figure 16



Supplementary Figure 16) Alterations in the microbiome profile of mice fed either a low- or high-choline diet with or without provision of IMC

C57BL/6J female mice (12 weeks old) were maintained on the indicated diets ± IMC (0.06% w/w) for 2 weeks. Cecal contents were harvested and intestinal microbial community composition was assessed by Linear Discriminant Analysis (LDA) effect size (LEfSe) between each group. Results shown are from n=59 mice as described in Figure 5a,b, representing n=10 Chow mice, n=10 Chow + IMC mice, n=20 Choline mice, and n=19 Choline + IMC mice.

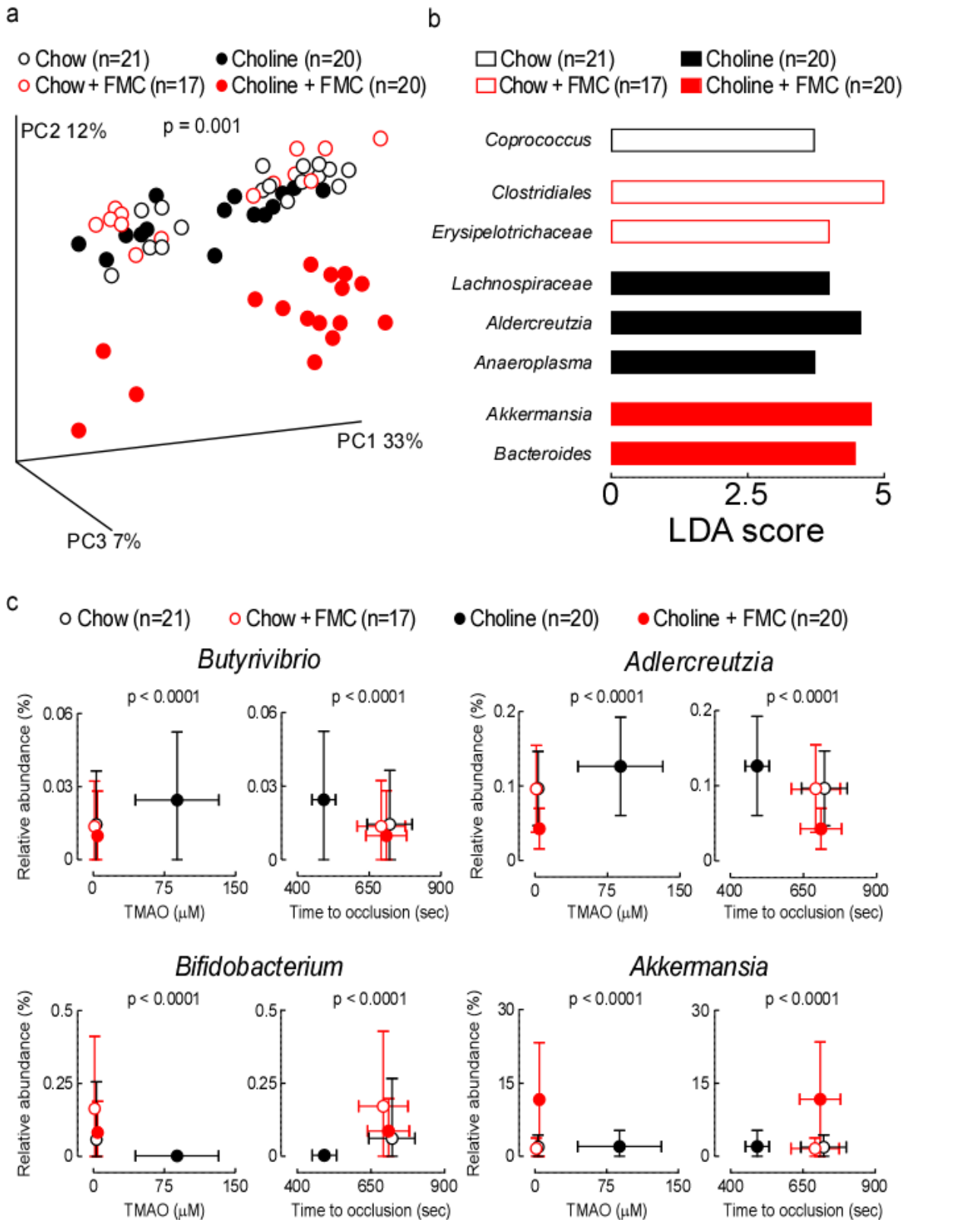
Supplementary Figure 17



Supplementary Figure 17) The choline TMA lyase inhibitor IMC reverses many choline diet-induced changes in cecal microbial community composition associated with plasma TMAO levels and time to occlusion

C57BL/6J female mice (12 weeks old) were maintained on the indicated diets ± IMC (0.06% w/w) for 2 weeks. Cecal contents were harvested from the indicated numbers of mice per group and cecal microbial taxa identified whose relative abundance are significantly associated with both plasma TMAO levels and occlusion time. Values in both X and Y directions are plotted as mean ± SEM. Significance was determined using Hotelling-Lawley t-test. Results shown are derived from sequence data from n=59 total mice as described in Figures 5a,b, representing n=10 Chow mice, n=10 Chow + IMC mice, n=20 Choline mice, and n=19 Choline + IMC mice.

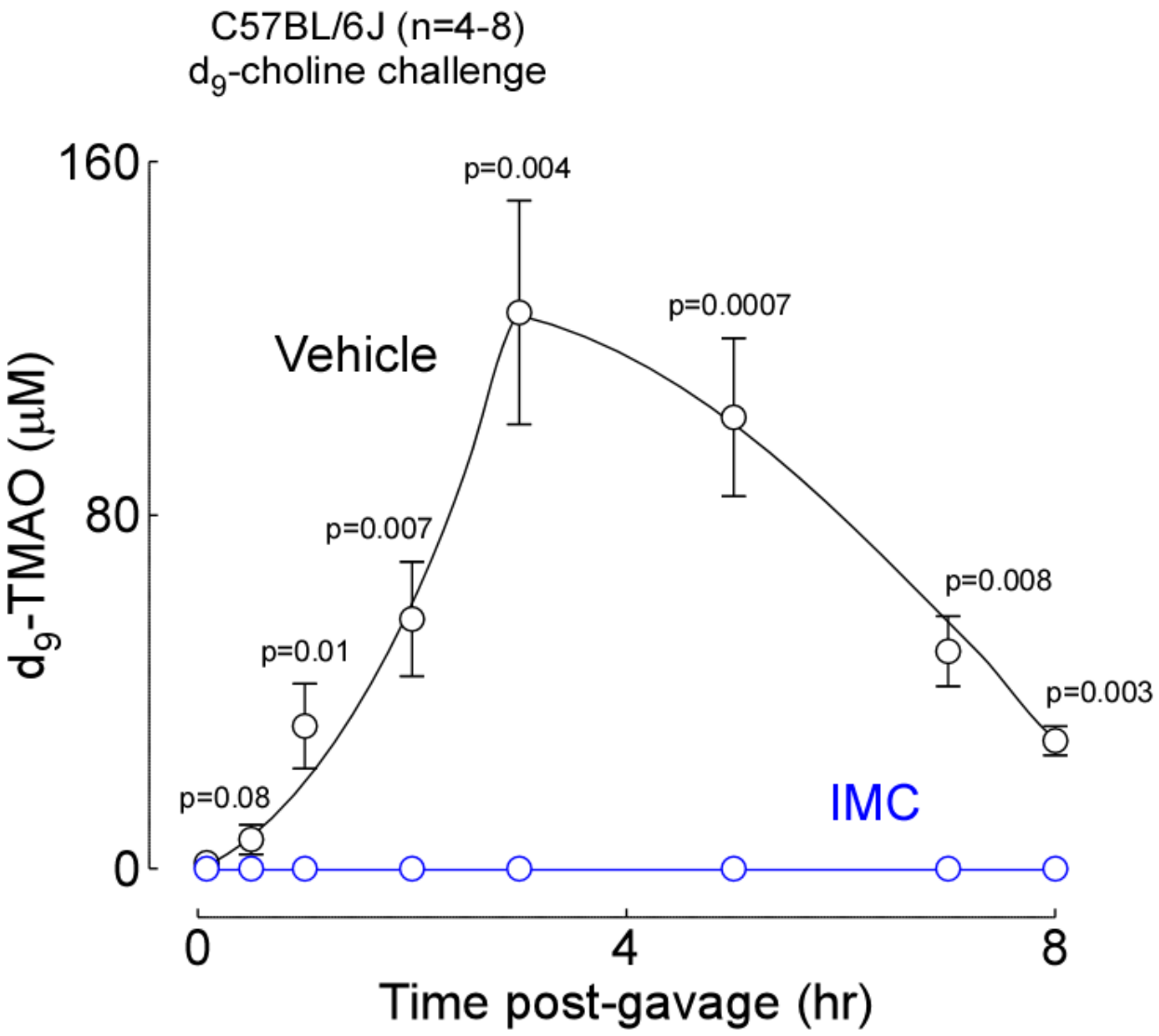
Supplementary Figure 18



Supplementary Figure 18) The choline TMA lyase inhibitor FMC reverses many choline diet-induced changes in cecal microbial community composition associated with plasma TMAO levels and thrombosis risk

C57BL/6J female mice (≥6 weeks old) were maintained on the indicated diets ± FMC (0.006% w/w) for 2 weeks. Cecal contents were harvested from the indicated numbers of mice per group and intestinal microbial community composition was assessed by **(a)** Principal Coordinates Analysis ($p=0.001$, $R^2=0.24$ by Adonis statistical test) or **(b)** Linear Discriminant Analysis (LDA) effect size (LEfSe). **(c)** Cecal microbial taxa identified whose relative abundance were significantly associated with both plasma TMAO levels and occlusion time. Values in both X and Y directions are plotted as mean ± SEM. Significance was determined using Hotelling-Lawley t-test.

Supplementary Figure 19



Supplementary Figure 19) Time-course for IMC effect on d_9-TMAO production from d_9-choline gavage

C57BL/6J female mice (>8 weeks old) maintained on a chemically-defined chow (0.08% w/w choline) diet were given a single dose via oral gavage of d_9-choline (10 mg) with either vehicle (water) or IMC (100 mg/kg). Blood was collected at the indicated time points and plasma was analyzed by LC/MS/MS for production of d_9-TMAO. Data points represent the mean ± SEM from at least 4 animals tested per treatment with cumulative results from all mice shown. Exact numbers of mice used for each data point can be found in the Source Data (n=4-8). P-values shown are for comparisons between the two groups at each time point using a two-tailed Student's t-test.

		Treatment and Result	
Toxicity Assay		**FMC**	**IMC**
Mitochondrial Toxicity	Viability of HepG2 cells in glucose-supplemented media	IC_{50} > 150 µM	IC_{50} > 150 µM
	Viability of HepG2 cells in galactose-supplemented media	IC_{50} > 150 µM	IC_{50} > 150 µM
Cytotoxicity	Viability of HK2 cells	IC_{50} > 150 µM	IC_{50} > 150 µM
hERG Inhibition	Inhibitory effect on hERG channel function in HEK293 cells	IC_{50} > 30 µM	IC_{50} > 30 µM
Mini-Ames Assay	Mutagenic effect in *Salmonella typhimurium*	Normal	Normal

Supplementary Table 1) Summary of pharmacological toxicity testing for FMC and IMC

Mitochondrial toxicity assays tested the viability of HepG2 human liver hepatocellular carcinoma cells exposed to FMC or IMC in either glucose-supplemented media or galactose-supplemented media using the CellTiter-Glo luminescent assay, as described in Methods. A mitochondrial toxicant is indicated by a greater than three-fold change in IC_{50} value observed in the galactose media compared to the glucose media. Thus, no mitochondrial toxicity was observed for FMC and IMC. Cytotoxicity assays tested the viability of HK2 cells in the presence of FMC or IMC using CellTiter-Glo luminescent assay, as described in Methods. The maximal concentration of drug used in the assay was 150 µM, and no cytotoxicity was observed for FMC and IMC. The cardiotoxicity of FMC and IMC was examined through their potential inhibitory effect on human Ether-à-go-go related gene (hERG) channel using the whole-cell patch-clamp technique. The FDA criterion for defining a drug as hERG-positive is when IC_{50} < 1 µM. The potency of FMC and IMC on inhibiting hERG channel is low (IC_{50} > 30 µM). The genotoxicity of FMC and IMC were tested using the bacteria reverse mutation assay (Ames Assay), as described in Methods. *S. typhimurium* strains TA98 and TA100 were used in this test. In addition to a mutation in the histidine or tryptophan operons, the tester strains contain rfa, uvrB mutations and pKM101 plasmid that enhance their sensitivity to some mutagenic compounds. The tester strains can be reverted from histidine dependence to histidine independence by mutagens. Compounds are judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than 2.0-fold of the mean solvent control value. No evidence of mutagenesis with either FMC or IMC were observed. All p-values were measured by two-tailed Student's t-tests.

Supplementary Methods

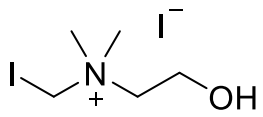
General Methods:

All solvents used in syntheses were distilled under a nitrogen atmosphere prior to use, and all materials were obtained from Sigma-Aldrich unless specified. Proton and carbon magnetic resonance (^{1}H or ^{13}C NMR) spectra were recorded on a Varian Inova AS400 spectrometer operating at 600 MHz. Chemical shifts are reported as parts per million (ppm). ^{1}H NMR spectral data were tabulated in terms of multiplicity of proton absorption (s, singlet; d, doublet; t, triplet; m, multiplet; br, broad), coupling constants (Hz), and number of protons. High resolution mass spectrometry was performed on recrystallized materials diluted to 10 µg/mL in 50% (v/v) water/methanol plus 0.1% (v/v) formic acid and infused at 10 µL/min into the ESI source of an AB Sciex 5600 triple TOF calibrated with CsI and ALILTLVS peptide in the positive ion and high resolution product ion modes. All other mass spectrometry studies were performed on Shimadzu 8050 triple quad mass spectrometers interfaced to Shimadzu UHPLC and autosampler devices.

Synthesis of halomethylcholine compounds

General procedure: dimethylethanolamine (1 equivalent) and the appropriate dihalomethane (1.2 equivalent) were added into acetonitrile (50 mL, Fisher Scientific). The reaction mixture was stirred at room temperature for 48 hours. The solvent was removed by rotary evaporation. The crude product was recrystallized in anhydrous ethanol.

Iodomethylcholine iodide (IMC)



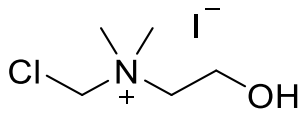
Reactants dimethylethanolamine (5.0 mL, 50 mmol), diiodomethane (4.8 mL, 60 mmol, Alfa Aesar), yield 47.8%.

^{1}H-NMR (600 MHz, D_2O): δ 5.13 (s, 2H), 3.90 (t, J = 4.8 Hz, 2H), 3.52 (t, J = 4.8 Hz, 2H), 3.16 (s, 6H);

^{13}C-NMR (150 MHz, D_2O): δ 65.7, 55.4, 52.4, 32.3.

HRMS: m/z calculated for $C_5H_{13}INO$ (M^+) 230.0036, found 230.0033.

Chloromethylcholine iodide (CMC)



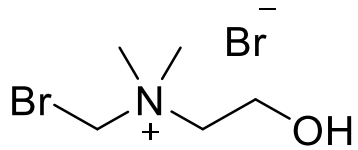
Reactants dimethylethanolamine (5.0 mL, 50 mmol), chloroiodomethane (4.1 mL, 56 mmol, Alfa Aesar), yield 82.2%.

^{1}H-NMR (600 MHz, D_2O): δ 5.10 (s, 2H), 3.91 (m, 2H), 3.52 (m, 2H), 3.13 (s, 6H)

^{13}C-NMR (150 MHz, D_2O): δ 69.5, 63.7, 55.1, 50.0.

HRMS: m/z calculated for $C_5H_{13}ClNO$ (M^+) 138.0680, found 138.0678.

Bromomethylcholine bromide (BMC)



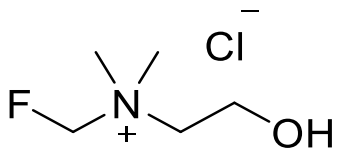
Reactants dimethylethanolamine (5.0 mL, 50 mmol), dibromomethane (4.2 mL, 60 mmol), yield 76.8%.

^{1}H-NMR (600 MHz, D_2O): δ 5.11 (s, 2H), 3.92 (t, J = 4.8 Hz, 2H), 3.54 (m, 2H), 3.17 (s, 6H);

^{13}C-NMR (150 MHz, D_2O): δ 64.6, 58.1, 55.2, 51.0.

HRMS: m/z calculated for $C_5H_{13}BrNO$ (M^+) 182.0175, found 182.0171.

Fluoromethylcholine chloride (FMC)



Fluoromethyl-4-methylbenzene-1-sulfonate (100 mg, 0.49 mmol, Ark Scientific) and dimethylethanolamine (49 µL, 0.49 mmol) were dissolved into acetonitrile (20 mL). The reaction mixture was stirred at room temperature for 48 hours. The reaction mixture was loaded on a pre-packed column of ion exchange resin (Amberlite IRA-400Cl, 50 mL, Alfa Aesar). The resin was eluted with methanol (100 mL), and the flow through was collected. Solvent was removed by rotary evaporation to provide the final product (60 mg, yield = 78%).

^{1}H-NMR (600 MHz, D_2O): δ 5.37 (s, 1H), 5.30 (s, 1H), 3.97 (m, 2H), 3.52 (m, 2H), 3.14 (s, 6H);

^{13}C-NMR (150 MHz, D_2O): δ 97.2, 95.7, 62.7, 54.9, 47.8.

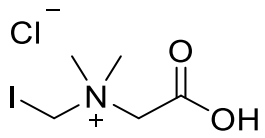
HRMS: m/z calculated for $C_5H_{13}FNO$ (M^+) 122.0976, found 122.0976.

General synthesis of halomethylbetaine compounds

Halomethylcholine (1 equivalent), periodic acid (3 equivalents) and pyridine chlorochromate (PCC, 5%) were dissolved into water (50 mL). The reaction mixture was stirred at room temperature (22 ºC) for 48 hours (or the depletion of halomethylcholine). The reaction mixture was washed with chloroform (100 mL x 3 times). The aqueous phase was collected, and the pH of the aqueous phase was adjusted to 8.0 (basic) by adding sodium hydroxide (1 M solution). Solvent was removed by rotary evaporation. The crude product was dissolved into hot anhydrous ethanol (100 mL, 70 °C), and the insoluble material (salts) was filtered out. The ethanol solution was cooled to room temperature. Concentrated hydrochloric acid was added until the apparent pH of the ethanol solution was 5.0 (acidic). Solvent was removed by rotary evaporation.

The crude product was recrystallized in methanol/acetone (1/5) solution. The crystallized product was isolated by filtration and vacuum dried overnight at 35 °C.

Iodomethylbetaine hydrochloric acid salt (IMB)



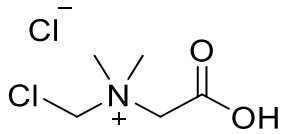
Reactant iodomethylcholine iodide (3.0 g, 8.4 mmol), yield 72%.

^{1}H-NMR (600 MHz, D_2O): δ 5.44 (s, 2H), 4.00 (s, 2H), 3.33 (s, 6H);

^{13}C-NMR (150 MHz, D_2O): δ 168.4, 64.6, 52.3, 31.2.

HRMS: m/z calculated for $C_5H_{11}INO_2$ (M^+) 243.9829, found 243.9818.

Chloromethylbetaine hydrochloric acid salt (CMB)



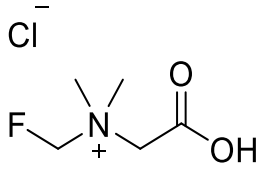
Reactant chloromethylcholine iodide (2 g, 7.5 mmol), yield 64%.

^{1}H-NMR (600 MHz, D_2O): δ 5.31 (s, 2H), 4.17 (s, 2H), 3.26 (s, 6H);

^{13}C-NMR (150 MHz, D_2O): δ 166.9, 68.7, 60.2, 50.3.

HRMS: m/z calculated for $C_5H_{11}ClNO_2$ (M^+) 152.0473, found 152.0469.

Fluoromethylbetaine hydrochloric acid salt (FMB)



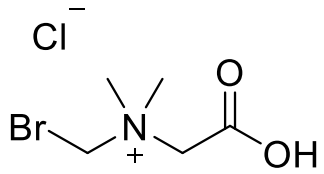
Reactant fluoromethylcholine chloride (1 g, 6.3 mmol), yield 90%.

^{1}H-NMR (600 MHz, D_2O): δ 5.49 (s, 1H), 5.42 (s, 1H), 4.14 (s, 2H), 3.22 (s, 6H);

^{13}C-NMR (150 MHz, D_2O): δ 166.8, 96.8, 95.3, 59.2, 48.0.

HRMS: m/z calculated for $C_5H_{11}FNO_2$ (M^+) 136.0768, found 136.0766.

Bromomethylbetaine hydrochloric acid salt (BMB)



Reactant bromomethylcholine bromide (3 g, 11.4 mmol), yield 58%.

^{1}H-NMR (600 MHz, D_2O): δ 5.40 (s, 2H), 4.33 (s, 2H), 3.38 (s, 6H);

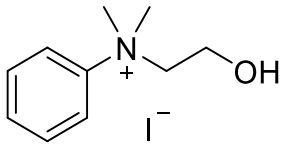
^{13}C-NMR (150 MHz, D_2O): δ 166.8, 61.2, 57.7, 51.7.

HRMS: m/z calculated for $C_5H_{11}BrNO_2$ (M^+) 195.9968, found 195.9963.

General procedure to synthesize other choline analogues:

Reagents were added in solvent and the reaction mixture was stirred at room temperature for 48 hours. The solvent was removed via rotary evaporation. Crude product was recrystallized in different solvents.

Phenylcholine iodide (PhC)



Used acetonitrile (100 mL), 2-(N-methylaniline)ethanol (5 mL, 35 mmol), and iodomethane (2.5 mL, 40 mmol). The reaction is quantitative.

^{1}H-NMR (600 MHz, D_2O): δ 7.83 (m, 2H), 7.67 (m, 3H), 4.08 (m, 2H) 3.75 (m, 2H) 3.73 (s, 6H);

^{13}C-NMR (150 MHz, D_2O): δ 144.0, 130.6, 130.5, 120.6, 70.3, 56.0, 55.4.

HRMS: m/z calculated for $C_{10}H_{16}NO$ (M^+) 166.1226, found 166.1220.

Isotope-labeled compounds:

Isotope-labeled substrates and standards (d_9-choline, d_9-betaine, d_9-TMA, d_9-TMAO) were purchased from Cambridge Isotope Lab. Isotope-labeled iodomethylcholine (d_2-IMC) was synthesized by substituting d_2-diiodomethane for diiodomethane in the synthesis procedure for iodomethylcholine iodide (see above).

Diet preparation:

IMC and FMC were incorporated into irradiated powdered diets (Envigo - 1% choline added (TD.09041) and chemically-defined chow (0.08% total choline) (TD.130401)) at 0.06% or 0.006% w/w of the salt, which corresponds to 100 mg/kg and 10 mg/kg respectively, assuming an average female mouse eats ~5 g/day and weighs ~20g. Animals were fed *ad libitum* using food cups fitted with feeder shields. Food was replaced every other day. DMB was added to the drinking water at 1.3% v/v. For the TMAO add-back experiments, TMAO (200 µL, 290 mM, pH

7.4) was injected intraperitoneally 2 hours prior. We had previously determined this amount would raise plasma TMAO levels to those seen in a 1% choline diet.

CutC/D plasmid construction:

The *cutC* and *cutD* genes from *Proteus mirabilis* ATCC 29906 (*cutC* ID: 6801039) and *Desulfovibrio alaskensis* G20 (*cutC* ID: 3926085) and their associated intergenic region were codon optimized for expression in *E. coli* and synthesized by Genscript (Piscataway, NJ) for cloning into pUC57 (Genscript) as described previously[1].

Protein expression and lysate preparation:

Full-length, recombinant CutC/D was expressed through the use of competent *E. coli* cells (either BL21(DE3)pLysS or TOP10, Invitrogen, Thermo Fisher Scientific, Carlsbad, CA) transformed with the expression plasmids encoding for the *cutC* and *cutD* genes from either *P. mirabilis* or *D. alaskensis*. CutC/D-containing cells were grown in nutrient-rich LB medium with the addition of ampicillin (100 µg/mL) overnight. The next day, cells were diluted 1:100 in LB medium and shaken vigorously at 37 °C with the addition of ampicillin until an OD_{600}=0.5 was reached. At this point, either 0.3 mM IPTG (for BL21(DE3)pLysS cells) or 0.2% arabinose (for TOP10 cells) was added to the cell culture in order to induce expression of CutC/D. Cells were then shaken for ~16 hours at room temperature. Cells were collected by centrifugation at 5,000 rpm for 1 hour. The cell pellets were kept on ice and re-suspended in 50 mL lysis buffer: 200 mM HEPES, 50 mM NaCl, 10 mM $MgCl_2$, pH 8.0. A small amount of lysozyme was added, and the cells were agitated for ~30 minutes at 4 °C. The cell mixture was lysed through use of a Microfluidizer (M-110Y, Microfluidics, Newton, MA). The lysate was clarified by centrifugation at 10,000 rpm for 30 minutes. Clarified lysate was aliquoted and flash frozen for storage at -80 °C.

Wild-type *P. mirabilis* lysate assay:

Starter cultures of *Proteus mirabilis* ATCC 29906 were grown in 0.8% Difco Nutrient broth (Becton Dickinson) at 37 °C for 22 hours with shaking. Starter cultures were diluted 16-fold into fresh media containing 20 mM choline chloride (Sigma-Aldrich) and incubated at 37 °C for 24 hours with shaking. Cell pellets were collected by centrifugation (6,000 x g at 4 °C for 12 minutes), concentrated 15-fold relative to initial culture volume in cold Dulbecco's phosphate buffered saline (D-PBS) (Corning), and treated with lysozyme (Sigma-Aldrich) for 30 minutes at 4 °C. Cells were disrupted with a French pressure cell press (internal psi equivalent ~16,000), followed by centrifugation to collect the soluble protein fraction of the cell lysate. Protein concentration of the clarified cell lysates was determined by a BCA protein assay kit (Thermo Fisher Scientific Co.) Lysate concentrations were adjusted to 3 mg/mL with DPBS and frozen at -80^{0}C. *P. mirabilis* 29906 lysate was thawed and diluted to 1.5 mg/mL total protein with D-PBS and incubated at 37°C for 22 hours with 2.5 mM choline chloride. Following incubation, deuterium labeled choline chloride (choline chloride (d_9-trimethyl), Cambridge Isotope Laboratories, Inc.) was added to aliquots of lysate (150 µL) to a final concentration of 50 µM. Halomethylcholine and halomethylbetaine compounds, dissolved in DMSO, were subsequently added in appropriate dose ranges via a HP D300e digital dispenser (HP Development Company). Sealed reaction mixtures were incubated at 37°C for 2 hours and stopped by addition of formic acid to 1% (v/v). All steps described above were performed aerobically.

Formic acid treated samples were spiked with stable isotope labeled internal standard (22.5 µL of 6 µg/mL of $^{13}C_3$-trimethylamine ($^{13}C_3$-TMA) was added to each sample), then d_9-trimethylamine (d_9-TMA), trimethylamine (TMA), and $^{13}C_3$-TMA were isolated from the lysate after protein precipitation as described below. Acetonitrile acidified with 0.1% formic acid, 600 µL, was added to each sample, which was then centrifuged (2,100 x g for 20 minutes) to pellet the protein and other precipitates. The TMA, d_9-TMA, and $^{13}C_3$-TMA in the isolated supernatant samples were subjected to gradient High Performance Liquid Chromatography (HPLC) analysis on a Waters Atlantis HILIC Silica column (Waters Corporation) (2.1 x 50 mm, 3 µm particles) with an Atlantis

Silica HILIC Sentry guard column (Waters Corporation) (100 Å, 3 µm, 2.1 mm X 10 mm), 10 mM ammonium formate in water with 0.1% formic acid as mobile phase A, and 0.1% formic acid in acetonitrile as mobile phase B. Detection and quantitation was achieved by tandem mass spectrometry operating under multiple reaction monitoring (MRM) MS/MS conditions (m/z 60.1 $\rightarrow$ 44.1 for TMA; m/z 69.1 $\rightarrow$ 49.1 for d_9-TMA; m/z 63.0 $\rightarrow$ 46.1 for $^{13}C_3$-TMA). TMA and d_9-TMA calibration standards (STD), prepared in 80/20/0.1%, acetonitrile/water/formic acid (v/v/v), were used to construct a calibration curve by plotting the response ratio (peak area TMA/peak area $^{13}C_3$-TMA) versus concentration for each standard. The concentrations of TMA and d_9-TMA in the cell lysate were determined by interpolation from the quadratic ($1/x^2$) regression curve. Calculation of IC_{50} values was performed with Dotmatics Studies software (Woburn, Massachusetts).

Human commensal growth curve and TMA determination:

Known TMA-producing human commensals, *P. mirabilis, E. fergusonii*, and *P. penneri*, were purchased from ATCC. Cells were grown to saturation overnight with vigorous shaking at 37 °C in nutrient-rich LB media. These cells were diluted 1:100 and shaken at 37 °C. For each commensal, one batch included the addition of inhibitor (1 mM) while one did not. At each time point, a 1 mL aliquot was removed and an OD_{600} measurement was taken. When the cells reached OD_{600}=0.5 an aliquot was removed for the activity assay using d_9-choline as substrate, monitoring d_9-TMA generation as outlined above[1]. For a longer duration study, *P. mirabilis* was grown and monitored similarly, except the experiment was continued for >2 weeks. After that period, the log-phase growth curve was monitored following a 1:100 dilution in fresh LB media. Activity was also similarly measured from aliquots taken while OD_{600}=0.6.

Docking and quantum mechanical calculations:

3-D molecular models of choline, IMC, and FMC were prepared with the molecular builder Avogadro[2] and docked to the crystal structure of *D. alaskensis* CutC enzyme (PDB ID: 5FAU)[3] by using the small-molecule docking program Autodock4[4]. Choline docking was performed as a control experiment. Docking of IMC and FMC to the 5FAU active site positions these substrates in the same orientation as choline, with the Asp_{216} residue anchoring the trimethylammonium moiety of the substrate and Glu_{491} making an H-bond with the OH group of the substrate. The binding free energies (in kcal/mol) calculated by Autodock4 were -4.1 (choline), -3.9 (IMC) and -4.5 (FMC). In addition to docking, quantum mechanical (QM) calculations using the *ab initio* program Gamess[5] (HF/6-31G(d) level of theory) were performed on 3-D molecular models of choline, IMC, and FMC surrounded by relevant amino acid residues from the enzyme active site (Tyr_{208}, Asp_{216}, Cys_{489}, Glu_{491}, Thr_{502}, Tyr_{506}), known to play a key role in substrate binding and the following chemical transformation[3]. To speed up calculations, the amino acids were replaced by surrogate chemical structures mimicking the main chemical function (i.e., Asp, Glu $\rightarrow$ acetate, Tyr $\rightarrow$ phenol, Cys $\rightarrow$ methylthiol, Thr $\rightarrow$ isopropanol).

The reaction mechanism emerged from the potential energy surface (PES) mapped from QM calculations for choline transformation into trimethylamine (TMA) and acetaldehyde, revealing a multistep reaction composed of three short lived reaction intermediates and four transition states (TS). The reaction step (TS_3) in which a choline radical intermediate splits into TMA and an acetaldehyde radical intermediate is very fast and exists also for FMC, but not when IMC is the substrate. The PES shows that due to the proximity of a negatively charged residue (Asp_{216}), QM calculations predict that an iodide ion (I^-) is eliminated in concert with the N-C bond breaking of IMC without producing a similar product (I-TMA) like choline (TMA) and FMC (F-TMA). Consequently, when IMC is a substrate, QM calculations predict that the residue Asp_{216}, in the enzyme active site, is chemically modified by covalent bond formation between one oxygen atom of Asp_{216} side chain and the nitrogen atom of TMA.

Mitochondrial toxicity

HepG2 human hepatocellular carcinoma cells in serum free glucose medium (25 mM glucose, 1mM sodium pyruvate, 5 mM HEPES) or serum free galactose medium (10 mM galactose, 1 mM sodium pyruvate, 6 mM glutamine, 5 mM HEPES) were incubated with FMC, IMC, and reference compounds (Nefazodone and Digitonin) at a range of different concentrations for 24 hours. At the end of the incubation period the cells were loaded with pre-mixed Cell Titer-Glo monitoring reagent and scanned using an automated plate reader with luminescence detection to determine the number of active cells. The data was fit to a sigmoidal dose-response curve with a variable slope using GraphPad Prism 5.0. IC_{50} calculations for FMC, IMC and reference compounds were calculated from the related curves.

HK-2 Cytotoxicity

Pre-cultured HK-2 cells (in DMEM/F12 (1:1) medium supplemented with 10% FBS, 5 mM HEPES and 1x penicillin-streptomycin mixture) were incubated with FMC, IMC and tamoxifen (as a positive control) at a range of different concentrations for 24 hours. At the end of the incubation period the cells were loaded with pre-mixed Cell Titer-Glo monitoring reagent and scanned using an automated plate reader with luminescence detection to determine the number of active cells. The data was fit to a sigmoidal dose-response curve with a variable slope using GraphPad Prism 5.0. IC_{50} calculations for FMC, IMC and tamoxifen were calculated from the related curves.

hERG inhibition

HEK 293 cells that stably express hERG were cultured in 85% DMEM, 10% dialyzed FBS, 0.1 mM NEAA, 25 mM HEPES, 100 U/mL penicillin-streptomycin, 5 µg/mL blasticidin and 400 µg/mL geneticin. FMC and IMC were initially prepared in DMSO at different concentrations (10.0, 3.33, 1.11, 0.37 mM), and finally diluted 1000 fold in extracellular solution (132 mM NaCl, 4 mM

KCl, 3 mM $CaCl_2$, 0.5 mM $MgCl_2$, 11.1 mM glucose, and 10 mM HEPES (pH adjusted to 7.35 with NaOH)). Currents were recorded at room temperature using the whole-cell patch-clamp technique. In the presence of test compounds at different concentrations, hERG current was measured for IC_{50} determination. The dose response curve of test compounds was plotted with percent inhibition against the concentration of test compounds using GraphPad Prism 6.0, and fit the data to a sigmoid dose-response curve with a variable slope.

Mutagenicity test using Mini-Ames assay

Salmonella typhimurium strains TA98, TA100 were used in this test. In addition to a mutation in the histidine or tryptophan operons, the tester strains contain *rfa*, *uvrB* mutations and a pKM101 plasmid that enhance their sensitivity to some mutagenic compounds. The tester strains can be reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy) by mutagens. Frozen working stocks (40 µL) were added into 10 mL of broth to prepare overnight cultures. For tester strains TA98, TA100, 31.25 µL of 0.8% ampicillin solution was added into every 20 mL of Oxoid Nutrient Broth No. 2. The mixtures were then incubated with shaking at 160 rpm at 37 °C until an OD_{650} of 0.6-1.6 was achieved. L-histidine/biotin solution (0.5 mM, 10 mL) or tryptophan solution (0.5 mM, 5 mL) was added to 100 mL melted top agar. This solution was aliquoted at 1.6 mL per tube and held at approximately 45 °C. Next, 80 µL of FMC and IMC at various concentrations, control solutions (400 µL of S9 mix (for S9 activated) or phosphate buffered saline (for non-activated)), and 80 µL of the bacterial culture were added to the 1.6 mL of top agar. After mixing the top agar mix, every 540 µL of the resulting mix was added into one well of the 6-well plates (34.8-mm dishes) containing solidified approximately 5 mL of minimal glucose agar media (1.5% agar, 2% glucose, in Vogel-Bonner medium E). Each treatment was plated in triplicate except solvent/negative control, which was plated in sextuple. As soon as the soft agar solidified, the 6-well plates were incubated at 37 °C for 48 to 72 hours. Plates were counted immediately following the incubation period. Compounds are judged positive

if the increase in mean revertants at the peak of the dose response is equal to or greater than 2.0-fold of the mean solvent control value.

Mouse plasma von Willebrand factor assay

Mouse plasma vWF levels were assessed by using Abcam's (cat# ab208980) vWF A2 (von Willebrand factor A2) in vitro SimpleStep ELISA (Enzyme-Linked Immunosorbent Assay) kit using the manufacturer's instructions.

Analysis of prothrombotic markers

C57BL/6J female mice (9-12 weeks of age, obtained from Jackson Laboratory #664) were started on either minimal choline or choline-supplemented (1% w/w) diets for more than 4 days**.** Mice were humanely euthanized (300 mg/kg ketamine and 30 mg/kg xylazine). Blood was collected via cardiac puncture through a 26G needle using a 1 cc syringe that contained 0.1 mL of 0.109 M sodium citrate. Needle was removed and blood was evacuated gently into a collection tube. Platelet-rich plasma (PRP) was obtained by centrifugation at 150 x g for 15 minutes. Diluted platelet poor plasma was prepared by further centrifugation at 11,000 x g for 2 minutes. Platelets were counted using a hemocytometer and concentrations adjusted to 2 x 10^8/mL with platelet poor plasma. To obtain washed platelets, an equal volume of modified Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 5 mM HEPES, 5.6 mM glucose, 0.35% BSA, pH 7.4) supplemented with 0.3 µM prostaglandin E1 (PGE1) was added to PRP and centrifuged at 1,000 x g for 5 minutes. $CaCl_2$ and $MgCl_2$ (both 1 mM final concentration) were added immediately before stimulation with 2 µM ADP as indicated. For immunoaffinity staining p-selectin analysis, samples were incubated with FITC-CD62p (BD Pharmingen, cat# 561923) for 10 minutes, and the reaction was stopped by addition of 2% paraformaldehyde. Fluorescence intensity was quantified on the LSRII at a flow rate of low on 10,000 events. Analysis was performed in FlowJo. For microparticle analysis, platelet poor plasma was centrifuged at 20,000

x g for 30 minutes, and the microparticle pellet was re-suspended in 400 µL double filtered modified Tyrode's buffer. Aliquots of 20 µL per tube were used for staining. All buffers used were double filtered (to reduce background noise) and reagent vials were centrifuged at 15,000 x g for 10 minutes to pellet potential aggregates before pipetting aliquots for staining. Presence of true microparticles was validated by dissolving vesicles by treating samples with Triton-X 100 detergent (0.1% final concentration). Briefly, for immunoaffinity staining, a 20 µL sample aliquot was re-suspended in annexin V binding buffer (140 mM NaCl, 10 mM HEPES, 2.5 mM $CaCl_2$, double filtered) containing PE-CD41 (BD Pharmingen, cat# 558040) and FITC-annexin V (BD Pharmingen, cat# 556547) antibodies and stained for 30 minutes. Fluorescence intensity was quantified on the Apogee A50 using a flow rate of 6 µL/min on 120 µL of sample. A compensation matrix was generated in FlowJo and then applied to the data in the Apogee software.

Human platelet aggregation studies:

Whole blood was collected from consenting healthy donors using 0.109 M sodium citrate as anticoagulant. Platelet-rich plasma (PRP) was separated by centrifuging at 100 x g for 10 minutes at 22 °C. Platelet poor plasma (PPP) was prepared by further centrifugation at 11,000 x g for 2 minutes. Platelets were counted using a hemocytometer and for aggregometry assays, concentrations were adjusted to 2 x 10^8/mL with PPP. PRPs were then incubated with either 100 µM TMAO or 20 µM inhibitors for 25 minutes at room temperature before stimulation with 2 µM ADP. In some experiments, PRPs were directly stimulated with either 100 µM TMAO or 20 µM inhibitors in the aggregometer.

Whole-blood, microfluidic platelet studies:

C57BL/6J female mice (10-12 weeks of age, obtained from Jackson Laboratory #664) were started on diets of either minimal choline, choline-supplemented (1% w/w), or choline and IMC (0.06% w/w) for 2 weeks**.** Mice were humanely euthanized (300 mg/kg ketamine and 30

mg/kg xylazine). Blood was collected via cardiac puncture through a 26G needle using a 1 cc syringe that contained 0.1 mL of 0.109 M sodium citrate. Needle was removed and blood was evacuated gently into a collection tube. Samples were then gently mixed then incubated for 30 minutes at 22 °C with FITC-conjugated rat-anti-mouse CD41 (clone MWreg30, BD Biosciences) to label platelets before being perfused over chips coated with or without immobilized type 1 collagen (200 μg/mL). Microfluidics experiments were performed using the Cellix Microfluidics System (Cellix Ltd., Dublin, Ireland). Images of fluorescently labeled platelets adhering to the collagen coating were captured every 5 seconds on a Leica DMI6000 inverted microscope equipped with an environmental chamber and cooled CCD camera, and computer assisted image quantification used for analyses. Each micro channel of a Vena8 Fluoro+ biochip was either coated with 15 μL of type 1 collagen (200 μg/mL) or PBS and the biochip was then placed in a humidified box overnight at 4 °C. Each channel of the Vena8Fluoro+ biochip was washed with 1X PBS using the Mirus Nanopump before placing the biochip on the microscope. Images were collected using an HC Plan Apo 20X/0.7NA lens on a Leica DMI6000 inverted microscope equipped with an environmental chamber and a Hamamatsu ImagEM cooled CCD camera. Briefly, fluorescently labeled whole blood was perfused over chips coated with or without immobilized type 1 collagen (200 μg/mL) using a multi-channel microfluidic device for 3 minutes at a flow rate of 60 dynes/cm^2, which is representative of the physiological rate in mice[6]. Images of fluorescent platelets adhering to the collagen coating were captured every 5 seconds during that time. At the end of the experiment, the tube containing the platelets was removed and the 1X PBS in the biochip reservoir was drawn through the channel at 20 dynes/cm^2. Five images were captured along the length of the channel during that time. Platelet activation and adherence to the collagen surface was then quantified with computer assisted tomographic analyses as previously described[7].

Cecal microbiota composition analyses:

16S rRNA gene sequencing methods were adapted from the methods developed for the NIH-Human Microbiome Project[8]. Briefly, genomic DNA was extracted from mouse ceca using a MoBio Power Soil DNA extraction kit (Omega, Norcross, GA). The 16S rRNA V4 region was amplified and sequenced. 16S microbial data were analyzed using QIIME version 1.9. Barcodes were matched to fastq files and then removed[9]. Similar sequences (97%) were combined into operational taxonomic units (OTU) using SUMACLUST. Representative sequences for each OTU were aligned using PyNAST[10]. The lanemaskPH was used to screen out the hypervariable regions and OTUs were classified with the greengenes database[11]. 13,631,206 total reads were generated post-quality filtering, with an average of 174,759 reads per sample, for the IMC data set, and 23,802,833 total reads post-quality filtering, with an average of 326,066 reads per sample, for the FMC data set. Samples were rarefied to a depth of 50,000 sequences/sample. Beta diversity was assessed using unweighted UniFrac in QIIME[9]. Adonis statistical test with 1,000 permutations was used to determine the strength and statistical significance of sample groupings by diet and treatment. LEfSe was used with default parameters on OTU tables to determine taxa that best characterize each population[12]. Only features with LDA scores >2.0 were kept. Relative abundance of OTUs were compared to plasma TMAO levels and time to cessation of blood flow ("Time to occlusion") in XY-plots. Statistical significance was determined using the Hotelling-Lawley t-test.

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