Supplemental Figures

Figure S1

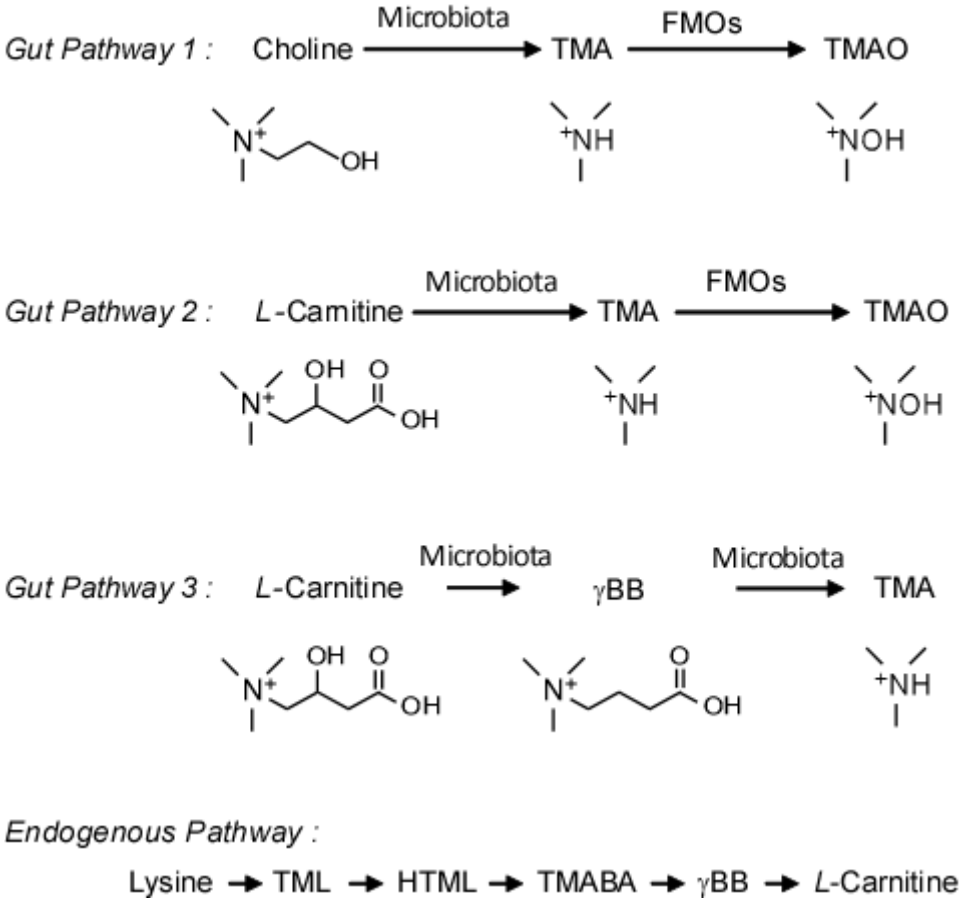

Figure S1, related to Figure 1. Background - Relevant pathways and structures

Gut Pathway 1 – The metaorganismal pathway for conversion of dietary choline into TMA and TMAO by sequential gut microbial and host hepatic FMO enzyme reactions.

Gut Pathway 2 – A metaorganismal pathway for the direct catabolism of carnitine via microbial TMA lyase activity forming TMA, and subsequent host hepatic FMO mediated generation of TMAO.

Gut Pathway 3 – The conversion of L-carnitine into TMA via a hypothesized sequential set of gut microbial reactions involving the initial formation of γ-butyrobetaine, followed by a microbial γ-butyrobetaine TMA lyase activity to produce TMA.

Endogenous Pathway – L-carnitine synthesis in mammals is achieved via multiple sequential reactions starting from the amino acid lysine as outlined.

Abbreviations: TML = N^6-trimethyllysine, 3-hydroxy-HTML = N^6-trimethyllysine HTML, TMABA = 4-N-trimethylaminobutyraldehyde.

Figure S2

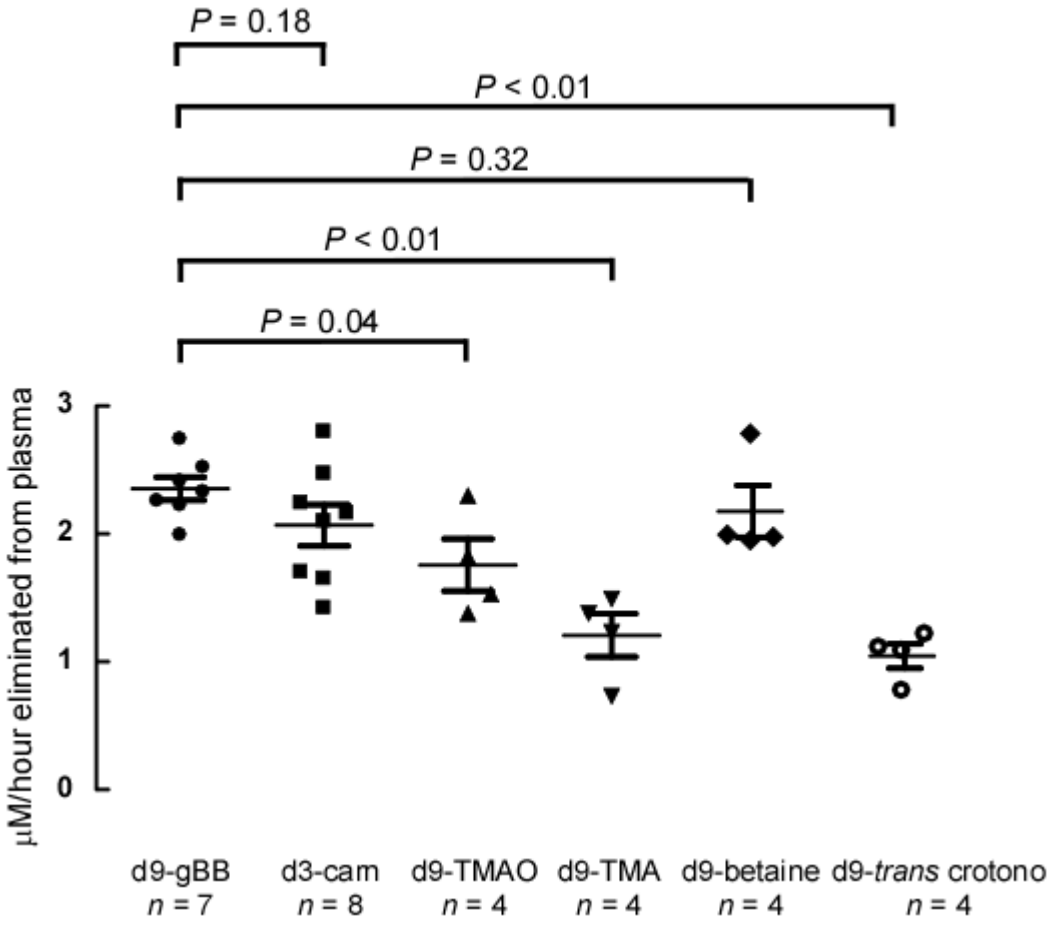

Figure S2, related to Figure 2. Plasma clearance rates of trimethylamines. Female C57BL/6J mice were injected intravenously with d9-γBB, d3-carnitine (d3-carn), d9-TMAO, d9-TMA, d9-betaine and d9-*trans* crotonobetaine (d9-*trans* crotono) and the rate of elimination from plasma of each trimethylamine was determined as described in Experimental Methods. Bars represent median ± SEM and *P* values were determined by Wilcoxon ranks sums test.

Figure S3

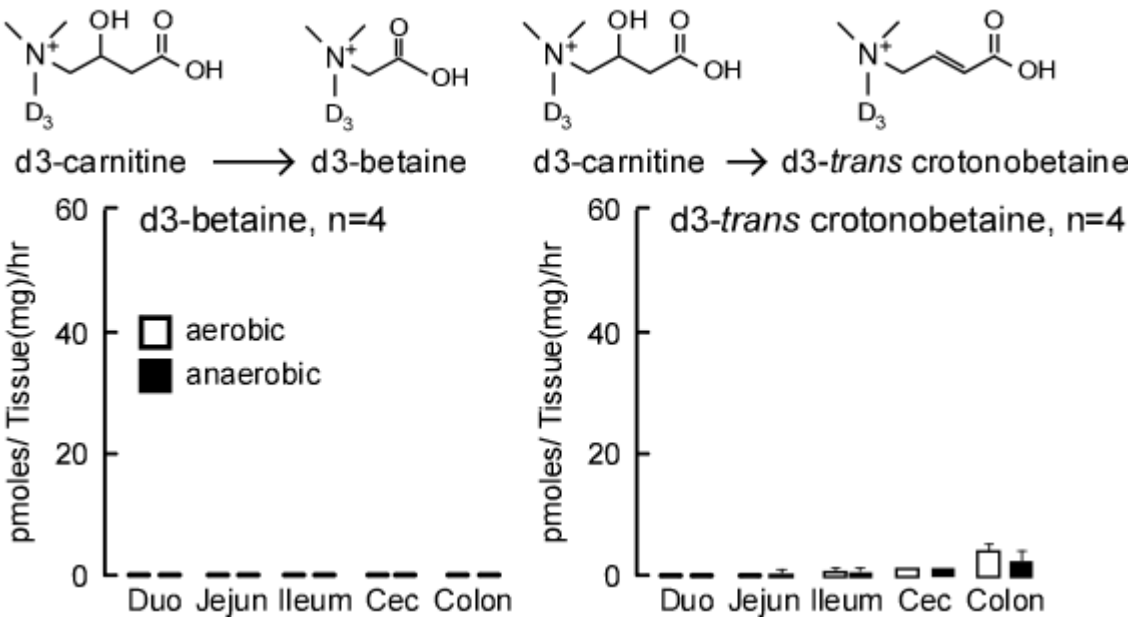

Figure S3, related to Figure 3. Incubation of gut segments with d3-*L*-carnitine reveals no detectable d3-betaine and trace d3-*trans* crotonobetaine generation. Female C57BL/6J mouse intestines (n=4) were sectioned into the indicated segments, filet open, and then incubated at 37 ^{o}C for 18 hours with d3-*L*-carnitine under either aerobic (open bars) or anaerobic (closed bars) conditions. Deuterated trimethylamine analytes were quantified by stable isotope dilution LC/MS/MS as described in Experimental Procedures. Data represent mean ± SEM. The isotopologue of the precursor incubated with the gut segments, and the product monitored by LC/MS/MS, are shown at the top of each graph.

Abbreviations: Duo =Duodenum, Jejun=Jejunum, Cec=Cecum.

Figure S4

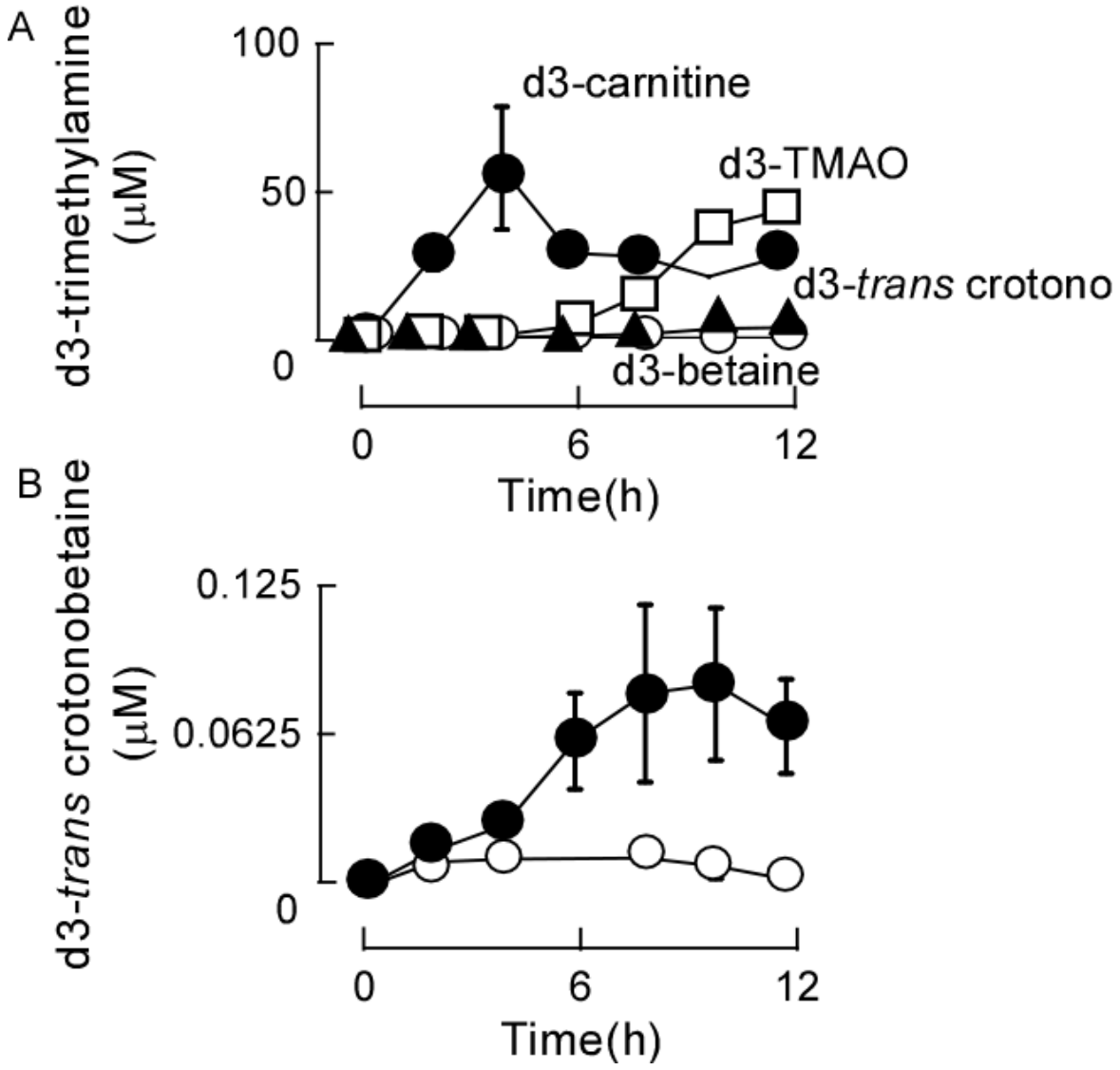

Figure S4, related to Figure 3. Plasma levels of trimethylamines produced following d3-*L*-carnitine ingestion, and demonstration that the trace levels of *trans* crotonobetaine formed following *L*-carnitine ingestion are of microbial origin. (A) C57BL/6J female mice (n=4) were administered d3-carnitine by gastric gavage, and post prandial plasma levels of d3-carnitine, d3-betaine, d3-TMAO and d3-*trans* crotonobetaine, were quantified in serial venous blood draws by stable isotope dilution LC/MS/MS. Data is expressed as means ± SE. (B) Female germ-free Swiss Webster mice (n=5) were administered d3-carnitine via gastric gavage before and after conventionalization. Post challenge levels of d3- *trans* crotonobetaine were examined in serial venous blood draws by stable isotope dilution LC/MS/MS. Data is expressed as means ± SE).

Figure S5

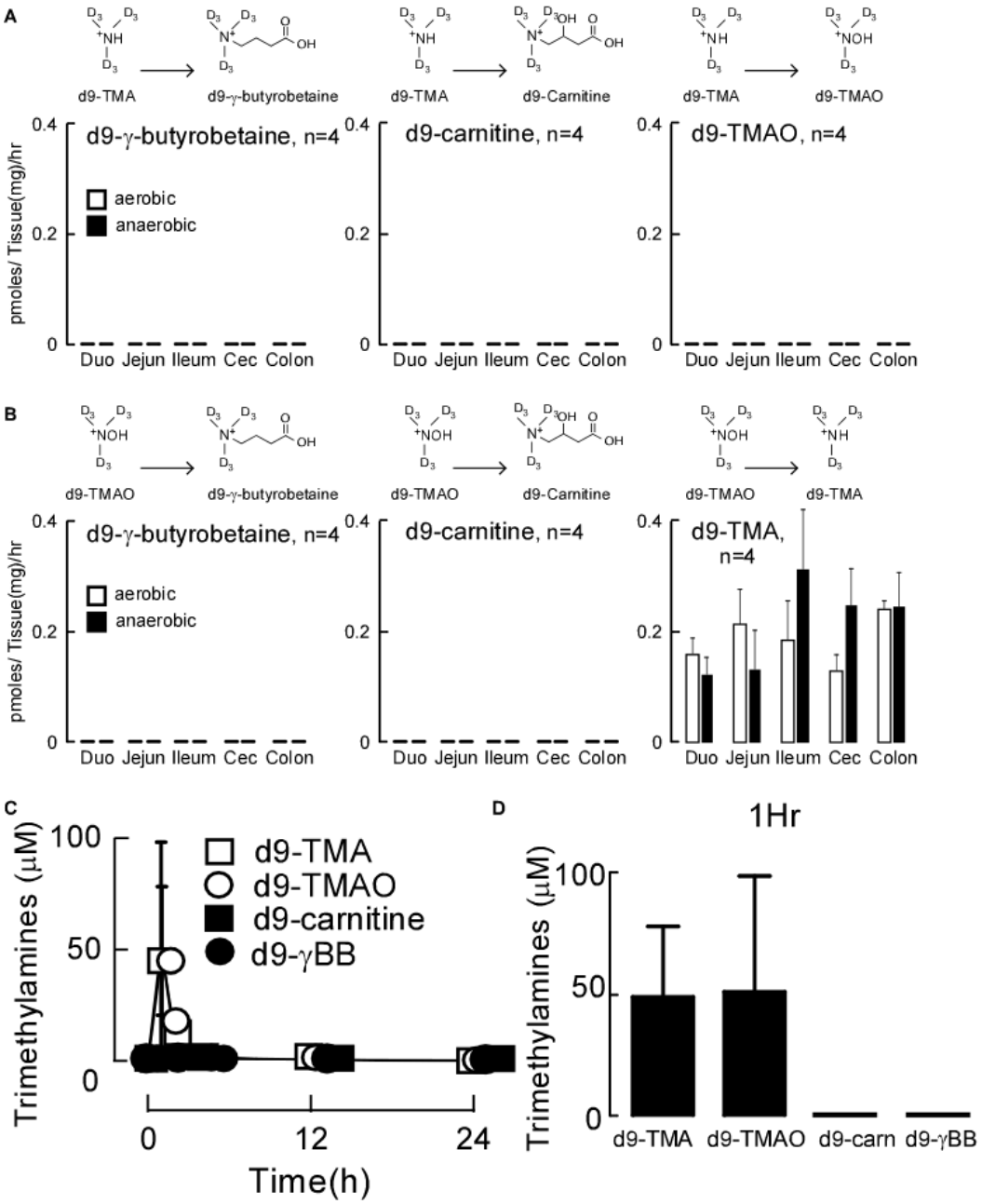

Figure S5, related to Figure 3. Gut microbiome does not convert TMA/TMAO into carnitine or γBB. C57BL/6J Female mouse intestines (n=4) were sectioned into the indicated segments, filet open, and then incubated at 37 ^{o}C for 18 hours in the presence of d9-TMA (A) or d9-TMAO (B) under either aerobic (open bars) or anaerobic (closed bars) conditions, as indicated. The indicated deuterated trimethylamine analytes were quantified by stable isotope dilution LC/MS/MS as described in Experimental Procedures. The isotopologue of the precursor incubated with the gut segments, and the product monitored by LC/MS/MS, are shown at the top of each graph. (C, D) C57BL/6J Female mice (n=2) were challenged (gastric gavage) with d9-TMA and then d9-TMA, d9-TMAO, d9-carnitine, and d9-γBB were quantified post gavage in mouse plasma at the indicated times. Only data from the 1 hour time point is shown in panel D. All data represent mean ± SEM. Duo =Duodenum, Jejun=Jejunum, Cec=Cecum.

Figure S6

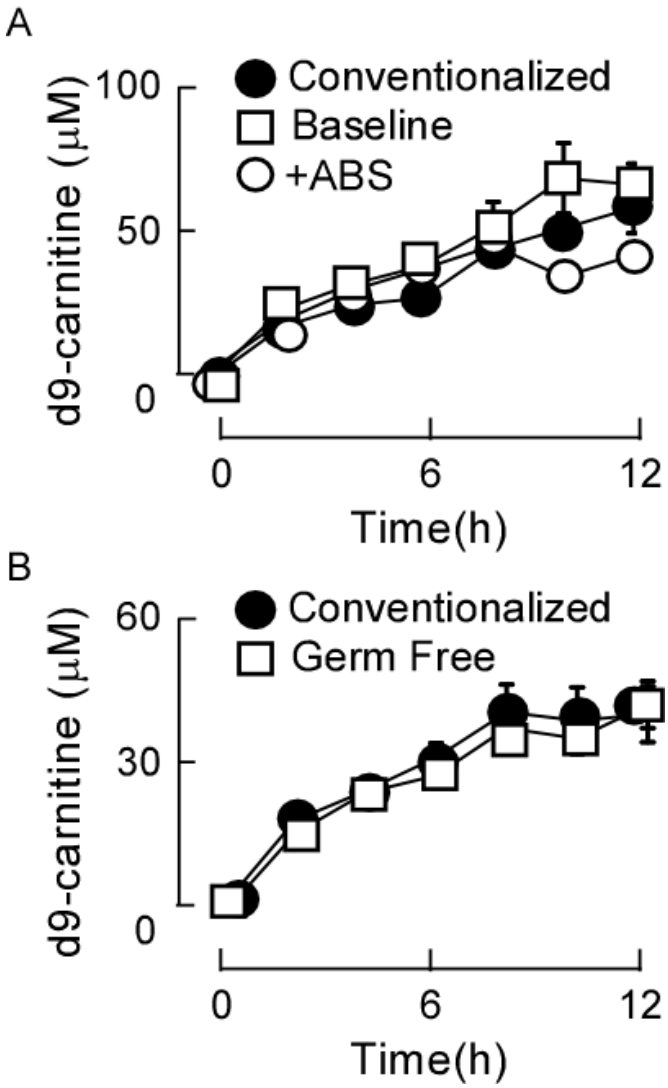

Figure S6, related to Figure 2. Carnitine produced from orally ingested γBB is not a gut microbiota dependent product. (A) Conventionally housed C57BL/6J female mice (n=5) or (B) Female Swiss Webster Germ Free mice (n=4) were provided d9-γBB via gastric gavage and then plasma levels of d9-carnitine quantified by stable isotope dilution LC/MS/MS. In panel A, the conventional mice were examined at baseline, then following 1 month of continuous exposure to antibiotic cocktail (+ABS) provided in the drinking water, and then after another month of no antibiotics and conventional housing (Conventionalized). In panel B, results shown are for plasma levels over time following the d9-γBB gavage in the germ free mice, and then again post gavage in the same mice but one month later following conventionalization by housing in conventional cages. Statistical analysis of the areas under the curves for d9-carnitine levels in (A) mice before (Baseline) vs. following suppressed gut microbiota challenged (+ABS) (*P* =0.12) or with mice with suppressed gut microbiota challenged (+ABS) vs. conventionalized mice (*P*=0.69) or (B) Germ free vs. Conventionalized (*P*=1.0) post d9-γBB gastric gavage showed no significant differences. Data represent mean ± SEM.

Figure S7

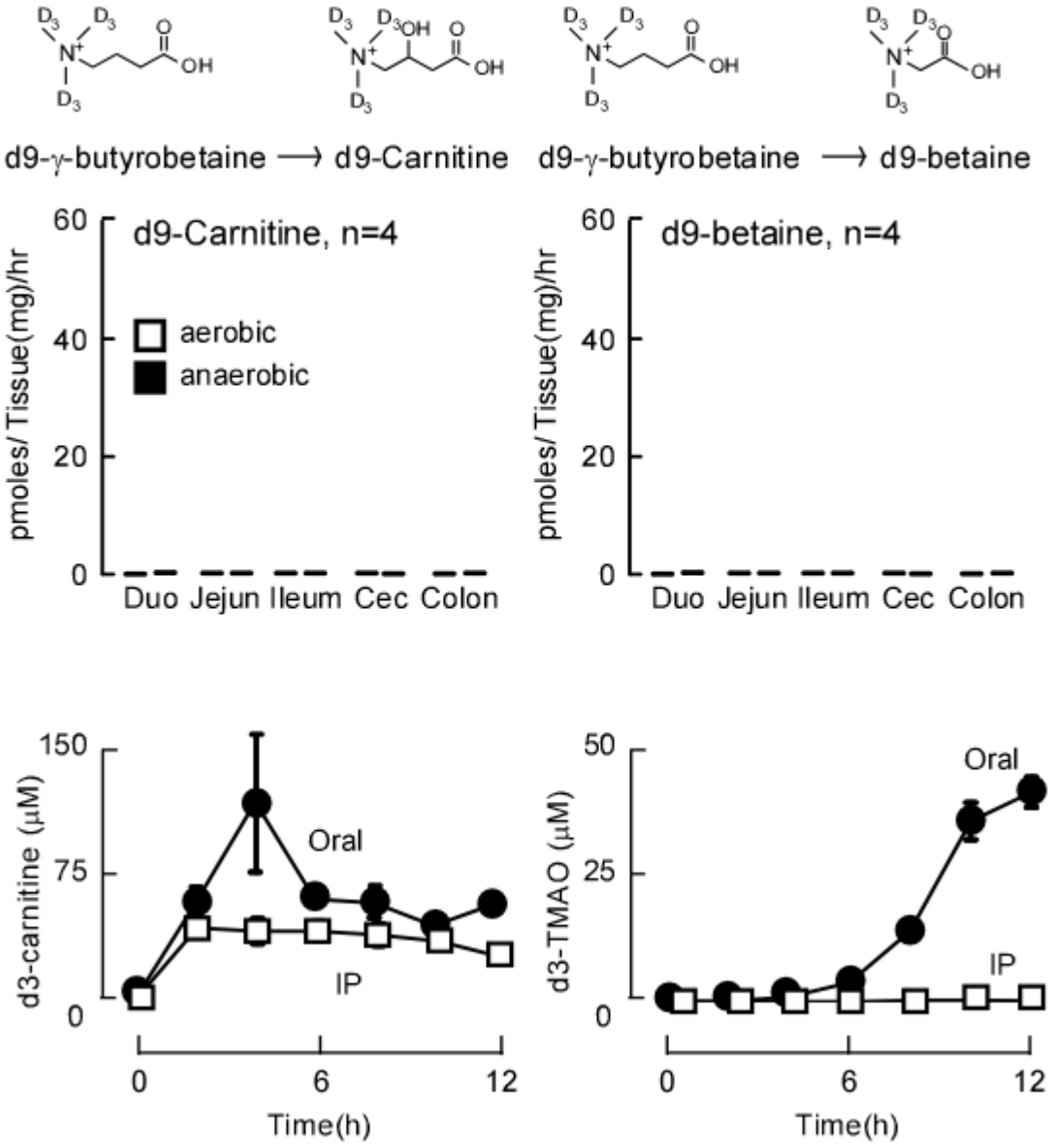

Figure S7, related to Figure 3. Gut microbes do not convert γBB into carnitine or betaine, and circulating carnitine does not contribute significantly to TMAO production. (Top panels) C57BL/6J Female mouse intestines (n=4) were sectioned into the indicated segments, filet open, and then incubated at 37°C for 18 hours in the presence of d9-γBB under either aerobic (open bars) or anaerobic (closed bars) conditions, as indicated. The isotopologue of the precursor incubated with the gut segments, and the product monitored by LC/MS/MS, are shown at the top of each graph. Duo =Duodenum, Jejun=Jejunum, Cec=Cecum. **(Bottom panels)** C57BL/6J female mice were administered 150 μl of 150 mM d3-carnitine in NS vehicle by either gastric gavage (Oral) vs. parenteral (IP) route. Serial venous sampling at the indicated times and plasma levels of d3-carnitine and d3-TMAO were then determined by LC/MS/MS analyses. Results shown are the mean ± SE of n=4 animals each group.

Supplemental Tables

Table S1

Plasma Lipids	Chow (n=20)	γ–butyrobetaine (1.3%) (n=17)	*P*
Triglyceride (mg/dL)	113 ± 26	139 ± 25	<0.01
Total Cholesterol (mg/dL)	304 ± 53	300 ± 56	0.84
HDL (mg/dL)	32 ± 10	33 ± 22	0.82
Total Glucose (mg/dL)	217 ± 45	194 ± 35	0.10

Plasma Lipids	Chow, + ABS (n=21)	γ–butyrobetaine (1.3%), + ABS (n=18)	*P*
Triglyceride (mg/dL)	74 ± 6	90 ± 23	0.03
Total Cholesterol (mg/dL)	304.3± 12	419 ± 53	0.53
HDL (mg/dL)	32 ± 6	27 ± 7	0.73
Total Glucose (mg/dL)	211 ± 16	188 ± 43	0.25

Liver	Chow (n=20)	γ–butyrobetaine (1.3%) (n=17)	*P*
Triglyceride (mg/g)	46 ± 21	35 ± 11	0.09
Cholesterol (mg/g)	1.6 ± 0.32	1.1 ± 0.25	<0.01

Liver	Chow, + ABS (n=21)	γ–butyrobetaine (1.3%), + ABS (n=18)	*P*
Triglyceride (mg/g)	44 ± 7.4	28 ± 9.4	0.01
Cholesterol (mg/g)	2.1 ± 0.16	1.6 ± 0.21	<0.01

Table S1, related to Figure 4. Plasma and liver chemistries in C57BL/6J, *Apoe-/-* female mice used in the γBB diet atherosclerosis study. Animals were fed the indicated diets ad lib, in the presence vs. absence of antibiotic cocktail (ABS) in the drinking water as described under Methods. Data are expressed as means ± SD.

Table S2

Cecum Taxonomic class:	Precursor (Diet)	Product	FDR	R Value
Genus: *Parasutterella*	Carnitine	γBB	0.059	0.545
Family: *Lachnospiraceae*	Carnitine	γBB	0.091	-0.513
Genus: *Bacteroides*	Carnitine	γBB	0.095	0.503
Genus: *Prevotella*	Carnitine	γBB	0.029	0.605
Genus: *Akkermansia*	γBB	TMAO	0.009	0.634
Family: *Lachnospiraceae*	Carnitine	TMAO	0.084	0.779
Genus: *Other Ruminococcaceae*	Carnitine	TMAO	0.084	0.725
Ileum **Taxonomic class:**				
Genus: *Akkermansia*	γBB	TMAO	0.017	0.711
Jejunum **Taxonomic class:**				
Genus: *Staphylococcus*	Carnitine	γBB	0.091	0.749

Table S2, related to Figure 6. Gut microbial taxa whose proportions in cecum, ileum or jejunum were significantly associated with plasma levels of carnitine, γBB, TMA or TMAO in mice on carnitine or γBB supplemented diets. Plasma was isolated from 10 week old *Apoe*-/- female mice that had been maintained since weaning on a chemically defined diet comparable to chow except for supplementation with either *L*-carnitine (1.3 gm%) or γBB (1.3 gm%), as outlined under Methods. Microbial compositions in the indicated gut segments were assessed by sequencing 16S rRNA gene amplicons and in parallel, plasma levels of carnitine, γBB, TMA and TMAO were quantified by stable isotope dilution LC/MS/MS. The associations shown are between gut microbial taxa and plasma levels of the trimethylamines within the indicated dietary groups of mice. The studies were performed from the same founding colony of *Apoe*-/- mice that were co-housed (with harem breeding and transfer of fecal pellets amongst cages in the colony) prior to separation into individual cages at time of weaning and placement on the indicated diets: γBB (n=11), *L*-carnitine (n=13). R shown are Spearman correlations and the FDR value is the P value after adjustment for multiple testing for mice within the indicated diet arm.

Supplemental Methods

Mouse challenge and atherosclerosis studies.
10-week-old female Taconic Swiss Webster germ-free mice were challenged with γBB immediately upon arrival in a microisolator. The mice were then conventionalized by being housed in cages with non-sterile C57BL/6J female mice for approximately 1 month before the γBB challenge was again performed. The γBB challenge was also performed on 12-week old native C57BL/6J female mice, after gut microbe suppression with broad spectrum antibiotics for 1 month, and finally, after being cohoused with conventional mice for at least an additional 1 month period after antibiotic cessation (Hawrelak and Myers, 2004; Wang et al., 2011). Gut microbe inducibility studies were completed by performing *L*-carnitine or the γBB challenge on 12 week old C57BL/6J, *Apoe*-/- mice on *L*-carnitine (Chem Impex International; Wood Dale, IL) or γBB supplemented diets (1.3%, gm/gm) compared to mice on control chow diet for at least a 10 week period. For the atherosclerosis study, C57BL/6J, *Apoe*-/- female mice were placed on a standard chow control diet (Teklad 2018) or γBB-supplemented diet (mouse drinking water with 1.3% γBB; BOC Sciences; Shirley, NY) with and without antibiotics at time of weaning for a 15 week duration. The antibiotic regimen used was provided to the mouse in the drinking water as described above. Mouse aortic root plaque was prepared and quantified as previously described (Wang et al., 2011). Quantification of natural abundance and isotope labeled forms of *L*-carnitine, γBB, TMA and TMAO in mouse plasma was performed using stable isotope dilution LC/MS/MS as described below.

Mouse husbandry, and microbiota analyses.
All mice began from the same founder colony with harem breeding, moving males amongst all the breeder cages, and with fecal pellets continuously transferred between cages throughout the breeding colony. Starting with cohoused mice, at time of weaning, female littermates were split into individual cages where the chemically defined indicated diets were provided. Mice were maintained individually in isolation for the duration of the studies until experimental procedures (e.g. carnitine or γBB challenges) and sacrifice.

The V4 region of the 16S rRNA gene was amplified using bar-coded fusion primers (F515/R806) with the 454 a Titanium sequencing adapter. The barcoded primers were produced following the protocol described by Hamady et al (Hamady et al., 2008). Sample preparation was performed similarly to that described by Costello et al. (Costello et al., 2009). Each sample was amplified in triplicate, combined in equal amounts and cleaned using the PCR clean-up kit (Mo Bio). Cleaned amplicons were quantified using Picogreen dsDNA reagent (Invitrogen, Life Technologies, Grand Island, NY) before sequencing using 454 GS FLX titanium chemistry at the GenoSeq Facility at the University of California, Los Angeles. The raw data from the 454 pyrosequencing machine were first processed through a quality filter that removed sequence reads that did not meet the quality criteria. Sequences were removed if they were shorter than 200 nucleotides, longer than 1,000 nucleotides, contained primer mismatches, ambiguous bases, uncorrectable barcodes, or homopolymer runs in excess of six bases. The remaining sequences were analyzed using the open source software package Quantitative Insights into Microbial Ecology (QIIME (Caporaso et al., 2010b; Kuczynski et al., 2011)). A total of 49,458 quality filtered reads were obtained from 39 samples (three samples were removed due to low sequence count). Individual reads that passed filtering were distributed to each sample based on bar-code sequences. Demultiplexed sequences were assigned to operational taxonomic units (OTUs) using UCLUST with a threshold of 97% pair-wise identity. Representative sequences were selected and BLASTed against a reference Greengenes reference database

(gg 12 10 version). The taxonomic composition was assigned to the representative sequence of each OTU using Ribosomal Database Project (RDP) Classifier 2.0.1 (Wang et al., 2007). The relative abundances of bacteria at each taxonomic level (e.g., phylum, class, order, family and genus) were computed for each mouse. For tree-based analyses, a single representative sequence for each OTU was aligned using PyNAST (Caporaso et al., 2010a), then a phylogenetic tree was built using FastTree. The phylogenetic tree was used to measure the β-diversity (using unweighted UniFrac) of samples (Lozupone and Knight, 2008). Correlations between relative abundance of gut microbiota and TMA and TMAO levels and association testing were performed in R. False discovery rates (FDR) of the multiple comparisons were estimated for each taxon based on the p-values resulted from correlation estimates.

Synthesis of d9-trans crotonobetaine

Briefly, d9-*trans* crotonobetaine was prepared by gradually dissolving synthetic d9(trimethyl)-L-carnitine (prepared as previously described, (Koeth et al., 2013)) into concentrated sulfuric acid (Fisher) at 145^{o}C. The reaction was cooled and then poured over crushed ice. Sodium hydroxide (Fisher) was then added to bring the pH of the reaction to 7.0. The reaction mixture was frozen and then lyophilized to a tan powder. d9-*trans* Crotonobetaine was extracted into methanol; the resulting heavy suspension filtered under vacuum to remove most of the sodium sulfate. The filtrate was rotary evaporated to a beige crystalline semisolid. Residual water was removed azeotropically by repeated rotary evaporation of absolute ethanol (Fisher) from the crystalline mass. The impure d9- *trans* crotonobetaine was re-dissolved in a minimal amount of absolute ethanol and the suspension again clarified by vacuum filtration. Gradual addition of ethyl acetate (Fisher) and seed crystals of d9- *trans* crotonobetaine to the filtrate initiated crystallization. The d9- *trans* crotonobetaine crystals were isolated by vacuum filtration and washed with ethyl acetate. Residual ethyl acetate was removed in a vacuum oven at 55^{o}C for one hour and the compound further dried under oil pump vacuum over P2O5 (Alfa Aesar, Ward Hill, MA). d9- *trans* Crotonobetaine was stored at room temperature in polyethylene containers. The purity (>97%) and identity of the d9- *trans* crotonobetaine preparations were confirmed by NMR, mass spectrometry, and thin layer chromatography.

D9-γbutyrobetaine chloride preparation.

(3-Carboxypropyl)trimethyl(d^{9})ammonium Chloride (d9-γbutyrobetaine Chloride, d9-γBB) was prepared from γ-aminobutyric (GABA) acid in methanol with potassium hydrogen carbonate and d3-methyl iodide (all from Sigma-Aldrich, St. Louis, MO). After 72 hours, the entire reaction mixture was quantitatively transferred onto a bed of silica gel (60Å pore 40-60 μM particle, Angela Technologies, Wilmington, DE) equilibrated in methanol in a coarse fritted Buchner pressure funnel. Non-polar material was removed by elution of the column with the 1.25 column volumes of methanol. The product d9-γBB was eluted in 2.5 column volumes of 30%,v/v, water in methanol. Rotary evaporation of this second fraction gave the crude product as an oily semisolid which was dissolved in water and titrated to pH 7.2 with dilute hydrochloric acid. The water was removed by rotary evaporation and final traces of moisture were removed azeotropically by sequential rotary evaporations of absolute ethanol under reduced pressure. The white to off-white solid was dissolved in absolute ethanol and filtered to remove residual inorganic salts. The material was concentrated to dryness by rotary evaporation and dissolved in excess dilute (3N) hydrochloric acid. Rotary evaporation of the resulting straw-colored solution was followed by the re-dissolution of the semi-crystalline light amber-colored salt in a minimal amount of methanol. This methanolic solution was treated with 5 volumes of acetone; the resulting almost clear solution was allowed to sit at room temperature for several hours. The plate-like crystals that formed were isolated by suction filtration, transferred to a clean container, and dried under vacuum at 60^{o}C. This material darkened slightly to an off white free flowing

powder which was stored refrigerated over desiccant. Concentrations of stock solutions of this material were determined relative to a standard curve of authentic γBB (chloride salt), by LC/MS/MS as described below. ESI positive ion mode mass spectrum for d9-γbutyrobetaine chloride (5 μM in 50%, v/v, methanol in water plus 0.1%, v/v, formic acid) shows a base peak at m/z 155.2 amu $[M]^+$, a peak at m/z 178.2 amu corresponding to $[M+Na]^+$ and a peak at m/z 194.2 amu corresponding to $[M+K]^+$, MS^2 analyses in the positive ion mode for the peak at m/z 155.2 amu (collision energy 20) shows a base peak at m/z 87.2 amu corresponding to $[M-N(CD_3)_3]^+$ and a peak at 69.3 amu corresponding to $[HN(CD_3)_3]^+$. The purity (>98%) and identity of the d9-γBB preparations were confirmed by multinuclear NMR, high resolution MS^n analyses on LC triple TOF, and thin layer chromatography.

Quantification of trimethylamines.
Precursor to product ion transitions used for endogenous and d3-isotopologues of trimethylamines included m/z 76 to 58 amu (TMAO), m/z 79 to 61 amu (d3-TMAO), m/z 60 to 44 amu (TMA), m/z 63 to 47 amu (d3-TMA), m/z 104 to 60 amu (choline), m/z 107 to 63 amu (d3-choline), m/z 146 to 60 amu (γBB), 149 to 63 amu (d3-γBB), m/z 118 to 59 amu (betaine), m/z 121 to 62 amu (d3-betaine), m/z 160 to 60 amu (3-dehydrocarnitine), m/z 163 to 63 amu (3-dehydrocarnitine), m/z 162 to 60 amu (*L*-carnitine), and m/z 165 to 63 amu (d3-*L*-carnitine).

Precursor to product ion transitions used for d9-isotopologues of trimethylamines included d9(trimethyl)TMAO (d9-TMAO, m/z 85 to 66 amu), d9(trimethyl)TMA (d9-TMA , m/z 69 to 49 amu), d9(trimethyl) γBB, (d9-γBB ,m/z 155 to 69 amu), d9(trimethyl)L-carnitine (d9-L-carnitine, m/z 171 to 69 amu), d9(trimethyl)betaine (d9-betaine, m/z 127 to 68 amu), and d9(trimethyl)*trans* crotonobetaine (m/z 153 to 68 amu). The synthetic d9-isotopologues were used as standards and added to mouse plasma to quantify native compound concentrations (syntheses of isotopologues of various trimethylamines described above).

After control studies with standard additions (spike and recovery across multiple concentrations) using authentic d9-isotopologues to confirm identical results, d4-choline (Cambridge Isotope Labs, Andover, MA) (m/z 108 to 64 amu) was used as internal standard to quantify d9-γBB and d9 gut microbial generated mouse products (d9-TMA, d9-TMAO) from d9-γBB-challenge studies. Synthetic d9-*trans* crotonobetaine (m/z 153 to 68 amu) was used to quantify endogenous *trans* crotonobetaine (m/z 144 to 59 amu) and d3-*trans* crotonobetaine (m/z 147 to 62 amu) by stable isotope dilution LC/MS/MS analyses. Increasing concentrations of the trimethylamines with a fixed amount of internal standard were added to control plasma to generate calibration curves for determining plasma concentrations of each respective analyte isotopologue similar to methods previously described (Hawrelak and Myers, 2004; Wang et al., 2011; Wang et al., 2014).

Cloning, expression, isolation and characterization of yeaW/yeaX.
A gene cluster consistent with the features sought was identified including the genes yeaU (dehydrogenase), GeneID: 6060908; yeaV (putative BCCT transporter), GeneID: 6060973; yeaW (dioxygenase), GeneID: 6060925 and yeaX (oxidoreductase), GeneID: 6060982. The genes for yeaW and yeaX are contiguous and were PCR'd from genomic *E. Coli* DH10B DNA, GeneBank: CP000948.1, nucleotides 1973260-1975405 using the following forward and reverse primers, respectively, (gene-specific sequences are underlined)
5'AGGAGATACCATGAGCAATCTGAGCCCTGACTTTGTACTAC , and 5' AGGAGATATACCATGTCAGACTATCAAATGTTTGAAGTACAGGTG. PCR reactions were run in 50 μl aliquots using the following temperature program: 2 min at 95°C; 1x(20 sec at 95°C 30 sec at 57°C, 80 sec at 68°C); 30x(20 sec at 95°C, 30 sec at 62°C, 80 sec at 68°C); 3 min at

68°C and 4°C hold. PCR reactions were fractionated on a 1% agarose gel and the PCR fragment gel-purified, quantified and used for cloning into a modified pET20 vector at the Nco I/ HindIII sites using InFusion (Clontech). After DNA sequence verification of clones, pilot expression studies confirmed that the correct size protein(s) were being produced and that the lysate, but not lysate from induced native BL21 cells, had carnitine TMA-lyase activity (produced d9-TMA from d9-carnitine). The individual yeaW and yeaX genes were then PCR'd from this expression clone using gene-specific primers and cloned in-frame behind an 8X-His tag in a modified pET20 vector. Upon sequence verification, the individual epitope-tagged yeaW and yeaX alleles were transformed into E. Coli BL21 DE3 pLysS cells (Invitrogen) and individual colonies of each were expanded for growth, induction, harvesting and extract preparation, as previously described (Gogonea et al., 2010). Cells expressing 8X-His-tagged yeaW or yeaX were grown to an OD600 of 0.5, induced at room temperature with 0.4mM IPTG for 18h, and then harvested and protein extracts prepared using a microfluidizer, as previously described (Gogonea et al., 2010). Recombinant proteins were purified using IMAC Ni-charged resin, as previously described (Gogonea et al., 2010). Enzymatic activity was determined by incubation of the purified yeaW and eaX (30 μg each) with 100 μM deuterium labeled trimethylamine containing compounds in the presence of 200 μM NADH in 1 ml PBS at 37^{o}C in gas tight vials. The product, d9-TMA, was determined by LC/MS/MS as described above.

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