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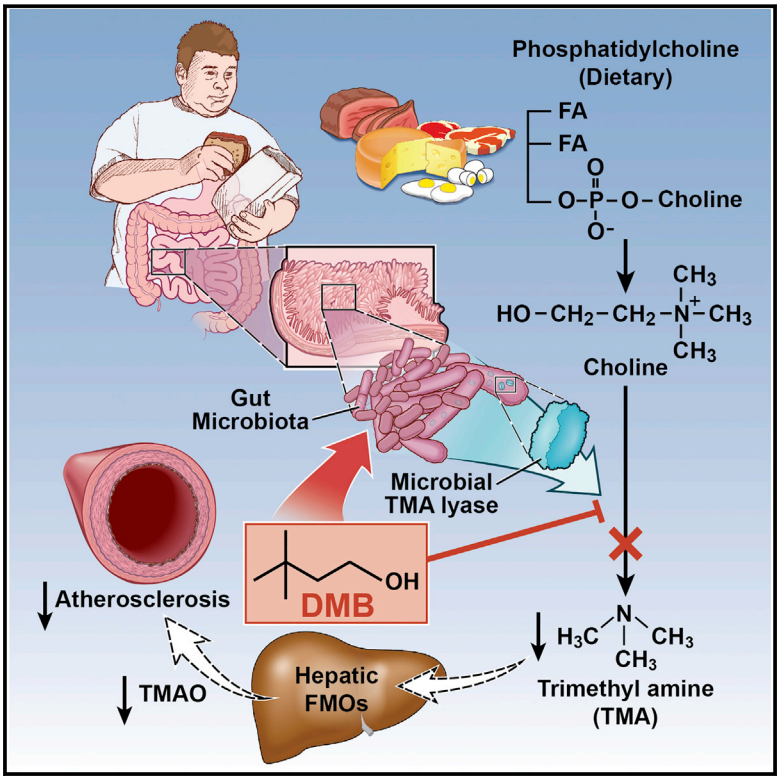
Non-lethal Inhibition of Gut Microbial Trimethylamine Production for the Treatment of Atherosclerosis

Graphical Abstract



Highlights

- Gut microbial trimethylamine lyases are a therapeutic target for atherosclerosis
- 3,3-dimethyl-1-butanol inhibits microbial trimethylamine formation
- 3,3-dimethyl-1-butanol attenuates choline diet-enhanced atherosclerosis
- Non-lethal gut microbial enzyme inhibition can impact host cardiometabolic phenotypes

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In Brief

Drugging the gut microbiota with a non-lethal inhibitor that blocks production of the metabolite trimethylamine reduces the formation of atherosclerotic lesions and represents the first step toward treatment of cardiometabolic diseases by targeting the microbiome.

Wang et al., 2015, Cell *163*, 1585–1595
December 17, 2015 ©2015 Elsevier Inc.
http://dx.doi.org/10.1016/j.cell.2015.11.055

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Non-lethal Inhibition of Gut Microbial Trimethylamine Production for the Treatment of Atherosclerosis

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http://dx.doi.org/10.1016/j.cell.2015.11.055

SUMMARY

Trimethylamine (TMA) N-oxide (TMAO), a gut-microbiota-dependent metabolite, both enhances atherosclerosis in animal models and is associated with cardiovascular risks in clinical studies. Here, we investigate the impact of targeted inhibition of the first step in TMAO generation, commensal microbial TMA production, on diet-induced atherosclerosis. A structural analog of choline, 3,3-dimethyl-1-butanol (DMB), is shown to non-lethally inhibit TMA formation from cultured microbes, to inhibit distinct microbial TMA lyases, and to both inhibit TMA production from physiologic polymicrobial cultures (e.g., intestinal contents, human feces) and reduce TMAO levels in mice fed a high-choline or L-carnitine diet. DMB inhibited choline diet-enhanced endogenous macrophage foam cell formation and atherosclerotic lesion development in apolipoprotein $e^{-/-}$ mice without alterations in circulating cholesterol levels. The present studies suggest that targeting gut microbial production of TMA specifically and non-lethal microbial inhibitors in general may serve as a potential therapeutic approach for the treatment of cardiometabolic diseases.

INTRODUCTION

Recent studies suggest that gut microbes are participants in atherosclerosis development. Specifically, choline, phosphatidylcholine, and carnitine—trimethylamine (TMA)-containing nutrients abundant in foods such as meat, egg yolks, and high-fat dairy products—serve as dietary precursors for TMA N-oxide (TMAO) generation in mice and humans, a metabolite that accelerates atherosclerosis in animal models (Koeth et al., 2013; Tang et al., 2013; Wang et al., 2011). Blood TMAO levels are associated with risks for both prevalent atherosclerotic heart disease and incident major adverse cardiac events in multiple independent cohorts (Koeth et al., 2013; Lever et al., 2014; Mente et al., 2015; Tang et al., 2013, 2014; Trøseid et al., 2015; Wang et al., 2011, 2014b). TMAO-lowering interventions are, thus, of considerable interest for their potential therapeutic benefit (Brown and Hazen, 2015).

TMAO formation in mammals occurs via a two-step metaorganismal pathway (Koeth et al., 2013; Wang et al., 2011). Specifically, following nutrient ingestion, gut microbes form TMA, and then host hepatic flavin monooxygenases (FMOs) catalyze the conversion of TMA into TMAO (Koeth et al., 2013; Wang et al., 2011). Recent studies highlight the importance of both TMAO and host hepatic FMO3, the primary FMO responsible for TMAO production, as important regulators in host lipid and sterol metabolism, as well as atherosclerosis development (Bennett et al., 2013; Koeth et al., 2013; Shih et al., 2015; Wang et al., 2011; Warrier et al., 2015). In addition, recent microbial transfer studies confirm that both choline-diet-enhanced atherosclerosis susceptibility and TMA/TMAO formation are transmissible traits (Gregory et al., 2015). Accordingly, the concept of "drugging the microbiome" to inhibit the metaorganismal pathway responsible for TMAO generation is of interest as a potential therapeutic approach for prevention of atherosclerosis. Toward that end, recent studies confirm that suppression of the host enzyme, FMO3, through an antisense oligonucleotide-based approach, results in the reduction of both circulating TMAO levels and diet-enhanced atherosclerosis in animal models (Miao et al., 2015; Shih et al., 2015). However, while numerous beneficial effects with FMO3 inhibition have been noted, FMO3 inhibition is also accompanied by several untoward side effects, including hepatic inflammation (Shih et al., 2015; Warrier et al., 2015). It is also well established that humans with genetic defects in FMO3 suffer from fish odor syndrome, due to the accumulation of TMA and its noxious odor (Cashman et al., 2003), reducing the appeal of FMO3 as a potential therapeutic target for inhibition. Reduction in TMAO levels by non-lethally targeting the gut microbial pathway(s) involved in TMA production has not yet been explored as a potential therapeutic approach to inhibit atherosclerosis.

RESULTS

Identification of an Inhibitor of Microbial TMA Formation from Choline

Anaerobic choline degradation is believed to serve as the major source of TMA formation within the intestines (Bain et al., 2005; Wang et al., 2011). Therefore, we focused initial drug screening efforts on the identification of potential inhibitors of microbial TMA production from choline- or so-called choline-TMA lyase activity. The cleavage of the C-N bond of choline to form TMA and acetaldehyde was reported with extracts of *Proteus mirabilis*, a known human commensal microorganism that is easily cultured, nearly 3 decades ago (Sandhu and Chase, 1986). Therefore, we initially used *P. mirabilis* in early drug screening studies. However, during the course of our drug discovery efforts, the identification of a microbial choline TMA lyase was reported as a unique glycyl radical enzyme complex composed of a catalytic polypeptide, CutC, and an associated activating protein, CutD, encoded by adjacent genes within a gene cluster (Craciun and Balskus, 2012). After this report, we subsequently cloned and expressed the *cutC/D* genes from *P. mirabilis* and confirmed that the expressed *cutC/D* gene complex serves as a microbial choline TMA lyase enzyme source (Figure S1A). For example, intact *E. coli* Top10 cells in culture lack choline TMA lyase activity, but following transformation with both *P. mirabilis cutC/D* genes, they acquire the capacity to enzymatically cleave choline to produce TMA. To further confirm that microbial TMA lyase enzyme activity monitored with this system required the presence of both CutC and CutD, we examined multiple additional controls, including the demonstration of the lack of choline TMA lyase activity following transformation with only *cutC* or *cutD* individually or following transformation with *cutD* but in combination with point mutants of *cutC* previously reported (Craciun and Balskus, 2012) to lack TMA lyase activity (*cutC* C781A or G1117A of *P. mirabilis* were made, which correspond to mutation of the conserved thiyl and glycyl-radical active-site residues C489 and G821 in *D. alaskensis*) (Figure S1A).

We surveyed multiple different natural and synthetic structural analogs of choline for their effects on the two microbial choline TMA-lyase activity systems (microbial TMA production using *P. mirabilis* lysate or recombinant choline TMA lyase activity using *cutC/D* from *P. mirabilis* transformed into *E. coli*) and noted three distinct patterns. Similar results were observed with both systems, with data shown in Figure 1A being from *cutC/D*-transformed *E. coli* cell lysates and illustrative data from *P. mirabilis* lysates shown in Figure S1B. The majority of choline analogs showed no effect on TMA lyase activity, which was monitored by quantifying the impact of the analogs on d9-TMA formation from a fixed amount of [d9-*N,N,N-trimethyl*]choline (d9-choline) substrate. One notable example in this category is betaine (Figure 1A; Figure S1B), a choline oxidation metabolite and food component that, we recently showed, can serve as a substrate for gut-microbe-dependent TMA and TMAO generation in vivo, albeit at a rate approximately 100-fold less than that observed with a comparable amount of ingested choline (Wang et al., 2014b). Two of the choline analogs examined, 3,3-dimethyl-1-butanol (DMB) and 2-(trimethylsilyl)ethanol (TMSi-ETOH), in which the nitrogen in choline is replaced with either a carbon or silicon atom, respectively, showed roughly comparable *cutC/D* choline TMA lyase inhibitory activity with a half-maximal inhibitory concentration (IC_{50}) in the ~10 μM range in the recombinant *cutC/D* system (Figure 1A). Interestingly, the phosphonium analog of choline (P-choline) showed a unique effect by augmenting *cutC/D* choline TMA lyase activity (Figure 1A; Figure S1B), suggesting that this choline analog acts as an agonist and may promote more effective interaction between the catalytic (CutC) and activating (CutD) polypeptides. Of the choline TMA lyase activity inhibitors identified, we suspected that DMB, given its structure, might be relatively non-toxic, and possibly even found as a natural product in existing foods or alcoholic beverages. Indeed, screening of multiple fermented liquids, oils, and distilled products confirmed that it is present in some alcoholic beverages and oils to varying levels (e.g., DMB was detected in some balsamic vinegars, in red wines, and in some cold-pressed extra virgin olive oils and grapeseed oils; highest levels observed were 25 mM; data not shown). Therefore, we elected to take DMB forward as a potential tool drug with which to test the hypothesis that inhibiting microbial production of TMA might serve as a potential therapeutic approach for the treatment of atherosclerosis.

In further in vitro studies, DMB was shown to block TMA production from live microbes, inhibiting the rate of intact *P. mirabilis* conversion of d9-choline into d9-TMA (Figure 1B). Moreover, parallel studies showed no effect of DMB on choline uptake by the live microbes, indicating that DMB does not block choline uptake into the cells (Figure 1C). Interestingly, while DMB inhibited the choline TMA lyase activity of *E. coli* transformed with *cutC/D* from *P. mirabilis* (Figure 1D), the sensitivity of CutC/D from different microbial species to DMB inhibition varied. For example, transforming *E. coli* with *cutC/D* from *D. alaskenesis*—whose homologous CutC and CutD amino acid sequences show 63% and 47% identity to CutC/D from *P. mirabilis*, respectively—showed decreased extent of inhibition with DMB (Figure 1E; Figure S1C). An obligatory role of CutC in the enzymatic cleavage of choline to produce TMA (and DMB inhibition) in *E. coli* transformed with the *cutC/D* gene complex from either *P. mirabilis* or *D. alaskensis* was supported in both cases by control studies in which the respective *cutC* gene was mutated to replace previously reported (Craciun and Balskus, 2012) critical active-site residues involved in catalysis (either C781A or G1117A for CutC from *P. mirabilis* (Figure 1D) or G489A for CutC from *D. alaskensis*) (Figure S1C). Examination of alternative TMA substrates with the recombinant *cutC/D* system (*E. coli* transformed with *P. mirabilis cutC/D*) revealed DMB inhibited conversion of both d9-choline and [d9-*N,N,N-trimethyl*] glycerophosphocholine (d9-GPC) substrates into d9-TMA (Figures S2A–S2E). Moreover, when affinity epitope-tagged CutC and CutD (from *E. coli* Stellar cells transformed with *P. mirabilis* cutC/D) were individually isolated to homogeneity, addition of DMB to anaerobic reaction mixtures containing both purified polypeptides showed dose-dependent inhibition in TMA production from either choline or GPC substrates (Figures S2F–S2H). Further studies showed choline and GPC also serve as preferred substrates over [d9-*N,N,N-trimethyl*]phosphatidylcholine (d9-PC) and [d9-*N,N,N-trimethyl*]carnitine (d9-carnitine) for

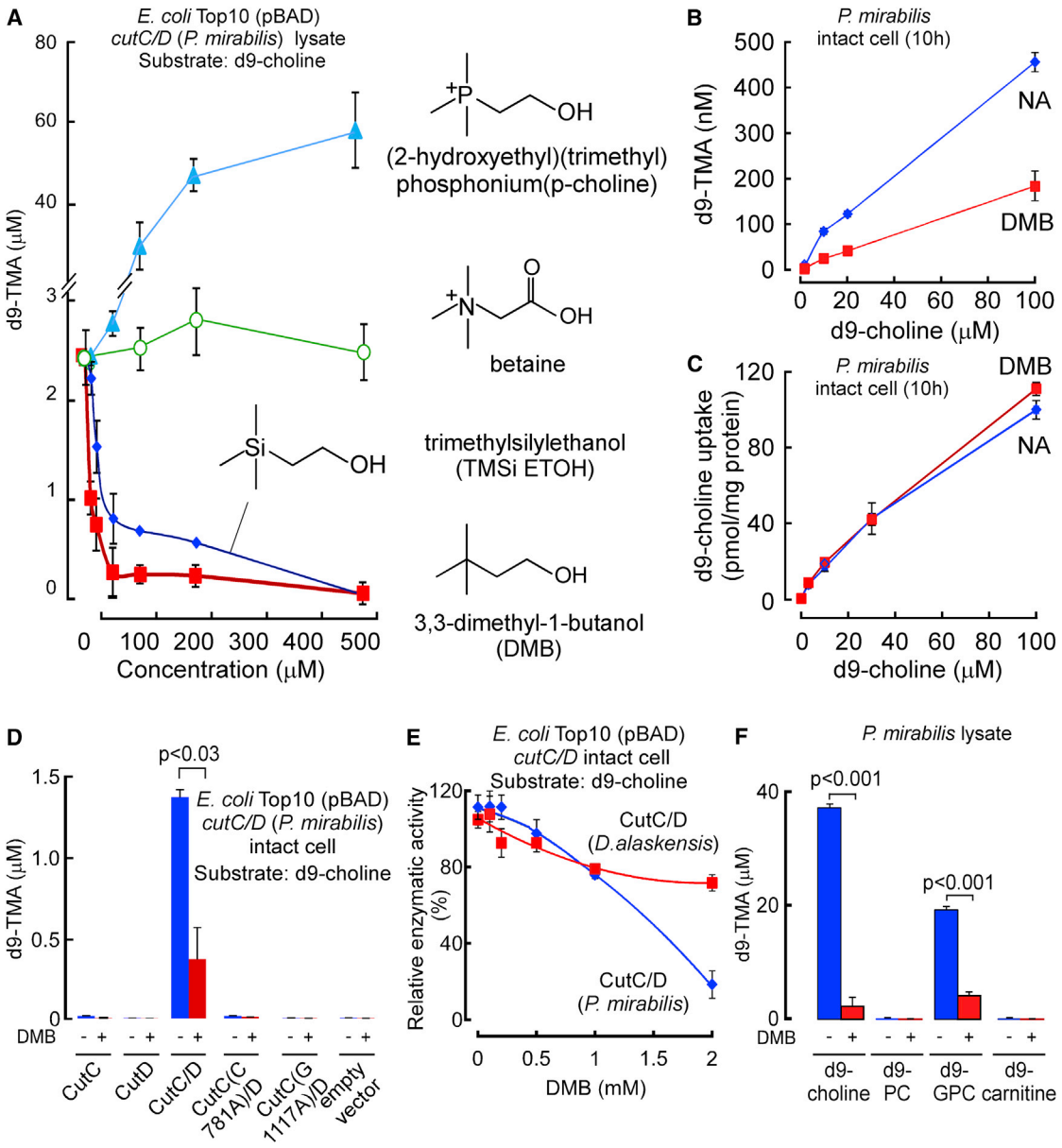

Figure 1. The Choline Analog DMB Inhibits Microbial Choline TMA Lyase Activity

(A) Effect of the indicated choline analogs on microbial TMA lyase activity (measured as d9-TMA production from 100 μM of the indicated d9-labeled substrate) from the lysate of *E. coli* Top10 cells transformed (pBAD vector) with *cutC/D* genes (from *P. mirabilis*).

(B) Effect of DMB on choline TMA lyase activity in intact *P. mirabilis* incubated with the indicated concentrations of d9-choline substrate with or without DMB (1 mM) at 37°C. NA, no addition.

(C) DMB effect on choline uptake. *P. mirabilis* ($OD_{600\ nm}$ = 0.5) cells were pelleted and then re-suspended in minimal media supplemented with the indicated concentrations of d9-choline with or without DMB (2 mM) for 15 min at 37°C. Intracellular d9-choline was then quantified as described in the Experimental Procedures.

(D) Choline TMA lyase activity from intact *E. coli* Top10 cells transformed with the indicated constructs in the presence versus absence of DMB.

(E) DMB dose-response curves for inhibition of choline TMA lyase activity in intact *E. coli* Top10 cells transformed with *cutC/D* genes from either *D. alaskensis* (pUC57 vector) or *P. mirabilis* (pBAD vector).

(F) TMA lyase activity for the indicated substrates in *P. mirabilis* lysate with or without DMB.

Data are presented as mean ± SE from three independent replicates.

See also Figures S1, S2, and S6.

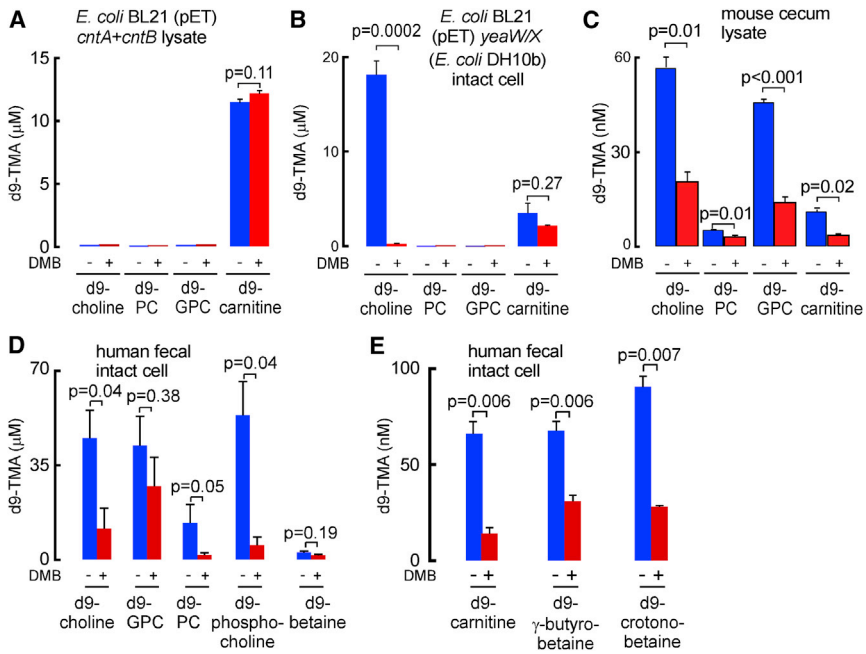

Figure 2. Inhibitory Effect of DMB on Alternative Microbial TMA Lyases and Both Mouse Cecal and Human Fecal Microbial TMA Lyase Activities with Multiple Substrates

(A) TMA lyase activity for the indicated substrates (375 μM each) in combined lysates from *E. coli* BL21 cells transformed (pET22 vector) with *cntA* and *cntB* genes (from *A. baumannii*) incubated in the presence or absence of DMB.

(B) TMA lyase activity with the indicated substrates (375 μM, with or without DMB) in intact *E. coli* BL21 cells transformed (pET22 vector) with *yeaW/X* genes (from *E. coli* DH10B).

(C–E) TMA lyase activity with the indicated substrates (with or without DMB) incubated with (C) mouse cecum lysate (1.3 mg/ml protein) or (D and E) human fecal microbes (equivalent to 100 mg/ml). The DMB concentration in all reaction systems was 10 mM (in A and B) or 2 mM (in C–E).

Data are presented as mean ± SE from three independent replicates.

See also Figures S3, S6C, and S6D.

TMA production from *P. mirabilis* lysates, and in both instances, addition of DMB inhibited TMA formation (Figure 1F).

DMB Inhibits Some, but Not All, Microbial TMA Lyases

Since the CutC/D microbial TMA lyase complex does not effectively use either d9-carnitine or d9-PC as substrates (Figures S2C and S2E), yet both TMA-containing nutrients can be converted into TMA by microbes, we sought to explore whether DMB might serve as an inhibitor to alternative microbial TMA lyase activities. Toward this end, we first cloned the *cntA* (oxygenase) and *cntB* (reductase) genes from *Acinetobacter baumannii*, a recently reported microbial (carnitine-specific) TMA lyase enzyme complex that belongs to the Rieske-type iron-sulfur protein family (Zhu et al., 2014). Examination of the wild-type *E. coli* BL21 strain showed no carnitine TMA lyase activity, so we transformed these cells with either *cntA* or *cntB* (from *A. baumannii*). While neither transformed *E. coli* possessed carnitine TMA lyase activity individually, combining the lysates from both *cntA*- and *cntB*-transformed *E. coli* showed the expected acquired enzymatic activity of cleaving d9-carnitine to form d9-TMA (Figure 2A; Figure S3A). Further confirmation that carnitine TMA lyase activity required the CntA polypeptide was achieved by showing that the lysate from *E. coli* transformed with a *cntA* point mutant that was previously shown to lack carnitine TMA lyase activity—E205D (Zhu et al., 2014)—failed to show activity when combined with *cntB*-transformed *E. coli* lysates and incubated with d9-carnitine (Figure S3A). DMB showed no inhibitory effect on CntA/B-catalyzed cleavage of d9-carnitine, and examination of the substrate preference of CntA/B revealed no evidence of TMA production in the presence of alternative potential precursor substrates such as choline, PC, or GPC (Figure 2A).

We recently reported the cloning and characterization of an alternative TMA lyase enzyme complex from *E. coli* DH10B strain, YeaW/X, which also appears to be a two-component Rieske-type oxygenase/reductase that, at the amino acid level, shows 71% and 50% sequence identity to CntA and CntB, respectively (Koeth et al., 2014). Despite the homology to CntA/B, YeaW/X was found to possess broader substrate usage and could produce TMA from either choline or carnitine as substrate (Koeth et al., 2014). To examine the impact of DMB on YeaW/X TMA lyase activity, we first transformed *E. coli* BL21 cells, which show no detectable TMA lyase activity, with either or both of the *yeaW/X* genes. We observed that DMB inhibits choline TMA lyase enzyme activity in the presence of both YeaW and YeaX (Figure 2B; Figure S3B). Purified YeaW/X showed enzymatic cleavage of d9-carnitine, [d9-*N,N,N-trimethyl*]γ-butyrobetaine (d9-γ-butyrobetaine) and [d9-*N,N,N-trimethyl*]crotonobetaine (d9-crotonobetaine), producing d9-TMA. DMB inhibited the conversion of carnitine and crotonobetaine, but not γ-butyrobetaine, into TMA (Figures S3C and S3D).

DMB Inhibits TMA Formation from Multiple Substrates in Physiological Polymicrobial Cultures

Next, we examined mouse cecal contents as a physiological polymicrobial source of TMA lyase activities with which the potential inhibitory effect of DMB could be further characterized. Mouse cecal contents were homogenized and lysed under anaerobic conditions and then were incubated with a fixed amount of individual potential d9-labeled TMA precursors (d9-choline, d9-PC, d9-GPC, or d9-carnitine) in the absence versus presence of DMB. All TMA-containing nutrients that we examined served as substrates for TMA production by the polymicrobial enzyme source. Moreover, the addition of DMB inhibited TMA formation from each substrate, suggesting broad inhibitory activity across multiple microbial TMA lyase enzyme activities (Figure 2C). In a further set of studies, we used human feces from healthy donors

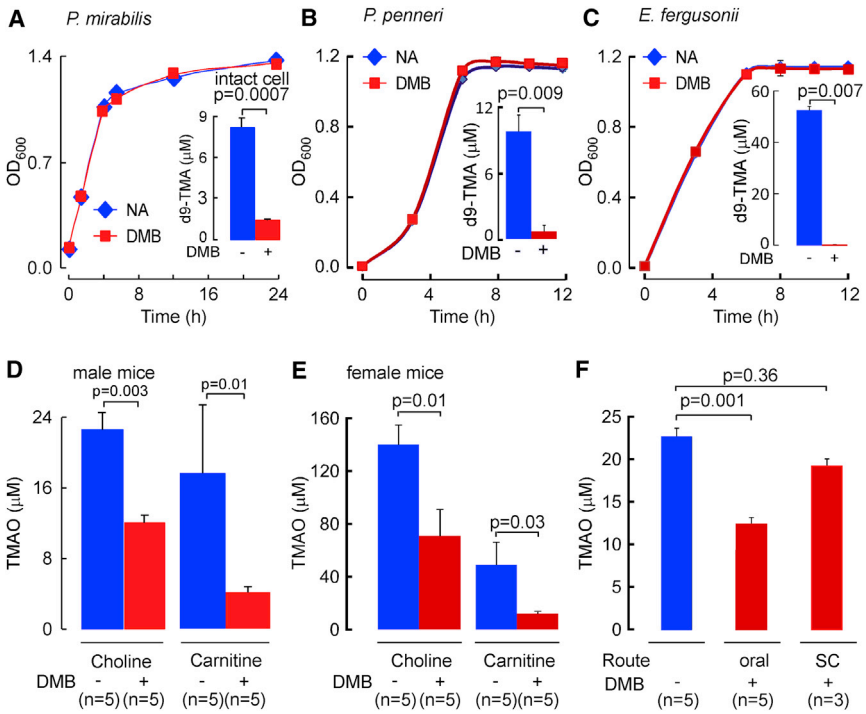

Figure 3. DMB Serves as a Non-lethal Inhibitor

(A–C) *P. mirabilis* (A), *P. penneri* (B), and *E. fergusonii* (C) cells were grown in nutrient broth ± DMB (0.1% v/v) and both the growth rate (OD_{600}) and total amount of d9-TMA produced from d9-choline (100 μM) (insert) were monitored. NA, no addition.

(D and E) Demonstration that DMB decreases plasma TMAO levels in male (D) and female (E) C57BL/6J *Apoe*$^{-/-}$ mice placed chronically on either a 1.0% choline-supplemented or a 1.0% carnitine-supplemented diet in the presence versus absence of DMB (1%, v/v in drinking water).

(F) Three groups of male C57BL/6J *Apoe*$^{-/-}$ mice (8 weeks old) were placed on a chemically defined diet equivalent to chow supplemented with choline (1.0% total choline). Two groups of mice were administered 1.2 μmol of DMB daily (provided in corn oil; total volume, 150 μl) by either (oral) gastric gavage or subcutaneous (SC) route, and the third group of mice received sham (vehicle) gavages daily. After 2 weeks, plasma TMAO levels were determined by LC/MS/MS.

Data are presented as mean ± SE from three independent replicates (A–C) or the indicated numbers of independent replicates (D–F). See also Figure S4C and Tables S1 and S2.

as another physiological polymicrobial source of TMA lyase activity. Intact fecal bacteria were recovered and then incubated with a fixed amount of individual d9-labeled potential nutrient precursors (d9-choline, d9-GPC, d9-PC, [d9-*N,N,N-trimethyl*] phosphocholine (d9-phosphocholine), [d9-*N,N,N-trimethyl*] betaine (d9-betaine), d9-carnitine, d9-γ-butyrobetaine, and d9-crotonobetaine) in the absence versus presence of DMB. Both the substrate preference and the inhibitory effect of DMB on human fecal microbial TMA production were assessed. First, all nutrient precursors examined produced TMA, with greater extent of production from nutrients structurally related to choline (Figure 2D) compared to carnitine (Figure 2E). Moreover, DMB significantly inhibited TMA production from each substrate except for betaine and GPC, both of which showed a trend toward inhibition that was not statistically significant (Figures 2D and 2E).

DMB Is a Non-lethal Inhibitor of TMA Production by Microbes in a Rich Nutrient Environment

Importantly, DMB was shown to serve as a non-lethal inhibitor (i.e., not an antibiotic) in cultures of *P. mirabilis* grown in a rich nutrient broth under conditions that showed no adverse impact on cell growth despite marked inhibition in microbial TMA lyase activity (Figure 3A). Recently, several human bacterial isolates were identified that are capable of producing TMA from choline in culture, as well as in vivo, when used to colonize germ-free mice (Romano et al., 2015). Therefore, we explored the impact of DMB on TMA production and bacterial growth using some of these additional human commensals. Both *Proteus penneri* and *Escherichia fergusonii* were confirmed to possess choline TMA lyase activity, readily consuming choline and producing TMA. Similar to results observed with *P. mirabilis*, addition of DMB to a rich nutrient broth in cultures (as exists in the gut lumen) of either *Proteus penneri* or *Escherichia fergusonii* markedly inhibited choline utilization to produce TMA but showed no inhibitory effect on bacterial growth (Figures 3B and 3C).

DMB Lowers Plasma TMAO Levels In Vivo

To examine the impact of DMB on microbial TMA production in vivo, several studies were performed. First, different groups of C57BL/6J mice were placed on chemically defined diets that were identical in composition except for supplementation with either choline (total choline 1.0%, w/w) or carnitine (1.0%, w/w), diets previously shown to raise TMAO levels and accelerate atherosclerosis in mice (Koeth et al., 2013; Wang et al., 2011). In addition, at the start of the study, each dietary group of mice was further divided by exposing half to DMB, provided in the drinking water. Provision of DMB resulted in significant reduction in plasma TMAO levels in both the choline-supplemented and the carnitine-supplemented groups, with similar results observed for both male and female mice (Figures 3D and 3E). In further studies, comparison of the effect of route of administration revealed that while orally administered DMB resulted in significant reduction in plasma TMAO levels, a comparable amount of DMB administered subcutaneously failed to reduce plasma TMAO levels (Figure 3F). Next, atherosclerosis-prone apolipoprotein E null mice (*Apoe*$^{-/-}$) at the time of weaning were placed chronically (16 weeks) on the same chemically defined diets comparable to chow (0.07% total choline) versus the same diet supplemented with choline (1.0% total choline) in the presence versus absence of DMB provided in the drinking water. Examination of plasma TMAO levels among the groups of

mice showed that while TMAO was markedly elevated in the mice on the choline-supplemented diet (11.8-fold, $p < 0.001$), addition of DMB again substantially inhibited the choline-diet-induced increase in plasma TMAO concentrations (means ± SD, in micromolar, for the four groups: chow, choline, chow + DMB, and choline + DMB, are 1.02 ± 0.09, 22.1 ± 2.1, 0.69 ± 0.06, and 15.9 ± 0.9, respectively; $p < 0.01$ for comparison of choline versus choline + DMB). Moreover, mice showed no evidence of toxicity to the chronic (16-week) DMB exposure, with no significant increases in circulating lipid levels, plasma choline, glucose or insulin levels, and normal indices of both renal function (creatinine) and liver function (transaminase activities, bilirubin) (Table S1). Further tissue biochemical studies confirmed that chronic exposure to DMB did not adversely impact liver lipid (cholesterol, cholesteryl ester, and triglyceride) levels, and histology (including Oil-Red-O and H&E) staining showed no signs of steatohepatitis (data not shown).

DMB Attenuates Choline-Diet-Enhanced Macrophage Foam Cell Formation and Atherosclerosis

High total choline content is a characteristic feature of a Western diet. We previously reported that dietary choline supplementation enhanced macrophage cholesterol accumulation, foam cell formation, and atherosclerosis in *Apoe*$^{-/-}$ mice via microbial TMA/TMAO formation (Wang et al., 2011). The effect of chronic DMB exposure (in the drinking water) on endogenous peritoneal macrophage foam cell formation was, therefore, next examined in the *Apoe*$^{-/-}$ mice maintained chronically (16 weeks from time of weaning) on the chow (0.07% total choline) versus choline-supplemented (1.0% total choline) diet. As was previously observed (Wang et al., 2011), a choline-enriched diet promoted marked increase in endogenous macrophage foam cell generation (approximately 2-fold on choline-supplemented diet; $p = 0.02$), yet the addition of DMB to the drinking water significantly reduced the observed diet-enhanced macrophage foam cell formation ($p < 0.05$ for comparison of choline versus choline + DMB; Figures 4A and 4B). To assess the impact of DMB on high-choline-diet-dependent enhancement in atherosclerosis in the *Apoe*$^{-/-}$ mice, parallel studies in male and female mice were performed in similarly treated *Apoe*$^{-/-}$ mice maintained chronically (16 weeks) from the time of weaning on the chemically defined chow diet (0.07% total choline) versus the choline-supplemented diet (1.0% total choline), in the presence versus absence of DMB (in drinking water). Comparable results (DMB-mediated attenuation of atherosclerosis) were observed in both male and female mice ($p = 0.003$ and $p = 0.01$, respectively), with data from males shown in Figures 4C and 4D, and results with females shown in Figure S4A. Briefly, aortic root lesion plaque quantification revealed that mice on a chronic choline-supplemented diet, as expected, showed significant enhancement in atherosclerotic plaque area (approximately 2-fold increase; $p = 0.003$ males, Figures 4C and 4D; $p = 0.03$ females; Figure S4A) despite no atherogenic changes in plasma lipids (Table S1). Remarkably, the addition of DMB significantly inhibited the choline-diet-dependent enhancement in aortic root lesion development in both males ($p = 0.006$, Figure 4D) and females ($p = 0.02$; Figure S4A). Since observed reductions in plasma TMAO in DMB-treated *Apoe*$^{-/-}$ mice could also occur via changes in hepatic FMO activity, we also examined total hepatic FMO enzymatic activity in liver homogenates generated from the different groups of mice. Contrary to what one might expect with the reduced TMAO plasma levels, mice exposed to DMB showed not a reduction but, instead, a modest but statistically significant increase in total FMO hepatic enzyme activity ($p < 0.0001$; Figure S4B).

Despite extensive efforts, we were unable to detect DMB in the plasma or urine of mice chronically exposed to DMB in the drinking water. Moreover, when equivalent amounts of DMB were provided to mice either via intraperitoneal or oral route, DMB was detected in both plasma and urine 30 min following administration via the intraperitoneal route but not following bolus administration by gastric gavage (Figure S4C). This suggested the possibility that very rapid hepatic metabolism of the agent may account for the low systemic circulating levels observed following oral administration, absorption, and portal blood delivery of DMB to the liver. Consistent with this hypothesis, DMB was found to be rapidly metabolized by liver alcohol dehydrogenase at a rate comparable to that for the substrate ethanol (Figures S5A and S5B). We could not detect the initial predicted metabolite, 3,3-dimethylbutyric aldehyde, in plasma or urine, but instead found evidence of further rapid metabolism to the carboxylic acid, with detection of the metabolite, 3,3-dimethylbutyrylcarnitine, in urine (structure confirmed by high-resolution mass spectrometry; data not shown).

It has previously been reported that 30 min after intraperitoneal administration of DMB, through competitive inhibition of choline dehydrogenase, the choline content of liver tissues increases (Barlow and Marchbanks, 1985). Choline dehydrogenase is expressed mainly in the liver and kidney and catalyzes conversion of choline into betaine aldehyde, an intermediate in the generation of betaine (Cafiero, 1951; Tan et al., 1981). Therefore, in additional studies, we measured levels of choline, betaine, TMA, and TMAO in liver and kidney, as well as plasma and urine, from C57BL/6J *Apoe*$^{-/-}$ mice chronically administered DMB in the drinking water. No evidence of tissue choline elevation was noted in either liver or kidney following chronic oral DMB treatment in mice; however, modest elevations in plasma and kidney betaine levels were observed in mice on choline diet with DMB (versus choline diet without DMB) (Table S2). Notably, as expected with provision of an inhibitor of microbial TMA production, the most significant effects detected with DMB exposure were reductions in TMA and TMAO levels in all tissues and fluids examined (liver, kidney, plasma, and urine), especially under conditions of choline diet supplementation (Table S2).

DMB Reduces the Proportions of Some Taxa Associated with TMAO Levels

One theoretical advantage of a therapeutic agent that functions as a non-lethal, microbial-enzyme-targeted inhibitor, as opposed to an antibiotic, is that there should be less selective pressure for development of resistance. Nonetheless, the gut microbiome is known to be remarkably dynamic and adaptive, including changes in response to different dietary inputs, as well as the presence versus absence of distinct diseases (Cox et al., 2014; Koren et al., 2011; Wu et al., 2011). Therefore, we sought to examine whether chronic exposure to DMB may alter

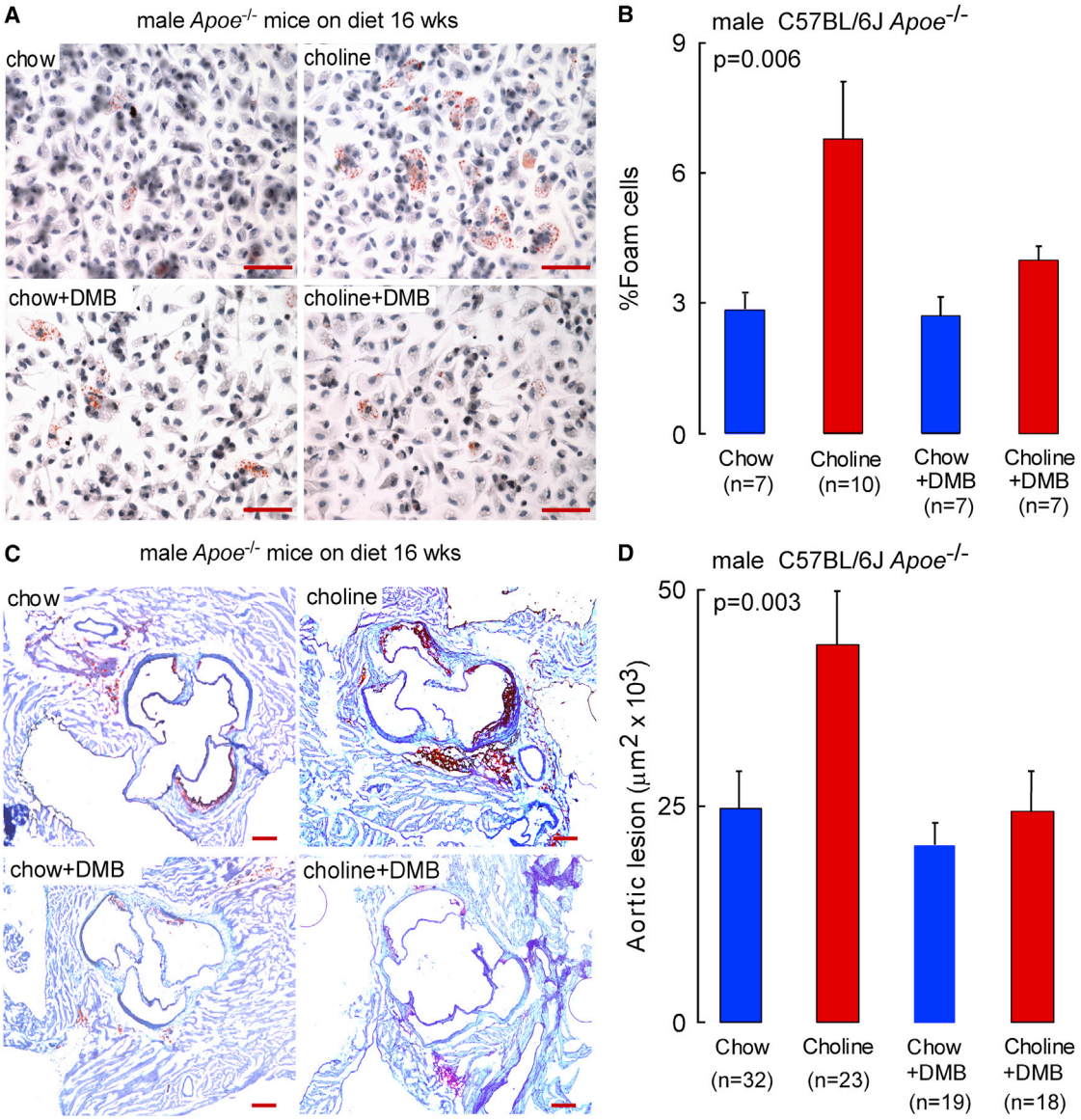

Figure 4. DMB Attenuates Choline-Enhanced Foam Cell Formation and Atherosclerosis

(A and B) Representative Oil-Red-O/hematoxylin staining of peritoneal macrophages (A) and foam cell quantification (B) from 20-week-old male C57BL/6J *Apoe*$^{-/-}$ mice fed chemically defined chow (0.07% total choline) or 1% choline-supplemented diets from the time of weaning (4 weeks). Scale bars in (A), 50 μm. Data are presented as mean ± SE.

(C) Representative Oil-Red-O/hematoxylin staining of aortic root sections from 20-week-old male C57BL/6J *Apoe*$^{-/-}$ mice fed chemically defined chow (0.07% total choline) or choline-supplemented (1.0% total choline) diets in the presence versus absence of DMB provided in the drinking water, as described in the Experimental Procedures. Scale bars, 200 μm.

(D) Aortic root lesion area was quantified in 20-week-old male C57BL/6J *Apoe*$^{-/-}$ mice from the indicated diet and DMB treatment groups. Data are presented as mean ± SE. p values shown were calculated by ANOVA. See also Figures S4 and S5.

TMAO levels not only via inhibition in microbial TMA production but also by potentially inducing reorganization of the gut microbial community. Cecal microbial compositions were analyzed in both male and female mice in the atherosclerosis studies by sequencing 16S rRNA gene amplicons in the various *Apoe*$^{-/-}$ mice groups. Global analyses of community structure showed differences between the chow-fed (0.07% choline) versus high-choline-diet-fed (1.0%) groups, which became more pronounced in the presence of DMB (Figures 5A and 5C). Further examination revealed that the proportions of several taxa within all groups of mice within each gender were associated with aortic root lesion area and plasma TMA and TMAO levels to some extent (Tables S3 and S4). For example, among male mice, proportions of the taxon *Clostridiaceae* were highly positively correlated with plasma TMA and TMAO levels, as well as with atherosclerotic lesion area (Table S3). Furthermore, male

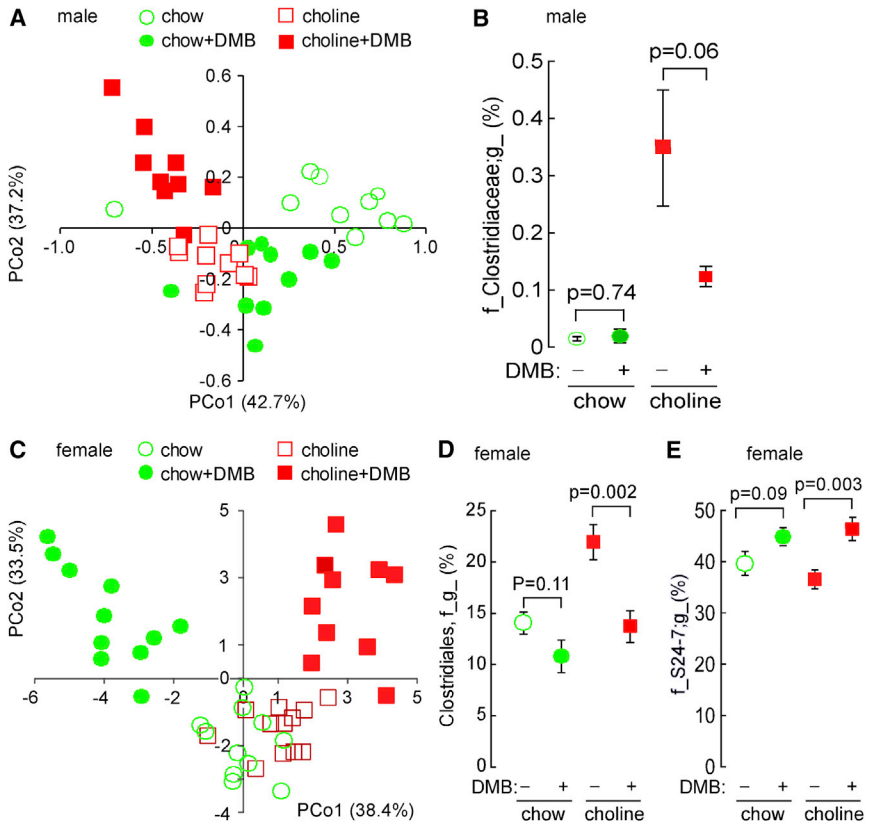

Figure 5. DMB Alters Gut Microbial Composition

(A–E) Unweighted UniFrac distances plotted in principal-component analysis (PCoA) space comparing cecal microbial communities in male (A) or female (C) C57BL/6J *Apoe*$^{-/-}$ mice fed chow versus choline-supplemented diet in the presence versus absence of DMB. Each data point represents a sample from a distinct mouse projected onto the first two principal coordinates (percent variation explained by each principal component [PCo] is shown in parentheses). (B, D, and E) Impact of diet and DMB on the proportion of several taxa.

Data are presented as mean ± SE ($n \geq 9$ mice per group). See also Tables S3 and S4.

mice placed on a choline-supplemented diet showed a significant increase in the cecal proportion of this taxon, and exposure to DMB promoted a strong tendency ($p = 0.06$) to decrease the proportion of this taxon in mice fed a choline-supplemented diet (Figure 5B).

For female mice, the order *Clostridiales* and the genus *Ruminococcus* were each highly positively correlated with both plasma TMA and TMAO levels, as well as atherosclerotic lesion area (Table S4). Similarly, the prevalence of the taxon *Lachnospiraceae* was highly associated with TMA and TMAO levels and showed a trend toward increased aortic root plaque area. It is also interesting to note that the proportion of the taxon *Clostridiales*, the most abundant bacterial order observed in cecum, was positively correlated to plasma TMA levels ($r = 0.35$, $p = 0.03$), and many *Clostridiales* species have been reported to cleave choline (Möller et al., 1986). Furthermore, female mice placed on a choline-supplemented diet showed a significant increase in the proportions of this taxon, and exposure to DMB induced a significant decrease in the proportions of this taxon (Figure 5D; Table S4). In contrast, proportions of *S24-7*, an abundant family from *Bacterioidetes*, showed significant inverse associations with both atherosclerotic plaque area and TMA levels and a trend toward reduced plasma TMAO (Figure 5E; Table S4). Indeed, within the female mice, examination of the impact of DMB on multiple taxa associated with TMA or TMAO levels and atherosclerotic plaque extent indicates that provision of the agent induced a reduction in the levels of many bacterial taxa positively associated with levels of TMA, TMAO, or aortic root lesion area and, conversely, a rise in the proportions of many of the bacterial taxa negatively associated with TMA, TMAO, or aortic lesion area (Table S4).

DISCUSSION

Recent advances identify a pivotal role for gut microbiota in multiple phenotypes associated with cardiometabolic diseases. A causal role for the involvement of gut microbes is, in part, based on the fulfillment of one of Koch's postulates: namely, the transmission of a disease-related phenotype with the transplantation of gut microbial organisms to a new host (Cho and Blaser, 2012; Cox et al., 2014; Dumas et al., 2006; Gregory et al., 2015; Koch, 1880; Sandhu and Chase, 1986). The recognition of gut microbes as participants in cardiovascular and metabolic disease pathogenesis suggests that pharmacological interventions aimed at "drugging the microbiome" may potentially serve as a therapeutic approach for the treatment of cardiometabolic diseases. Toward this end, in the absence of knowledge of specific microbial enzyme activities or their metabolites mechanistically linked to disease phenotypes, therapeutic approaches (aside from dietary restrictions) have, thus far, instead focused on altering gut microbial community composition through the use of either defined microbial compositions (probiotics) or non-microbial substances that may secondarily foster alteration in microbial community structure (prebiotics) (Petschow et al., 2013). Multiple lines of evidence show that the meta-organismal pathway involved in TMAO production is mechanistically linked to atherosclerosis susceptibility (Bennett et al., 2013; Gregory et al., 2015; Koeth et al., 2013; Wang et al., 2011). However, the effect of small-molecule antagonists for the critical initiating microbial TMA lyase step on atherosclerosis development has not yet been reported.

The present studies provide proof of concept for the idea that inhibiting microbial TMA production, through microbial TMA lyase inhibition, may serve as a potential therapeutic approach for the prevention or treatment of atherosclerosis (Figure 6). DMB, a structural analog of choline, served as a preliminary

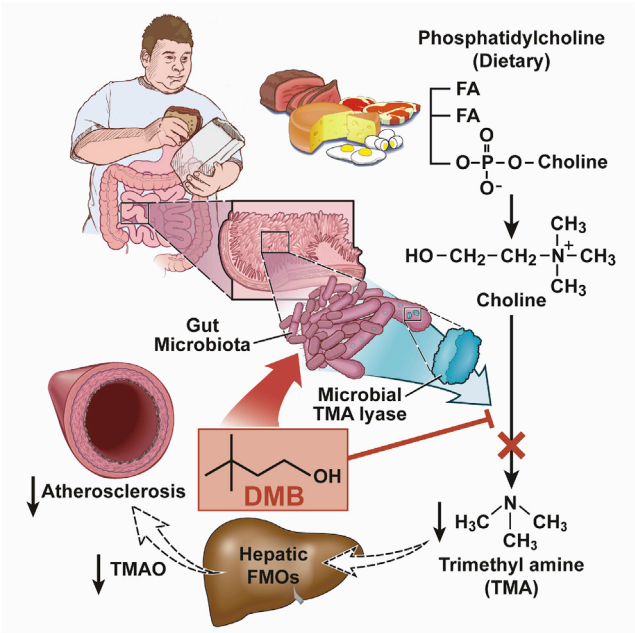

Figure 6. Schema Showing Use of DMB to Inhibit Gut Microbial TMA Production for the Treatment of Atherosclerosis

DMB is a structural analog of choline and an inhibitor of microbial TMA production (a choline TMA lyase inhibitor). Provision of DMB in the drinking water of atherosclerosis-prone *Apoe*$^{-/-}$ mice inhibits choline-diet-dependent enhancement in TMAO, endogenous macrophage foam cell formation, and atherosclerosis development.

tool compound to inhibit microbe-dependent TMA and host TMAO formation from multiple distinct TMA-containing nutrients in vitro and in vivo. Moreover, DMB provision, despite no significant effects on circulating cholesterol, choline, and other pro-atherogenic risk factors, inhibited choline-diet-dependent accumulation of both macrophage cholesteryl ester accumulation (foam cell formation) and aortic root atherosclerotic plaque development. In addition to attenuating plasma levels of TMAO, DMB promoted reduction in proportions of some microbial taxa that are associated with plasma TMA and TMAO levels, as well as aortic plaque extent. While the impact of DMB on microbial composition was noticeable, and while one might anticipate that there is less selective pressure for development of resistance against a non-lethal enzyme inhibitor compared to an antibiotic, the fact that there is any change in microbial composition induced by DMB suggests that some degree of selective pressure is occurring with exposure to the agent and thus raises the possibility for the development of resistance.

Gut microbes serve as a filter for our largest environmental exposure—what we eat. They also function, fundamentally, as a dynamic endocrine organ, generating biologically active metabolites in response to specific nutrient inputs that can enter the circulation and elicit biological effects at distant sites within the host (Brown and Hazen, 2015). The present studies suggest that the development of selective, non-lethal microbial enzyme inhibitors that target causative pathways, once they are revealed, may serve as an effective and complementary strategy to current approaches for the prevention and treatment of cardiometabolic diseases.

EXPERIMENTAL PROCEDURES

More complete experimental procedures are provided in the Supplemental Experimental Procedures.

General Procedures and Reagents

Reagents were purchased from Sigma unless otherwise stated. Routine blood tests were measured on a Roche Cobas Analyzer. Insulin was measured by ELISA with the Mouse Insulin ELISA Kit (Mercodia). Protein concentration was determined by bicinchoninic acid (BCA) assay. Hepatic FMO activity was quantified using liver homogenate and d9-TMA substrate, monitoring NADPH-dependent production of d9-TMAO, as described previously (Bennett et al., 2013).

Ethical Considerations

All animal model studies were approved by the Institutional Animal Care and Use Committee at the Cleveland Clinic. All study protocols and informed consent for human subjects were approved by the Cleveland Clinic Institutional Review Board. Informed consents were obtained for all subject samples.

Chemical Syntheses

P-choline was synthesized by reaction of trimethylphosphine and chloroethyl alcohol, using a modification of methods described previously (Renshaw and Bishop, 1938). d9-PC, d9-γ-butyrobetaine, d9-crotonobetaine, and d9-carnitine were synthesized as previously described (Koeth et al., 2013, 2014; Tang et al., 2013; Wang et al., 2011). d9-GPC was synthesized by reacting glycerophosphoethanolamine (CordenPharma) with CD_3I and purified by silica gel column chromatography (Koeth et al., 2013). Structural characterization, including high-resolution mass spectrometric analyses of all synthetic material, can be found in the Supplemental Experimental Procedures and Figure S6.

Mass Spectrometry Quantification of Analytes

Stable isotope dilution liquid chromatography with on-line tandem mass spectrometry (LC-MS/MS) was used for the quantification of plasma and tissue TMAO, choline, and betaine as previously described (Wang et al., 2011, 2014a). Samples used for TMA quantification were acidified prior to storage, and d9-TMA was used as an internal standard for quantification by LC/MS/MS. Liver cholesterol was quantified by gas chromatography/mass spectrometry (GC/MS) (Wang et al., 2011). DMB was quantified by GC/MS from plasma/urine after extraction with hexane. Cellular uptake of d9-choline was quantified from washed intact bacteria following freeze-thaw cycle-induced lysis by LC/MS/MS using [1,1,2,2-d4]choline as an internal standard. Complete details can be found in the Supplemental Experimental Procedures.

Determination of TMA Lyase Activity

TMA lyase activity was typically quantified by incubating (for 10–16 hr) the indicated enzyme source (intact cell [optical density at 600 nm, or $OD_{600\ nm}$, ~1.0], cell lysate [3 mg protein], or purified protein [30 μg]) with the indicated co-factors in the presence or absence of DMB (2 mM), unless otherwise stated. Reactions were initiated by the addition of d9-labeled substrate (100 μM, unless otherwise indicated) in gas-tight reaction vials at 37°C. d9-TMA production was quantified by LC/MS/MS. Complete details can be found in the Supplemental Experimental Procedures.

Cloning and Expression of *cutC/cutD*, *cntA/cntB*, and *yeaW/yeaX*

P. mirabilis (ATCC 29906) whole-genome sequence, contig00092 (ACLE01000068.1), was used to acquire the nucleotide sequence of *cutC* (locus tag: HMPREF0693_2863), *cutD* (locus tag: HMPREF0693_2862), and the 104 bases of intergenic DNA between *cutC* and *cutD*. The open reading frames (ORFs) for *cutC/D* were codon optimized, synthesized contiguously by GenScript (http://www.genscript.com), and then cloned into pBAD (Invitrogen) and transformed into *E. coli* Top10 cells (Invitrogen). For expression of amino-terminal 8x-His-tagged CutC and carboxy-terminal Strep-Tag

II-tagged CutD, the codon-optimized *cutC*-intergenic region-*cutD* from *P. mirabilis* was PCR amplified and cloned in-frame into the plasmid pGK::nucMCS that was isolated from *L. lactis* (ATCC 8731) behind an N-terminal 8x-His tag and in front of a Strep-tag II affinity tag. Sequence-verified clones were transformed into *E. coli* Stellar cells (Clontech). The ORFs for *Desulfovibrio alaskensis* G20 *cutC/D* genes (Ddes_3282 and Ddes_1357, respectively) and their associated intergenic region were codon optimized for expression in *E. coli* and synthesized by GenScript, for cloning into pUC57. Constitutive expression of the *D. alaskensis* G20 CutC/D proteins in *E. coli* Top10 cells (Invitrogen) was performed using the synthetic *em7* constitutive bacterial promoter with ribosome-binding site, as described in the Supplemental Experimental Procedures. The genes for *yeaW/yeaX* were cloned from *E. coli* K-12 DH10B genomic DNA by PCR, inserted into a modified pET22 vector backbone, and expressed in *E. coli* BL21(DE3) (Invitrogen) (Koeth et al., 2014). For expression of 8x-His-tagged YeaW and 8x-His-tagged YeaX proteins, the relevant genes were cloned in-frame behind an 8x-His-tag in *E. coli* BL21(DE3). *CntA/cntB* genes from *Acinetobacter baumannii* (ATCC 19606) (Zhu et al., 2014) were codon optimized and synthesized (GenScript) and then cloned into pUC57, inserted separately into a modified pET22 vector backbone, and expressed as un-tagged proteins in *E. coli* BL21(DE3), as described in Supplemental Experimental Procedures. The catalytically inactive C489A and G821A mutants in *D. alaskensis* G20 CutC (Craciun and Balskus, 2012), C781A and G1117A mutants of *P. mirabilis* (ATCC 29906) CutC, E205D mutant of cntA *Acinetobacter baumannii* (ATCC 19606) (Zhu et al., 2014), and E208D mutant of *E. coli* K-12 DH10B YeaW were all made by site-directed mutagenesis, as described in the Supplemental Experimental Procedures.

Growth and Induction Conditions for Recombinant TMA Lyases

Recombinant clones transformed in *E. coli* Top10 and BL21(DE3) were grown in Luria broth (LB) medium with 100 μg/ml ampicillin, and arabinose or isopropyl β-D-1-thiogalactopyranoside (IPTG) was added at a final concentration of 0.2% or 0.4 mM for induction of TMA lyase subunits constructed in pBAD or pET vectors, respectively. The *cutC/D* recombinant clones in the pGK or pUC57 vectors were grown in LB medium with erythromycin (250 μg/ml) or ampicillin (100 μg/ml), respectively, at 37°C overnight to constitutively express the targeted TMA lyase subunit. Further details can be found in the Supplemental Experimental Procedures.

Mouse Models

For atherosclerosis and endogenous foam cell formation studies, at the time of weaning (4 weeks of age), apolipoprotein E knockout mice (C57BL/6J *Apoe*$^{-/-}$; >40 generations backcrossed) were fed either a chemically defined standard chow (Harlan Teklad #2018) (total choline, 0.07%) or the same diet supplemented to 1.0% choline (Harlan Teklad #TD.07864) (total choline confirmed by LC/MS/MS). Where indicated, mice were also treated with DMB (1.0%, v/v) and/or L-carnitine (1.0%) added to the drinking water. Aortic lesion quantitation was performed as described previously (Baglione and Smith, 2006; Wang et al., 2011). Endogenous macrophage foam cell formation was assayed following peritoneal lavage collection 72 hr after 4% thioglycolate elicitation (Wang et al., 2007).

Bacterial, Mouse Cecum, and Human Feces TMA Lyase Sample Preparation

Mouse cecum from C57BL/6J *Apoe*$^{-/-}$ mice or human feces was gently homogenized, followed by centrifugation under argon atmosphere. The upper phase was collected followed by centrifugation under argon blanket. The bottom pellet was collected, resuspended, and used as intact bacterium cell suspensions under either nitrogen or argon atmosphere in gas-tight vials. Lysates of bacteria were generated either by French press using argon gas or by homogenization with 100-μm glass beads using a TissueLyser II (QIAGEN) kept within an argon- or a nitrogen-filled chamber.

Microbiota Studies

Bacterial DNA was isolated from mouse cecum, and 16S rRNA gene V4 hypervariable regions were amplified, pyrosequenced, assigned into different taxa, and analyzed as previously described (Gregory et al., 2015; Koeth et al., 2013). We conducted a principal-component analysis (PCA) using MarkerView Software (AB SCIEX) to compare the effects of different diets and DMB exposure on microbiota composition.

Statistical Analyses

We analyzed data using the Student's t test and ANOVA, followed by post hoc t test with Holm-Bonferroni correction, to compare differences in mean value between different groups for most studies, as indicated. Pearson's correlation was used to calculate the association between two variables after demonstration of normal distribution of data. For all statistical tests, $p < 0.05$ was considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2015.11.055.

AUTHOR CONTRIBUTIONS

Z.W. helped with the design, performance, and analyses of all studies and the drafting of the manuscript. A.B.R., J.A.D., X.G., Y.H., M.Z.-D., A.J.D., and X.F. assisted in gene cloning, protein purification, choline transport, and TMA lyase activity assay. J.A.B., W.Z., M.K.C., D.K., and J.E.H. assisted with animal model studies. B.S.L. assisted in chemical syntheses. E.O. and A.J.L. performed microbial composition analyses. S.L.H. conceived of the project idea and assisted in the design of experiments and data analyses and in the drafting of the manuscript. All authors critically reviewed the manuscript.

ACKNOWLEDGMENTS

We thank B. Sullivan and M. Wagner for technical assistance. This research was supported by NIH and Office of Dietary Supplements grants R01 HL103866, P20 HL113452, R01106000, and P01H28481. Z.W. and W.Z. were partially supported by American Heart Association Scientist Development grants. S.L.H. is also partially supported by a gift from the Leonard Krieger fund. MS studies were performed on instrumentation housed within a facility partially supported by a Center of Innovation Award by AB SCIEX.

Z.W., B.S.L., and S.L.H. are named as co-inventors on patents held by the Cleveland Clinic relating to cardiovascular diagnostics and/or therapeutics. S.L.H. reports having been paid as a consultant for the following companies: Esperion and Procter & Gamble. S.L.H. reports receiving research funds or support from Astra Zeneca, Pfizer, Procter & Gamble, Roche, and Takeda. Z.W., B.S.L., and S.L.H. report having the right to receive royalty payments for inventions or discoveries related to cardiovascular diagnostics and/or therapeutics from the Cleveland Heart Laboratory, and S.L.H. also from the following companies: Siemens, Esperion, and Frantz Biomarkers, LLC.

Received: October 20, 2015
Revised: November 19, 2015
Accepted: November 23, 2015
Published: December 17, 2015

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