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ORIGINAL RESEARCH

Effect of Varying Quantities of Lean Beef as Part of a Mediterranean-Style Dietary Pattern on Gut Microbiota and Plasma, Fecal, and Urinary Metabolites: A Randomized Crossover Controlled Feeding Trial

Zachary S. DiMattia, BS; Jingcheng Zhao, PhD; Fuhua Hao, PhD; Sergei Koshkin, PhD; Jordan E. Bisanz, PhD; Andrew D. Patterson, PhD; Jennifer A. Fleming, PhD, RD; Penny M. Kris-Etherton, PhD, RD; Kristina S. Petersen, PhD

BACKGROUND: Consumption of trimethylamine *N*-oxide (TMAO) precursors, such as carnitine found in lean beef, raises circulating TMAO concentrations; however, a healthy dietary pattern may attenuate these effects.

METHODS: This randomized, 4-period crossover, controlled-feeding study investigated the effects of Mediterranean-style (MED) diets (carbohydrate 42%, protein 17%, fat 41%) with 14 (MED0.5; 0.5 oz), 71 (MED2.5; 2.5 oz), and 156 (MED5.5; 5.5 oz) g/day/2000 kcal of lean beef, compared with an average American diet (AAD; carbohydrate 52%, protein 15%, fat 33%; 71 g/day/2000 kcal beef), on gut microbiota composition and plasma, urinary, and fecal metabolites including TMAO and precursor molecules. Thirty generally healthy individuals consumed each diet for 4 weeks with a ≥1-week washout. Fasting blood samples, 24-hour urine samples, and fecal samples were collected at baseline and at the end of each 4-week diet period. Metabolites were measured by proton nuclear magnetic resonance and liquid chromatography/mass spectrometry. Gut microbiota composition was measured using amplicon sequencing of the 16S rRNA gene.

RESULTS: The 3 MED diets increased gut microbiota diversity compared with the AAD. Plasma TMAO was higher following the AAD compared with the MED0.5 (mean fold difference, 1.78 [95% CI, 1.05–3.06]) and MED2.5 (2.04 [95% CI, 1.18–3.52]). Urinary TMAO was higher following the AAD compared with the MED0.5 (1.88 [95% CI, 1.19–2.97]), MED2.5 (2.15 [95% CI, 1.37– 3.39]), and MED5.5 (1.76 [95% CI, 1.12–2.77]).

CONCLUSIONS: Compared with an AAD, inclusion of up to 71 g/day of lean beef in a Mediterranean-style diet increased gut microbiota diversity and lowered TMAO concentrations in healthy adults.

REGISTRATION: URL: https://clinicaltrials.gov; Unique identifier: NCT02723617.

Key Words: cardiovascular disease ■ lean beef ■ Mediterranean diet ■ microbiome ■ trimethylamine N-oxide

Correspondence to: Kristina S. Petersen, Department of Nutritional Sciences, 110 Chandlee Laboratory, Pennsylvania State University, University Park, PA 16802, USA. Email: kup63@psu.edu

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CLINICAL PERSPECTIVE

What Is New?

- In a controlled feeding trial, Mediterranean-style diets containing 14 and 71 g/day/2000 kcal of lean beef lowered plasma and urinary trimethylamine *N*-oxide concentrations compared with an average American diet with 71 g/day/2000 kcal of beef; however, plasma trimethylamine *N*-oxide concentrations remained well below the clinical threshold for increased cardiovascular disease risk across all diets.
- Gut microbiota diversity increased after the Mediterranean-style diets and was not affected by beef dose, whereas substantial interindividual differences in trimethylamine *N*-oxide concentrations were observed, regardless of diet.

What Are the Clinical Implications?

- In healthy individuals, moderate lean beef consumption as part of a Mediterranean-style does not elevate trimethylamine *N*-oxide to concentrations considered clinically relevant for cardiovascular disease risk.

Nonstandard Abbreviations and Acronyms

^{1}H NMR	proton nuclear magnetic resonance
AAD	average American diet containing 71 g (2.5 oz) lean beef/day/2000 kcal
ASV	amplicon sequence variant
LC/MS	liquid chromatography/mass spectrometry
MED	Mediterranean-style
MED0.5	Mediterranean-style diet with 14 g (0.5 oz) lean beef/day/2000 kcal
MED2.5	Mediterranean-style diet with 71 g (2.5 oz) lean beef/day/2000 kcal
MED5.5	Mediterranean-style diet with 156 g (5.5 oz) beef/day/2000 kcal
PERMANOVA	permutational multivariate analysis of variance
PLS-DA	partial least squares discriminant analysis
TMA	trimethylamine
TMAO	trimethylamine *N*-oxide

Epidemiological evidence shows that higher red meat consumption is associated with increased risk of cardiovascular disease (CVD).[1,2] However, establishing causality from observational data is challenging because of the potential for residual confounding[3] and measurement errors inherent to commonly used self-reported diet assessment methods.[4] Evidence from clinical trials shows that lean, unprocessed red meat can be included as a part of heart-healthy dietary patterns without adversely impacting traditional CVD risk factors.[5,6] Nonetheless, concerns remain about the novel CVD risk factor, trimethylamine *N*-oxide (TMAO), which is produced during the metabolism of animal foods and is implicated in atherosclerotic plaque formation.[7,8] Although an association between beef intake and increased TMAO production has been documented,[9] isolating the specific effects of beef consumption from those of the overall dietary pattern remains difficult. Only 1 clinical trial has examined TMAO response to a heart-healthy dietary pattern high in beef,[10] which demonstrated that a Mediterranean-style (MED) diet with a higher dose of red meat (500 g/week; ~18 oz-eq/week) increased circulating TMAO compared with a lower dose (200 g/week; ~7 oz-eq/week). Within the framework of the Dietary Guidelines for Americans, the recommended intake of meats, poultry, and eggs for a 2000-kcal diet is 26 oz-eq per week.[11] Further research is needed to clarify how different levels of beef consumption influence TMAO production within heart-healthy dietary patterns and how the effects of healthy versus less-healthy dietary patterns on TMAO differ when beef intake is held constant.

TMAO is a gut microbiota-dependent metabolite of dietary choline, carnitine, betaine, and phosphatidylcholine-rich animal foods, such as red meat, eggs, fish, and poultry.[7,8] These precursor molecules are metabolized by gut microbiota into trimethylamine, which is further oxidized to TMAO in the liver.[7,8] Animal experiments and in vitro studies suggest that TMAO contributes to atherosclerotic CVD by promoting foam cell formation, vascular inflammation, endothelial dysfunction, and decreasing reverse cholesterol transport.[12–14] In alignment, prospective cohort studies show higher plasma TMAO concentrations are associated with a higher risk of atherosclerotic CVD events and mortality.[7,15,16] However, diet quality may modify the association between TMAO and CVD risk. A prospective nested case–control analysis of the Nurses' Health Study cohort demonstrated that high diet quality attenuated the association between TMAO and coronary heart disease risk, whereas low diet quality strengthened the association.[16] In this analysis, diet quality scores were generally higher with lower intakes of animal products; therefore, the cardiovascular implications of higher quality diets containing animal products remain unclear. Additionally large interindividual variability in plasma TMAO concentrations is observed in response to dietary precursors.[14,17] This variability is partially mediated by the gut microbiota, as trimethylamine production relies on the action of microbes that may be altered (ie, type and amount) based on the overall dietary

pattern.[18,19] A cross-sectional international pooled analysis (*n*=32 166) demonstrated that dietary components typical of healthy dietary patterns, such as nuts and plant protein, were inversely associated with circulating TMAO, potentially because of modulation of the gut microbiome.[19]

This paper presents a post hoc exploratory analysis of a controlled-feeding, randomized crossover trial that assessed the effects of a Mediterranean diet containing 0.5 (MED0.5), 2.5 (MED2.5), or 5.5 (MED5.5) oz/day/2000 kcal of lean beef compared with an average American diet (AAD) with 2.5 oz/day/2000 kcal of beef.[6] The primary findings of the trial indicated that ≤2.5 oz/day/2000 kcal of lean beef can be included as part of a healthy MED dietary pattern without compromising its beneficial effects on lipids and lipoproteins. This exploratory analysis aimed to examine the effect of consuming varying amounts of lean beef (14, 71, 156 g/day/2000 kcal [0.5, 2.5, 5.5 oz/day/2000 kcal]) as part of a healthy MED diet on the gut microbiota and plasma, urine, and fecal metabolites compared with an AAD containing 71 g (2.5 oz) beef/day/2000 kcal. It was hypothesized that the MED diets would increase microbial diversity compared with the AAD. In addition, it was hypothesized that TMAO concentrations would be lower following the MED diets compared with the AAD because of changes in gut microbiota composition in response to the overall healthy dietary pattern. This study will contribute to understanding the diet-related modulation of TMAO and the influence of the gut microbiota composition on interindividual variability in TMAO production.

METHODS

Study Design

Data described in the article, code book, and analytic code will be made available upon request pending application and approval to the corresponding author. A full description of the methods and results for the prespecified primary and secondary outcomes have been published previously.[6] Briefly, a 4-period, randomized, crossover, controlled-feeding study was conducted at the Pennsylvania State University and the US Department of Agriculture Beltsville Human Nutrition Research Center. A sample size of 60 participants (*n*=30 per site) was determined based on low-density lipoprotein-cholesterol, the prespecified primary outcome.[6] Only samples from the Penn State site were used for analyses presented in this paper (*n*=30). Fecal microbiota were analyzed using 16S rRNA gene sequencing. Plasma, urine, and fecal metabolites were first analyzed using proton nuclear magnetic resonance (^{1}H NMR). TMAO and related precursor molecules were not detected in the plasma samples using ^{1}H NMR because of low concentrations (<3 μM) in most samples.[20] Therefore, plasma samples were analyzed with liquid chromatography/mass spectrometry (LC/MS) instead, which allowed detection of lower concentrations. Both untargeted and targeted LC/MS were used to obtain relative and absolute concentrations of TMAO-related metabolites, respectively. For all analyses, samples were collected at baseline and following each diet period. The Institutional Review Board at the Pennsylvania State University approved the study protocol before the initiation of the study. This trial is registered at clinicaltrials.gov as NCT02723617.

The parent study included 4 intervention diets: (1) a MED diet including 14 g (0.5 oz) lean beef/day/2000 kcal (MED0.5), (2) a MED diet including 71 g (2.5 oz) lean beef/day/2000 kcal (MED2.5), (3) a MED diet including 156 g (5.5 oz) lean beef/day/2000 kcal (MED5.5), (4) an AAD containing 71 g (2.5 oz) beef/day/2000 kcal. These controlled, weight-maintenance diets had a fixed macronutrient composition that varied only between the MED diets (41% fat, 42% carbohydrate, 17% protein) and the AAD (33% fat, 52% carbohydrate, and 15% protein). Lean beef, as used in this study, is defined by the US Department of Agriculture as <10 g fat/100 g beef, <4.5 g saturated fat/100 g beef, and <95 mg cholesterol/100 g beef.[21] The yogurt content of diets also differed such that the MED diets included Greek yogurt (Chobani) whereas the AAD included conventional yogurt (Dannon); these yogurts differ in their microbial composition. Participants received each diet for 4 weeks with a washout period of at least 1 week between diet periods. Study diets were prepared in the metabolic kitchen facility onsite and included provision of 3 meals and 2 snacks daily using a 7-day rotating menu for the complete duration of each 4-week intervention period. Menus were developed using FOOD PROCESSOR (ESHA Research) and the nutrient content of the diet was analyzed to verify the macronutrient composition and assure protocol accuracy. In brief, homogenized samples of each menu across 2 calorie levels were analyzed by Covance Laboratories, Inc. Chemical analysis of the nutrient composition of the test diets is presented in Table 1.

Research Participants

Participants were nonsmoking individuals with a body mass index 20 to 40 kg/m^2, aged 30 to 70 years, and recruited between October 2016 and November 2017 from the State College (PA) area. All participants provided written informed consent before screening and enrollment. Exclusion criteria included triglycerides >350 mg/dL; high-density lipoprotein cholesterol <15th percentile of US population (men <37 mg/dL, women <44 mg/dL); fasting glucose >126 mg/dL; blood pressure >160/100 mm Hg; or history of kidney disease, liver disease, gout, untreated or unstable hyper- or hypothyroidism, cancer, gastrointestinal

Table 1. Nutrient Targets and Chemical Analysis of Test Diets (Based on 2000 kcal/day) Prepared at Penn State University*

	MED diets nutrient targets	MED0.5	MED2.5	MED5.5	AAD nutrient targets	AAD
Protein, % E	17	19.7	19.6	18.8	15	17.5
Carbohydrate, % E	42	46.7	44.6	42.2	52	56.2
Fat, % E	41	40.8	44.7	43.1	33	34.0
Saturated FA, % E	8	6.5	7.1	7.8	12	10.0
Monosaturated FA, % E	26	24.0	25.0	22.7	13	11.9
Polyunsatuated FA, % E	8	7.4	7.2	6.8	8	6.0
α-linolenic acid, g	1.5	1.69	1.58	1.54	1.5	1.28
Marine n-3, g	0.5	0.32	0.28	0.27	<0.1	0.25
Cholesterol†, mg	<300				<300	
Sodium†, mg	<2300				~3500	
Fiber†, g		26	26	23		20
Beef†		14 g (0.5 oz)	71 g (2.5 oz)	156 g (5.5 oz)		~2.5 oz

AAD indicates average American diet; FA, fatty acid; MED, Mediterranean-style eating pattern used in the study; MED0.5, MED diet with 14 g (0.5 oz) per day of lean beef; MED2.5, MED diet with 71 g (2.5 oz) per day of lean beef; MED5.5, MED diet with 156 g (5.5 oz) per day of lean beef based on a 2000-kcal diet; and % E, percentage of total energy.

*On the basis of 2000 kcal/day. Average across a 7-day menu cycle. Values were determined by chemical analysis (Covance Laboratories, Inc.).

†Values were determined using FOOD PROCESSOR (ESHA Research).

disease, pancreatic disease, other metabolic diseases, malabsorption syndromes, or CVD. Use of cholesterol-lowering medication or refusal to discontinue intake of putative cholesterol-lowering supplements (psyllium, fish oil capsules, soy lecithin, niacin, fiber, flax, and phytoestrogens) were also exclusion criteria. To be eligible, discontinuation of the supplement for 2 to 4 weeks, depending on the supplement, before study enrollment was required. Individuals prescribed blood pressure-lowering medications were eligible if their screening blood pressure was <160/100 mm Hg on a stable medication dose for ≥6 months. Participants were randomly allocated to 1 of 12 diet sequences to ensure that diets were assigned in a balanced order. The block randomization code was generated by an independent US Department of Agriculture staff member using an orthogonal Latin-square design with 5 blocks (5 replicates) and 12 sequences per block. The participants were not blinded; however, the study coordinator, investigators, analysts, and statisticians were blinded for purposes of outcome assessment and statistical analysis. Compliance was monitored based on daily and weekly questionnaires asking about the consumption of study and nonstudy foods and beverages along with daily weigh-ins. Compliance was high (>90%) as previously reported.[6]

Sample Collection

Blood samples were collected in the clinic on 2 consecutive days at baseline (start of phase 1) and the end of each diet period. Participants were instructed to refrain from alcohol consumption and use of anti-inflammatory medications for 48 hours before each collection. Participants were asked not to engage in vigorous exercise for 24 hours before each collection and not consume any food or drink except water for 12 hours before their visit. ETDA plasma samples were aliquoted and stored at −80 °C until the time of analysis. For these analyses, samples collected on 1 day at each time point were used. Fecal samples were collected by participants at home at baseline and the end of each diet period. Participants were given a fecal sample collection kit that included a 30 mL Para-Pak Clean Vial (Meridian Bioscience, Cincinnati, OH), a stool collection hat, a long-handled spoon, a cooler, Ziploc bags, icepacks, and nonlatex gloves. Participants were instructed to store the sample in a freezer and return it the next day in the cooler with ice packs. Fecal samples were stored and remained frozen at −80 °C until the time of analysis. No preservative was added to the collected fecal samples. Twenty-four hour urine samples were collected by participants at home at baseline and the end of each diet period. Participants were provided with a 24-hour urine collection kit and detailed instructions for how to collect the sample. Participants were instructed to collect all their urine from immediately following the day 1 clinic visit through to their day 2 clinic visit. The urine collection container was provided in a cooler with ice packs. No preservative was added to the collected urine samples. Samples were aliquoted and stored at −80 °C until the time of analysis.

Microbiota Analysis

DNA was extracted from fecal aliquots using the ZymoBIOMICS 96 MagBead DNA Kit (Zymo D4308)

according to the manufacturer's protocol. According to a well-established amplicon library preparation protocol (https://github.com/BisanzLab/AmpliconSeq), samples were amplified using primers 515F (GTGYCAGCMGCCGCGGTAA) and 806R (GGACTACNVGGGTWTCTAAT) with partial overhangs for i7 and i5 adapters targeting the V4 region of the 16S rRNA gene. A series of 10x dilutions were used to acquire a late-exponential phase amplification for indexing. All polymerase chain reactions were carried out using KAPA HiFi hot start enzyme. Indexing polymerase chain reaction was performed by diluting the products and limited amplification with unique forward and reverse barcodes (12nt). After determining the concentration of the indexing product by Picogreen dye (Life Technologies), samples were pooled at equimolar ratios before size selection with Ampure XP beads. The pooled library was sequenced using MiSeq V3 600 cycle reagents at the Penn State Genomics Core Facility in 1 batch. QIIME2 v2023.5 and Dada2 v1.26.0 were used to demultiplex and denoise the data, respectively. Taxonomy assignments were performed based on a SILVA 138 database with a 99% similarity clustering via the Qiime2 classifier, followed by the establishment of the phylogenetic tree by using the Qiime2 phylogeny align-to-tree-mafft-fasttree pipline. Negative controls included extraction blanks that failed to generate products, but were still pooled at the maximum available reaction volume. These contained <300 reads whereas the number of reads in all samples was >10 000. Therefore, all samples were included for the analysis of alpha diversity, beta diversity, and differential abundance.

Alpha diversity was evaluated by using observed amplicon sequence variants (ASVs) (picante v1.8.284), Shannon's diversity (vegan v2.6-6.1), and phylogenetic diversity (picante v1.8.284) metrics. Beta diversity was estimated using the Bray-Curtis (vegan v2.6-6.1), centered log-ratio (CLR) Euclidean, that is, Aitchison distance (make_clr function in qiime2R v0.99.685), Jensen–Shannon divergence (phyloseq_1.44.0), phylogenetic isometric log-ratio Euclidean (philr v1.26.0), weighted UniFrac (unifrac function in rbiom v1.0.3), and unweighted UniFrac (unifrac function in rbiom v1.0.3) metrics. The sequencing data are available at National Center for Biotechnology Information Sequence Read Archive under accession PRJNA1199414. Functional potential of the gut microbiota was inferred using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) v 2.4.2.[22] Predicted metagenomes were generated based on 16S rRNA ASV abundances, with functional annotations assigned to Kyoto Encyclopedia of Genes and Genomes Orthology gene families and metabolic pathways.

Metabolomics Analysis

Untargeted metabolites in urinary, fecal, and plasma samples were first quantified using ^{1}H NMR-based metabolomics, following previously described methods[23] with modifications. Plasma ^{1}H NMR sample preparation is not described here, as results from plasma ^{1}H NMR samples are not presented. Instead, LC/MS was used for plasma analysis due to its superior detection limit. Approximately 50 mg of wet fecal samples were extracted using 1.2 mL of PBS (0.1 M, 50% D_2O, pH 7.4) containing 0.005% (w/v) of TSP as an internal standard. Following homogenization, freeze-thawing, and centrifugation steps, 550 μL of supernatant was transferred into 5 mm ^{1}H NMR tubes for ^{1}H NMR analysis. Then, 500 μL of urine samples were mixed with 14 μL of KF (5 M) in a 1.7 mL EP tube, after vortex and centrifugation, 450 μL of supernatant was transferred to 5 mm ^{1}H NMR tubes with preadded EDTA-d12. After adding 45 μL of PBS (1.5 M, 100% D2O) with 0.01% (w/v) of TSP as an internal standard, the samples were used for ^{1}H NMR analysis. The ^{1}H spectra of extracts were acquired at 298 K using a Bruker Avance NEO 600 MHz spectrometer equipped with a SampleJet sample changer (Bruker Biospin, Rheinstetten, Germany) in 1 batch. The noesygppr1d pulse sequence was used for recording ^{1}H 1D NMR experiments with presaturation water suppression during relaxation and mixing time. All ^{1}H NMR spectra were processed automatically with Chenomx NMR Suite (Chenomx Inc, Edmonton, Alberta, Canada, version 10), then each spectrum was checked and adjusted manually for phase and baseline. Metabolites were identified and quantified using the Chenomx software's built-in metabolite library and fitting algorithm, compared with the concentration of the internal standard (TSP).

Plasma samples were prepared for LC/MS by diluting them 1:3 with ice-cold methanol containing 1 μM chlorpropamide, followed by vortexing, incubating at –20 °C for 1 hour, and centrifuging at 14 000 *g* for 15 minutes at 4 °C. Supernatants were transferred to LC vials for analysis. For untargeted metabolite analysis, samples were analyzed on a Nexera 40 high-performance liquid chromatography system (Shimadzu) coupled to a ZenoTOF 7600 mass spectrometer (Sciex) in 1 batch with an electrospray ionization source in positive ion mode. Chromatographic separation was performed on a Waters Acquity BEH C18 column (2.1 × 150 mm, 1.7 μm) at 45 °C, with a mobile phase flow rate of 0.35 mL/min. The mobile phases consisted of water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B), with a gradient program: 0 to 1 minutes, 0% B; 10 to 15 minutes, 90% B; 16 to 20 minutes, 0% B. Sample injection volume was 5 μL. MS1 data were acquired in a 50 to 1000 m/z range with 50 msec accumulation time,

and MS2 data were collected in HRMRM mode for choline, betaine, carnitine, trimethylamine, and TMAO, using collision energies of 30 to 40 eV and specific m/z ranges for each compound. MS-DIAL (v5.1) was used for feature identification based on in-house and public reference libraries. For targeted metabolite quantification, samples were analyzed on the same high-performance liquid chromatography system coupled to a QTrap 6500+ mass spectrometer (Sciex) in 1 batch with similar chromatographic separation parameters, except for a flow rate of 0.30 mL/min. MS data were acquired in multiple reaction monitoring mode with the following transitions: 104.1→60.1 (choline), 118.1→59.1 (betaine), 162.1→103.1 (L-carnitine), 151.1→58.1 (TMAO), and 277.04→192.1 (chlorpropamide). Declustering potential was set to 60 V, with an ion spray voltage of 5500 V, curtain gas at 35 psi, nebulizer gas at 50 psi, heater gas at 70 psi, and a heater temperature of 600 °C. Sciex OS software was used for quantification. TMAO values below the lower limit of detection (0.1 μM) were set at 0.05 μM per EPA (Environmental Protection Agency) guidelines, as fewer than 15% of samples were below this threshold.[24] Both targeted and untargeted samples were analyzed in duplicate, with final values averaged.

Statistical Analysis

Statistical analyses were performed using R (v 4.4.0) primarily with packages vegan v2.6-6.1, Qiime2 v2023.5, lme4 v1.1.35.4, lmertest v3.1.3, and emmeans v1.10.2.

Microbiota Data

Microbiota data were prepared by checking for missing values, performing necessary cleaning, and normalizing data for statistical models. For alpha diversity calculations and for Bray–Curtis and UniFrac distance calculations, the data were rarefied to the sequencing depth of the sample with the lowest read count using subsampling with replacement. For differential abundance analysis, microbiota data were transformed using the CLR transformation with the count zero multiplicative method of zero replacement.[25] Linear mixed-effects models were fitted to assess diet effects on alpha diversity, beta diversity indices, and differential abundance of individual microbes, adjusting for phase, baseline values, and sex; significance of diet effects was evaluated using Type III ANOVA *F*-tests. All linear model outputs were visually inspected to ensure the assumptions of linearity, homogeneity of variance, and normality of residuals were met. To further assess the linear mixed model assumptions, a sensitivity analysis was performed using permutation testing. Models with significant diet effects were subjected to permutation testing in which the diet was permuted within subjects 1000 times in reduced models excluding covariates. Empirical *P* values were calculated from the resulting null distribution of *F*-statistics using the Phipson and Smyth correction.[26] All permutation tests yielded empirical *P* values <0.05, indicating that the observed diet effects were unlikely to arise by chance and supporting the reliability of the findings. Alpha diversity models and differential abundance models were adjusted for phase, baseline value, and sex; and beta diversity change-from-baseline models were adjusted for phase and sex. First-order carryover effects were tested in alpha and beta diversity models by adding a carryover variable and checking for main effect *P* values <0.05; carryover was not included in the final models because no significant carryover effects were observed. Diet was included as a fixed effect and participant was included as a random effect in all models. Sex by diet interactions were tested to evaluate sex differences in diet response.

For differential abundance analyses, diet main effect *P* values were corrected for multiple comparisons using the Benjamini–Hochberg procedure, with a false discovery rate (FDR) threshold of <0.1 as significant. For alpha and beta diversity indices, a main effect of diet was considered significant using an alpha of 0.05. When a main effect of diet was detected for any model, post hoc pairwise testing was performed and adjusted for multiple comparisons with the Tukey–Kramer method, using an alpha of 0.05 as significant. Least square means, SEs, and differences between diets with 95% CIs were calculated from linear mixed-effects models. To assess directionality of change in microbiota composition, change in principal coordinate 1 (PC1) from baseline was calculated for each diet using the CLR Euclidean distance matrix. Principal coordinates analysis was performed on the full CLR Euclidean distance matrix using pcoa (ape v5.8-1), embedding all samples in a shared ordination space. PC1 values were extracted, and within each subject, the baseline PC1 value was subtracted from the PC1 value for each diet condition. Sensitivity analyses tested the influence of specific yogurt probiotic microbes with significant differential abundance between diets by recalculating diversity indices after excluding these microbes and comparing the results to the full data set.

Metabolomics Data

Metabolomics data were first cleaned and assessed for missing values. Distributions of metabolite concentrations were evaluated visually for skewness, which is common in metabolomics data sets. To reduce skew and improve model performance, metabolite values were transformed using natural logarithmic (log) or square root transformations, as appropriate. For log transformations, a small constant equal to the smallest nonzero value for that metabolite was added to accommodate zero values. All metabolite concentrations were back-transformed to their original scale following analysis. Urinary metabolites were normalized to

creatinine concentrations to account for water content by dividing metabolite concentrations by creatinine concentration before transformation in each sample. Linear mixed-effects models were fitted to assess diet effects on plasma, fecal, and urinary metabolites, adjusting for phase, baseline values, and sex; significance of diet effects was evaluated using Type III ANOVA F-tests. Model assumptions, sex by diet interactions, and carryover effects were assessed as described for the microbiota data. Diet was included as a fixed effect and participant was included as a random effect in all models. For exploratory analyses of entire metabolite data sets, main diet effect *P* values were corrected for multiple comparisons using the Benjamini–Hochberg procedure, with an FDR threshold of <0.1 as significant. For targeted TMAO-related metabolites, diet main effects were considered significant at $P<0.05$. When a main effect of diet was detected in any model, post hoc pairwise testing was conducted with correction for multiple comparisons using the Tukey–Kramer method, with an alpha of 0.05. Fold differences between diets were reported for log-transformed metabolites to accurately reflect the multiplicative relationships in the log-transformed scale before back-transformation.

Microbiota and Metabolomics Profiles

To evaluate the overall differences in metabolomic and microbiota profiles between diets, Euclidean distances were calculated for samples using vegdist (vegan v2.6-6.1). A permutational multivariate analysis of variance (PERMANOVA)[27] was performed for distance matrices for both gut microbiota and plasma, urine, and fecal metabolites using adonis2 (vegan v2.6-6.1) with diet as the main factor. Models were adjusted for within-subject variability by including the participants in the model and stratifying permutations by participant. The significance of diet effects was assessed using 999 permutations and an alpha of 0.05. For visualization, multilevel partial least squares discriminant analysis (PLS-DA) was conducted on the original data using plsda (mixOmics v6.28).[28,29] The multilevel PLS-DA models were adjusted to account for within-subject variability. PLS-DA score plots were generated, with ellipses representing the 95% CIs for each diet group, including baseline.

Within-Diet Variability and TMAO Prediction

Assessment of the impact of diet, including baseline and the 4 diet periods, on the variability (dispersion) of microbial community structure and metabolomic profiles was done with betadisper (vegan v2.6.6.1). These models were analyzed using a permutation test for homogeneity of multivariate dispersions[30] with permutest (vegan v2.6.6.1) to evaluate dispersion across groups (999 permutations; $P<0.05$ as significant), where the diet variable included all 4 diets along with baseline. Dispersion was assessed for all 6 microbiota beta diversity indices. For metabolome data in plasma, urine, and feces, Euclidean distances were calculated for each data set and differences in dispersion by diet were evaluated.

Linear mixed-effects models were used to determine if alpha and beta diversity predicted plasma and urinary TMAO concentrations, applying a natural logarithmic transformation to TMAO and adjusting for phase. Diversity indices were included as a fixed effect and participant was included as a random effect. Participants were categorized as high or low TMAO producers/excreters based on median TMAO values in the plasma/urine across diets. Individuals >80th percentile were labeled as high-producers/excreters, and those <20th percentile were as labeled low-producers/excreters. To examine if producer status was influenced by fish intake the day before testing, a sensitivity analysis was conducted where data points corresponding to fish intake (cod or salmon) during the day before testing were removed from the analysis. Linear mixed-effect models were built to determine if alpha and beta diversity predicted plasma and urinary TMAO concentrations in the subset of high and low TMAO-producers/excreters as described. Models were also built to assess differences in microbiota diversity indices by producer/excreter status.

Microbial Functional Potential

Predicted functional abundances from PICRUSt2 were CLR transformed before statistical analysis. Linear mixed-effects models were used to assess the effect of diet on microbial enzyme abundances associated with trimethylamine and TMAO metabolism at both Kyoto Encyclopedia of Genes and Genomes Orthology and pathway levels, with diet, phase, and baseline values as fixed effects and participant as a random effect. Additionally, linear mixed-effects models were used to determine if CLR-transformed predicted enzyme abundances were associated with plasma and urinary TMAO concentrations, applying a natural logarithmic transformation to TMAO and adjusting for phase. Predicted trimethylamine-related enzyme abundances were included as fixed effects and participant was included as a random effect.

RESULTS

Participant Characteristics

The total analytical sample was 30 participants (16 women:14 men). Initially, 36 individuals were enrolled at the Penn State site, but 6 withdrew before completing

Table 2. Characteristics of the Study Participants Included in the Analytical Sample at Baseline (*n*=30)*

	Mean±SD
Age, y	46±12
Men:women, *n*	14:16
Body mass index, kg/m^2	26±3
Plasma TMAO, μM	1.36 (IQR 1.03–1.60)†
Urinary TMAO, μM/mM Creatinine	34 (IQR 22–50)†
Total cholesterol, mg/dL	188±43
Low-density lipoprotein-cholesterol, mg/dL	106±32
High-density lipoprotein-cholesterol, mg/dL	55±10
Triglycerides, mg/dL	90 (IQR 66–117)†
Systolic blood pressure, mm Hg	116±12
Diastolic blood pressure, mm Hg	80±7

*Baseline values were measured before consuming any study food. Values presented as mean±SD unless otherwise stated. IQR indicates interquartile range;and TMAO, trimethylamine *N*-oxide.

†Median (IQR).

the first diet period. Data from individuals who did not complete at least 2 full diet periods were excluded from all analyses. In total, 8 participants withdrew from the study, with 2 withdrawing after the first diet period. The primary reason for withdrawal was the inability to comply with the controlled-feeding protocol because of social obligations. The Consolidated Standards of Reporting Trials diagram for the analytical sample is presented in Figure S1. The participants were generally healthy at the start of the study (mean age±SD: 46±12 years; mean body mass index±SD: 26±3 kg/m^2). Median baseline plasma TMAO was 1.36 μM (interquartile range, 1.03–1.60) and median baseline urinary TMAO was 42.5 μM/mM Cr (interquartile range, 22–46). All baseline participant characteristics for the analytical sample are presented in Table 2. Characteristics for the analytical sample and the whole cohort were generally comparable (Table S1). Baseline characteristics are additionally presented by diet period 1 allocation in Table S2.

Mediterranean-Style Diets With Lean Beef Resulted in Increased Microbiota Diversity and Distinct Microbial Composition Compared With an Average American Diet

Study diet effects were observed for 2 alpha diversity indices, Shannon index (*P*=0.002) and phylogenetic diversity (*P*=0.023) (Table S3). Shannon index was higher following the MED0.5 (mean difference, 0.19 [95% CI, 0.05–0.34]; *P*=0.005) and MED2.5 (0.2 [95% CI, 0.06–0.34]; *P*=0.003) diets compared with the AAD (Figure 1A) based on post hoc pairwise testing. No other pairwise differences were observed for Shannon index. Phylogenetic diversity was higher following the MED0.5 diet (0.86 [95% CI, 0.08–2.90]; *P*=0.02) compared with the AAD (Table S3) based on post hoc pairwise testing. No other pairwise differences were observed for phylogenetic diversity. Diet effects were not detected for observed ASVs (*P*=0.051) (Table S3).

Diet effects were observed for 3 beta diversity indices, including CLR Euclidean (Aitchison distance) (*P*<0.001), Bray–Curtis (*P*=0.004), and Jensen–Shannon divergence (*P*=0.008), as distance to baseline after each diet (Table S3). CLR Euclidean distance (Aitchison distance) to baseline was higher following the MED0.5 (mean difference 4.56 [95% CI, 1–8.2]; *P*=0.008), MED2.5 (5.23 [95% CI, 1.73–8.74]; *P*=0.001), and MED5.5 (4.59 [95% CI, 1.05–8.14]; *P*=0.009) diets compared with the AAD (Figure 1B) based on post hoc pairwise testing. Bray–Curtis distance to baseline was higher following the MED2.5 (0.07 [95% CI, 0.01–0.12]; *P*=0.006) and MED5.5 (0.06 [95% CI, 0.01–0.11]; *P*=0.01) diets compared with the AAD based on post hoc pairwise testing. Jensen–Shannon divergence distance to baseline was higher following the MED2.5 (0.04 [95% CI, 0.01–0.06]; *P*=0.01) and MED5.5 (0.03 [95% CI, 0.00–0.06]; *P*=0.04) diets compared with the AAD based on post hoc pairwise testing. No other pairwise differences were observed for any index. These results indicate that the change in microbial composition from baseline was greater following the MED2.5 and MED5.5 compared with the AAD. Diet effects were not observed for phylogenetic isometric log-ratio Euclidean, unweighted UniFrac, and weighted UniFrac distance to baseline (Table S3). Change in PC1 from baseline, derived from principal coordinates analysis of CLR Euclidean distances, was higher following the MED0.5 (4.56 [95% CI, 1–8.2]; *P*<0.001), MED2.5 (5.23 [95% CI, 1.73–8.74]; *P*<0.001), and MED5.5 (4.59 [95% CI, 1.05–8.14]; *P*=0.02) diets compared with the AAD (Figure 1C) based on post hoc pairwise testing.

Out of 1828 ASVs detected, the abundance of 14 ASVs differed by diet (FDR-adjusted *P*<0.1 after Benjamini–Hochberg procedure) (Table 3). ASVs were from the families Lachnospiraceae (*n*=6), Ruminococcaceae (*n*=3), Lactobacillaceae (*n*=2), Streptococcaceae (*n*=1), Coriobacteriaceae (*n*=1), and Alcaligenaceae (*n*=1) with further classification of species and genus in Table 3. The abundance of 2 ASVs from the genus *Lacticaseibacillus* was higher following all 3 MED diets compared with the AAD (*P*<0.05) (Figures S2A and S2B) based on post hoc pairwise testing, which is consistent with the microbes found in Greek yogurt in the MED diets compared with conventional yogurt in the AAD. All other means and pairwise comparisons for ASVs are shown in Table 3 and Figure S2. In a sensitivity analysis to account for yogurt probiotics in the MED diets, removing the 2 ASVs for the genus *Lacticaseibacillus* from the

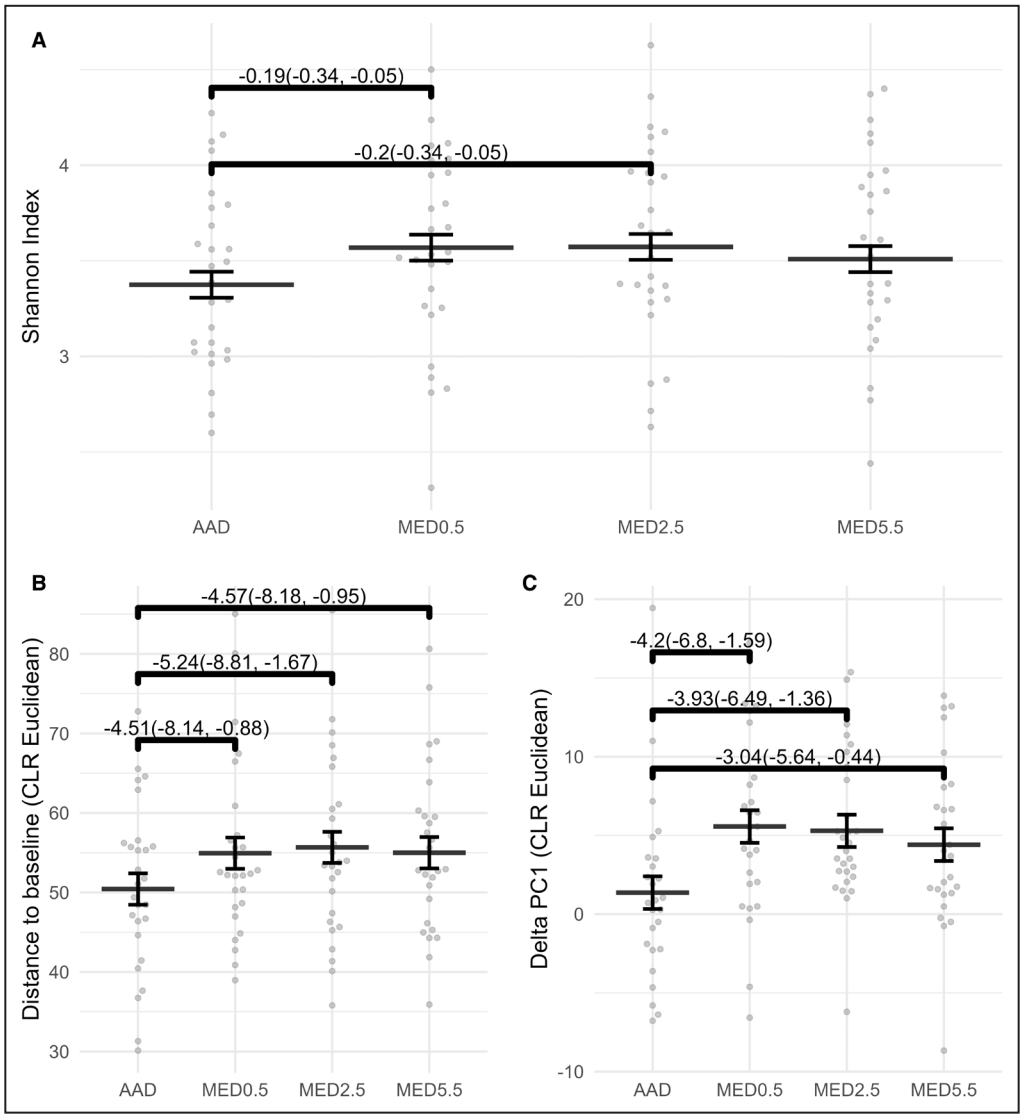

Figure 1. Microbiota diversity increased after 4 weeks on MED diets compared with AAD.
Microbial community structure differed after all MED diets compared with AAD. Statistical analysis was performed in R (v 4.4.0) with packages vegan v2.6–6.1, lme4 v1.1.35.4, lmertest v3.1.3, and emmeans v1.10.2. Black bars with error bars represent least square means with SEs derived from linear mixed-effect models. Individual raw values are displayed in light gray using beeswarm plots to show the distribution within each diet. Significant differences between diets (P<0.05 after pairwise comparisons with Tukey adjustment) are denoted by brackets as absolute difference (95% CI). Models were adjusted for phase and baseline value as fixed effects and participant as a random effect. **A**, Alpha diversity as Shannon index. **B**, Beta diversity as distance to baseline of CLR Euclidean distances (Aitchison distance). **C**, Change in principal coordinate 1 from baseline in ordination analysis (principal coordinates analysis) of distance matrices for CLR Euclidean distances (Aitchison distance). AAD indicates average American diet; CLR, centered log-ratio; MED, Mediterranean-style eating pattern used in the study; MED0.5, MED diet with 14 g (0.5 oz) per day of lean beef; MED2.5, MED diet with 71 g (2.5 oz) per day of lean beef; MED5.5, MED diet with 156 g (5.5 oz) per day of lean beef based on a 2000-kcal diet; PC, principal coordinate; and TMAO, trimethylamine *N*-oxide.

data set had no impact on either alpha or beta diversity indices (Table S4). The gut microbiota profiles differed by diet when comparing baseline and all diet periods, accounting for 2.2% of the variation (Aitchison distances; PERMANOVA P=0.001, R^2=0.022, 999 permutations). In the same model, participants explained the majority of variation at 71.3% (P=0.001, R^2=0.713, 999 permutations). To visualize microbiota sample clustering by diet, a multilevel PLS-DA model accounting for participant was used and a score plot was generated (Figure 2A), where test diets tended to cluster closer to each other than baseline.

Table 3. Differentially Abundant Microbes Between Diets After 4 Weeks Consuming Each Test Diet*

Family	Genus	Species	AAD	MED0.5	MED2.5	MED5.5	Diet main effect *P* value
Lactobacillaceae	*Lacticaseibacillus*	N/A	−2.23±0.66[a]	2.59±0.65[b]	1.03±0.64[b]	1.29±0.67[b]	<0.001
Lactobacillaceae	*Lacticaseibacillus*	N/A	−1.09±0.66[a]	2.12±0.65[b]	2.33±0.64[b]	3.19±0.67[b]	<0.001
Lachnospiraceae	*Dorea*	*Longicatena*	6.97±0.43[a]	6.04±0.43[b]	6.23±0.43[b]	6.34±0.43[b]	<0.001
Lachnospiraceae	*Lachnospiraceae UCG-001*	N/A	−1.44±0.62[a]	0.02±0.62[ab]	0.54±0.61[b]	0.14±0.63[b]	0.006
Lachnospiraceae	*[Eubacterium] ventriosum group*	*Ventriosum*	−1.29±0.38[a]	−2.12±0.37[ab]	−2.51±0.37[b]	−2.85±0.38[b]	0.002
Lachnospiraceae	N/A	N/A	1.66±0.67[a]	4.06±0.66[b]	3.84±0.65[b]	4.37±0.67[b]	<0.001
Lachnospiraceae	*Roseburia*	*Hominis*	−0.84±0.76[a]	1.95±0.74[b]	2.5±0.73[b]	1.41±0.76[ab]	0.007
Lachnospiraceae	*Coprococcus*	*comes*	2.68±0.33[a]	1.76±0.33[b]	1.82±0.32[b]	1.56±0.33[b]	0.006
Coriobacteriaceae	*Collinsella*	*Intestinalis/stercoris*	−1.33±0.37[a]	−2.19±0.37[ab]	−2.49±0.36[b]	−2.38±0.37[b]	0.006
Ruminococcaceae	*Subdoligranulum*	N/A	4.18±0.3[a]	5.05±0.3[b]	5.08±0.29[b]	5.43±0.3[b]	<0.001
Ruminococcaceae	*Incertae Sedis*	N/A	1.72±0.5[a]	0.29±0.49[b]	0.15±0.49[b]	0.11±0.5[b]	0.002
Ruminococcaceae	*Subdoligranulum*	N/A	6.29±0.34[a]	5.28±0.34[b]	5±0.34[b]	5.2±0.35[b]	0.001
Streptococcaceae	*Lactococcus*	*Garvieae/lactis/taiwanensis*	1.81±0.55[a]	0.1±0.53[ab]	−0.52±0.52[b]	−1±0.55[b]	0.002
Alcaligenaceae	*Bordetella*	*Aegrifaciens/animicus*	−2.11±0.32[a]	−3.3±0.31[b]	−2.56±0.31[ab]	−3.5±0.32[b]	0.004

*Effect estimates are centered log-ratio transformed microbe abundance as least square means ± SEMs (n=27) derived from linear mixed-effect models. Microbe counts were transformed using centered log-ratio with zero replacement before analysis. Models were adjusted for phase, sex, and baseline values as fixed effects and participant as a random effect. Main diet effects were obtained from Type III ANOVA F-tests of linear mixed-effect models. Post hoc pairwise comparisons were performed to test between diet differences using the emmeans function when diet main effects were significant (false discovery rate-adjusted P<0.1 after Benjamini–Hochberg procedure). Values in the same row with different superscript letters are significantly different (Tukey-adjusted P<0.05). Statistical analysis was performed in R (v 4.4.0) using packages lme4 v1.1.35.4, lmertest v3.1.3, and emmeans v1.10.2. Family, genus, and species are shown for each amplicon sequence variant, where any unknown value is marked with N/A. AAD indicates average American diet; MED, Mediterranean-style eating pattern used in the study; MED0.5, MED diet with 14 g (0.5 oz) per day of lean beef; MED2.5, MED diet with 71 g (2.5 oz) per day of lean beef; and MED5.5, MED diet with 156 g (5.5 oz) per day of lean beef based on a 2000-kcal diet.

Diet did not affect interindividual variation in any beta diversity distance matrix when compared with baseline (permutation test $P>0.05$) (Table S5), indicating that placing participants on any diet for 28 days did not reduce interindividual variability in the gut microbiota from baseline, as the dispersion of the microbiota at baseline was equivalent to the dispersion observed after each of the 4 diet periods.

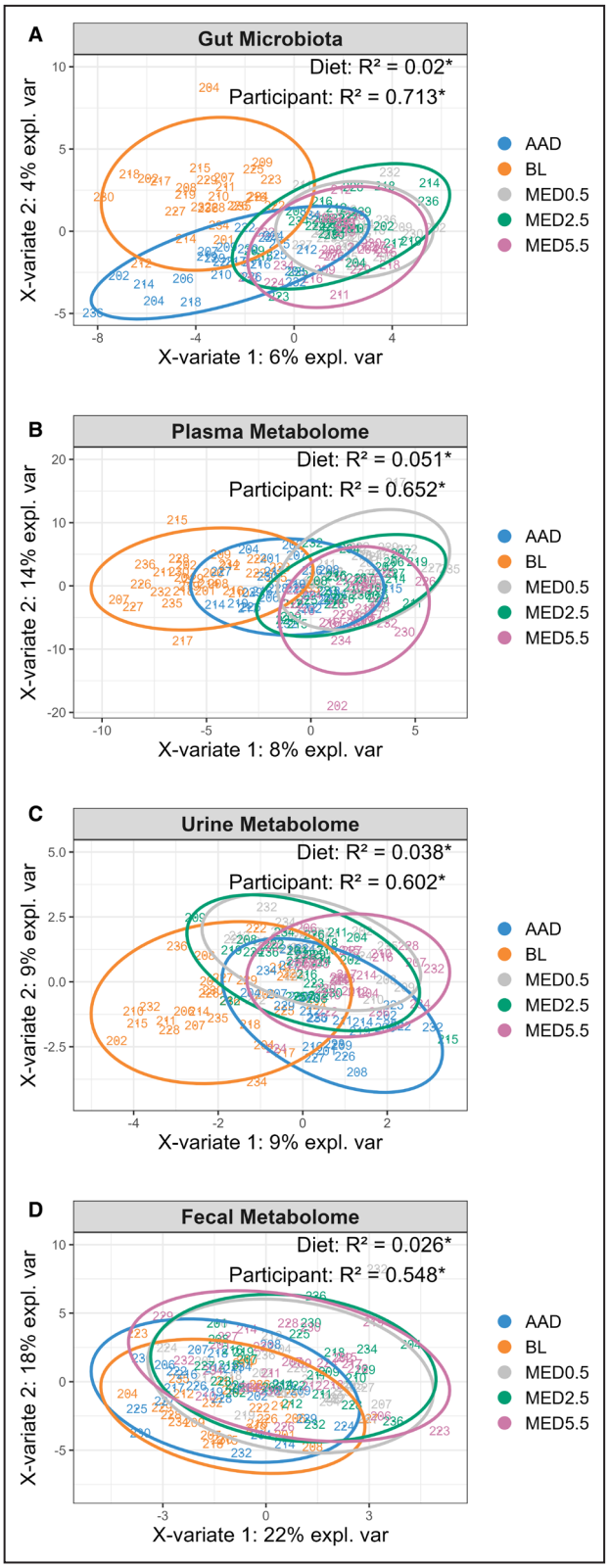

Figure 2. MED diet samples tended to cluster closer to each other than to baseline for both the gut microbiota and metabolomes.
Partial least squares discriminant analysis score plots depict the variance between samples in response to the four diets and baseline in the (**A**) gut microbiota, (**B**) plasma metabolome from untargeted LC/MS, (**C**) urine metabolome from untargeted ^{1}H NMR, and (**D**) fecal metabolome from untargeted ^{1}H NMR. Models were adjusted to account for within-subject variability. Groups include AAD, BL, MED0.5, MED2.5, and MED5.5. Confidence ellipses represent 95% CIs around the group centroids. Each sample is shown with the participant ID number. Statistical analysis was performed in R (v 4.4.0) using the mixOmics package (v6.28). Models were generated using the function plsda and plots were generated using function plotIndiv. *Diet and participant R^2 values obtained from PERMANOVA with 999 permutations on CLR-transformed Euclidean distances (Aitchison distance) for microbiota and natural log-transformed Euclidean distance for metabolomes. ^{1}H NMR indicates proton nuclear magnetic resonance; AAD, average American diet; BL, baseline; LC/MS, liquid chromatography/mass spectrometry; MED, Mediterranean-style eating pattern used in the study; MED0.5, MED diet with 14 g (0.5 oz) per day of lean beef; MED2.5, MED diet with 71 g (2.5 oz) per day of lean beef; and MED5.5, MED diet with 156 g (5.5 oz) per day of lean beef based on a 2000-kcal diet.

TMAO Concentrations Did Not Differ Between Mediterranean-Style Diets With Varying Doses of Lean Beef But Were Higher on an Average American Diet Compared With a Mediterranean-Style Diet With the Same Amount of Beef

A total of 161 known metabolites were identified in the untargeted plasma samples using LC/MS. In exploratory analyses, diet effects were observed for 39 metabolites (FDR-adjusted $P<0.1$ after Benjamini–Hochberg procedure), which included TMAO, L-carnitine, and several derivatives of carnitine, betaine, and phosphocholine (Table S6). For targeted analyses, plasma TMAO was higher following the AAD in comparison to the MED0.5 (mean fold difference, 1.78 [95% CI, 1.05–3.06]; $P=0.03$) and MED2.5 (2.04 [95% CI, 1.18–3.52]; $P=0.005$) diets (Figure 3A) based on post hoc pairwise testing. No other pairwise differences were observed for TMAO, but TMAO trended toward being higher following the AAD (mean fold difference, 1.59 [95% CI, 0.92–2.74]; $P=0.12$) compared with the MED5.5 (Table 4). Plasma L-carnitine was higher following the MED2.5 (mean difference, 1.39 μM [95% CI, 0.07–2.71]; $P=0.04$) and MED5.5 (1.43 μM [95% CI, 0.11–2.74]; $P=0.03$) diets compared with the AAD (Figure 3B) based on post hoc pairwise testing. No other pairwise differences were observed for

L-carnitine. No diet effects were observed for betaine and choline, the only other TMAO-related metabolites detected in plasma samples (Table 4). Carryover effects were not detected for any TMAO-related metabolites. Plasma metabolome profiles differed by diet when comparing baseline and all diet periods, accounting for 5.2% of variability (log-transformed Euclidean distances; PERMANOVA P=0.001, R^2=0.052, 999 permutations). In the same model, participants explained the majority of variation at 65.2% (P=0.001, R^2=0.652, 999 permutations). To visualize sample clustering by diet, a multilevel PLS-DA model accounting for participant was used and a score plot was generated (Figure 2B), where diet groups tended to cluster closer to each other than baseline.

A total of 30 known metabolites were identified in the urine samples using ^{1}H NMR, including creatinine, which was used for normalization of all other urinary metabolites. In exploratory analyses, diet effects were observed for 5 metabolites (FDR-adjusted P<0.1 after Benjamini–Hochberg procedure), which included TMAO, creatine, citrate, methylhistidine, and hydroxyisobutyrate (Table S6). Urinary TMAO was higher following the AAD in comparison to the MED0.5 (mean fold difference, 1.88 [95% CI, 1.19–2.97]; P=0.003), MED2.5 (2.15 [95% CI, 1.37–3.39]; P<0.001), and MED5.5 (1.76 [95% CI, 1.12–2.77]; P=0.008) (Figure 3C) based on post hoc pairwise testing. No other pairwise differences were observed for TMAO (Table 4). Diet effects were also observed for trimethylamine (P=0.03) and

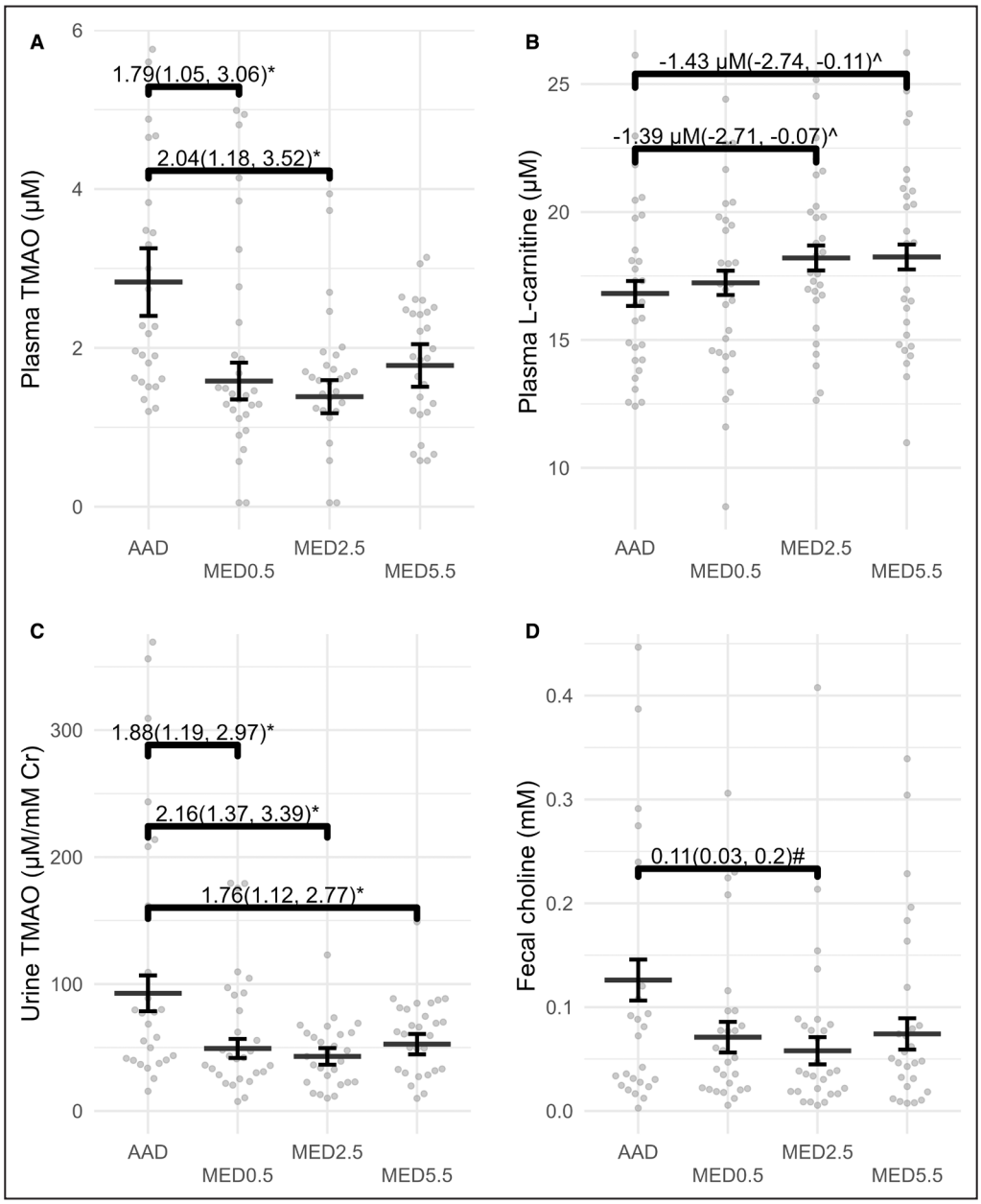

Figure 3. TMAO was higher in both plasma and urine after 4 weeks on AAD compared with MED diet with the same amount of lean beef.

TMAO did not differ between MED diets, despite increasing lean beef. Plasma L-carnitine was higher after the 2 MED diets with the highest doses of lean beef compared with the AAD. Fecal choline was higher after the AAD compared with the MED diet with the same amount of beef. Statistical analysis was performed in R (v 4.4.0) using packages lme4 v1.1.35.4, lmertest v3.1.3, and emmeans v1.10.2. Black bars with error bars represent least square means with SEs derived from linear mixed-effect models. Metabolites were back-transformed to their original scale to obtain the values shown. Individual raw values are displayed in light gray using beeswarm plots to show the distribution within each diet group. For log-transformed metabolites (*), differences between diets are reported as fold differences, reflecting the multiplicative relationships inherent in the log-transformed scale that are obtained before back-transformation. **A**, Plasma TMAO obtained by LC/MS. **B**, Plasma L-carnitine obtained by LC/MS. **C**, Urine TMAO obtained by ^{1}H NMR. **D**, Fecal choline obtained by ^{1}H NMR. *Significant differences between diets (P<0.05 after pairwise comparisons with Tukey adjustment) are denoted by brackets and are fold difference (95% CI). ^Significant differences between diets (P<0.05 after pairwise comparisons with Tukey adjustment) are denoted by brackets and are absolute difference (95% CI). #Significant differences between diets (P<0.05 after pairwise comparisons with Tukey adjustment) are denoted by brackets and represent differences on the square root–transformed scale (95% CI). Estimates are not back-transformed because contrasts on the original scale are not interpretable. ^{1}H NMR indicates proton nuclear magnetic resonance; AAD, average American diet; Cr, creatinine; LC/MS, liquid chromatography/mass spectrometry; MED, Mediterranean-style eating pattern used in the study; MED0.5, MED diet with 14 g (0.5 oz) per day of lean beef; MED2.5, MED diet with 71 g (2.5 oz) per day of lean beef; MED5.5, MED diet with 156 g (5.5 oz) per day of lean beef based on a 2000-kcal diet; and TMAO, trimethylamine *N*-oxide.

dimethylamine (P=0.03), the only other TMAO-related metabolites detected in urine samples. Urinary trimethylamine was higher following the AAD in comparison to the MED5.5 (mean fold difference, 1.15 [95% CI, 1.01–1.31]; P=0.03) based on post hoc pairwise testing. For TMAO-related metabolites, carryover effects were not detected, but carryover was close to reaching the prespecified significance threshold for dimethylamine (P=0.06) and trimethylamine (P=0.08). Urine metabolome profiles differed by diet when comparing baseline and all diet periods, accounting for 3.7% of variability (log-transformed Euclidean distances; PERMANOVA P=0.001, R^2=0.037, 999 permutations). In the same model, participants explained the majority of variation at 60.2% (P=0.001, R^2=0.602, 999 permutations). To visualize sample clustering, a multilevel PLS-DA model accounting for participant was used and a score plot was generated (Figure 2C), where diets tended to cluster closer to each other than baseline.

A total of 39 known metabolites were identified in the fecal samples using ^{1}H NMR. In exploratory analyses, diet effects were observed for 2 metabolites (FDR-adjusted P<0.1 after Benjamini–Hochberg procedure), which included choline and malonate (Table S6). Fecal choline was higher following the AAD in comparison to the MED2.5 (mean difference, 0.11 on the square root-transformed scale [95% CI, 0.03–0.20]; P=0.006) (Figure 3D) based on post hoc pairwise testing. No other pairwise differences were observed for choline (Table 4). No diet effects were observed for trimethylamine (P=0.11), the only other TMAO-related metabolite detected in the fecal samples (Table 4). Carryover effects were not detected for any TMAO-related metabolites. Fecal metabolome profiles differed by diet when comparing baseline and all diet periods, accounting for 2.7% of the variability (log-transformed Euclidean distances; PERMANOVA P=0.010, R^2=0.027, 999 permutations). In the same model, participants explained the majority of variation at 54.8% (P=0.017, R^2=0.548, 999 permutations). To visualize sample clustering, a multilevel PLS-DA model accounting for participant was used and a score plot was generated (Figure 2D), where diets tended to cluster closer to each other than baseline.

Diet did not affect interindividual variation in the plasma, urine, or fecal metabolomes for log-transformed Euclidean distances (permutation test P>0.05) (Table S5), indicating that variability of metabolomic profiles among individuals did not change when put on the same diet compared with dispersion at baseline.

High- and Low TMAO Producer Status May Be Associated With Gut Microbiota Diversity or Community Structure Index

In plasma, high-TMAO producers (n=6) had a median TMAO of 2.59 μM and low-TMAO producers (n=6) had a median TMAO of 1.14 μM across all 4 diets (Figure 4A). A sensitivity analysis removing any data points for participants who consumed fish (cod or salmon) the day before testing resulted in high-TMAO producers (n=6) with a median TMAO of 2.46 μM and low-TMAO producers (n=6) with a median TMAO of 1.12 μM across all 4 diets (Figure S3). Only 1 participant who was classified as a low-TMAO producer was >20th percentile after the removal of data points after fish intake and all other participants remained the same. Individual participant data for plasma TMAO are shown by diet as a waterfall plot (Figure S4A) and a spaghetti plot (Figure S4B). Microbial diversity (Shannon index) was not a predictor of plasma TMAO for the full data set (P=0.15) but was a predictor of plasma TMAO for the data subset of high-TMAO producers and low-TMAO producers (β=0.97, P=0.03) with an estimated TMAO increase of 164% per 1-unit increase in the Shannon index (Figure S5A). Similarly, microbial diversity differed

Table 4. Metabolite Concentrations of Molecules Related to TMAO After 4 Weeks Consuming Each Test Diet

Metabolite	AAD	MED0.5	MED2.5	MED5.5	Diet main effect *P* value
Plasma					
TMAO†, μM	2.83±0.43[a]	1.58±0.23[b]	1.39±0.21[b]	1.78±0.27[ab]	0.006
L-carnitine, μM	16.8±0.48[a]	17.2±0.48[ab]	18.2±0.48[b]	18.2±0.48[b]	0.01
Betaine†, μM	6.86±0.19	6.86±0.18	6.93±0.19	6.68±0.18	0.45
Choline†, μM	25.6±0.81	25.2±0.78	25.5±0.81	24.6±0.78	0.44
Urine					
TMAO†, μM/mM Creatinine	92.7±14.1[a]	49.3±7.51[b]	43.0±6.55[b]	52.6±8.02[b]	<0.001
Trimethylamine†, μM/mM Creatinine	4.78±0.25[a]	4.29±0.23[ab]	4.48±0.24[ab]	4.16±0.22[b]	0.03
Dimethylamine†, μM/mM Creatinine	48.1±3.42	43.9±3.13	38.2±2.72	38.1±2.72	0.03
Feces					
Choline‡, mM	0.13±0.02[a]	0.07±0.01[ab]	0.06±0.01[b]	0.08±0.01[ab]	0.008
Trimethylamine*, mM	0.09±0.01	0.07±0.01	0.08±0.01	0.10±0.02	0.11

*Data presented as least square means±SEMs (*n*=28–30) derived from linear mixed-effect models. Models were adjusted for phase and baseline values as fixed effects and participant as a random effect. Main diet effects were obtained from Type III ANOVA F-tests of linear mixed-effect models built using the lmer function. Post hoc pairwise comparisons were performed to test between diet differences using the emmeans function when diet main effects were significant (*P*<0.05). Values in the same row with different superscript letters are significantly different (Tukey-adjusted *P*<0.05). Statistical analysis was performed in R (v 4.4.0) using packages lme4 v1.1.35.4, lmertest v3.1.3, and emmeans v1.10.2. AAD indicates average American diet; MED, Mediterranean-style eating pattern used in the study; MED0.5, MED diet with 14 g (0.5 oz) per day of lean beef; MED2.5, MED diet with 71 g (2.5 oz) per day of lean beef; MED5.5, MED diet with 156 g (5.5 oz) per day of lean beef based on a 2000-kcal diet; and TMAO, trimethylamine *N*-oxide.

†Models used natural logarithm transformation. All displayed least square means are back-transformed to original scale.

‡Model used square root transformation. All displayed least square means are back-transformed to original scale.

by producer status in plasma data (*P*=0.03), where high-TMAO producers had higher microbial diversity (0.56 [95% CI, 0.09–1.03]) (Figure S5B). Microbial community structure (Aitchison distance to baseline) was not a predictor of plasma TMAO for either the full data set (*P*=0.29) or the data subset of high producers and low producers (*P*=0.21), but microbial community structure differed by producer status (*P*=0.04) where high producers had a greater change from baseline (9.87 [95% CI, 0.62–19.1]) (Figure S5C).

In urine, high-TMAO excreters (*n*=6) had a median TMAO of 88.09 μM/mM Cr and low-TMAO excreters (*n*=6) had a median TMAO of 22.71 μM/mM Cr across all 4 diets (Figure 4B). Individual participant data for urine TMAO are shown by diet as a waterfall plot (Figure S4C) and spaghetti plot (Figure S4D). Microbial diversity (the Shannon index) was not a predictor of urine TMAO for the full data set (*P*=0.13) but was a predictor of urine TMAO for the data subset of high-TMAO excreters and low-TMAO excreters (β=0.93, *P*=0.01) with an estimated TMAO increase of 154% per one-unit increase in the Shannon index (Figure S5D). Similarly, microbial diversity differed by producer status in urine data (*P*=0.04) where high producers had higher microbial diversity (0.58 [95% CI, 0.03–1.13]) (Figure S5E). Microbial community structure (Aitchison distance to baseline) was a not a predictor of urine TMAO for either the full data set (*P*=0.39) or the data subset of high producers and low producers (*P*=0.06), but microbial community structure differed by excreter status in urine data (*P*=0.04) where high-TMAO excreters had a greater change from baseline (11.8 [95% CI, 0.88–22.8]) (Figure S5F).

Diet Had No Effect on Trimethylamine-Related Predicted Microbial Genes or Functional Pathways, But Predicted Microbial Enzyme Abundance for One Enzyme Did Predict Urinary TMAO

Using the PICRUSt2 predicted functional data, diet effects were not observed for any trimethylamine-related predicted microbial functional genes or pathways (Table S7). Although diet-related shifts in microbial composition were observed, these changes did not translate into corresponding differences in predicted functional genes or pathways. However, CLR-transformed abundance of trimethylamine-corrinoid protein Co-methyltransferase was found to be a predictor of urinary TMAO (Figure S6). A 1-unit increase in CLR-transformed predicted abundance of trimethylamine-corrinoid protein Co-methyltransferase was associated with a 7.7% decrease in urinary TMAO (β=−0.08, *P*=0.009). Plasma TMAO had similar directionality and magnitude in its association with trimethylamine-corrinoid protein Co-methyltransferase but did not reach significance (β=−0.052, *P*=0.10). The other 2 trimethylamine-related enzymes detected in this

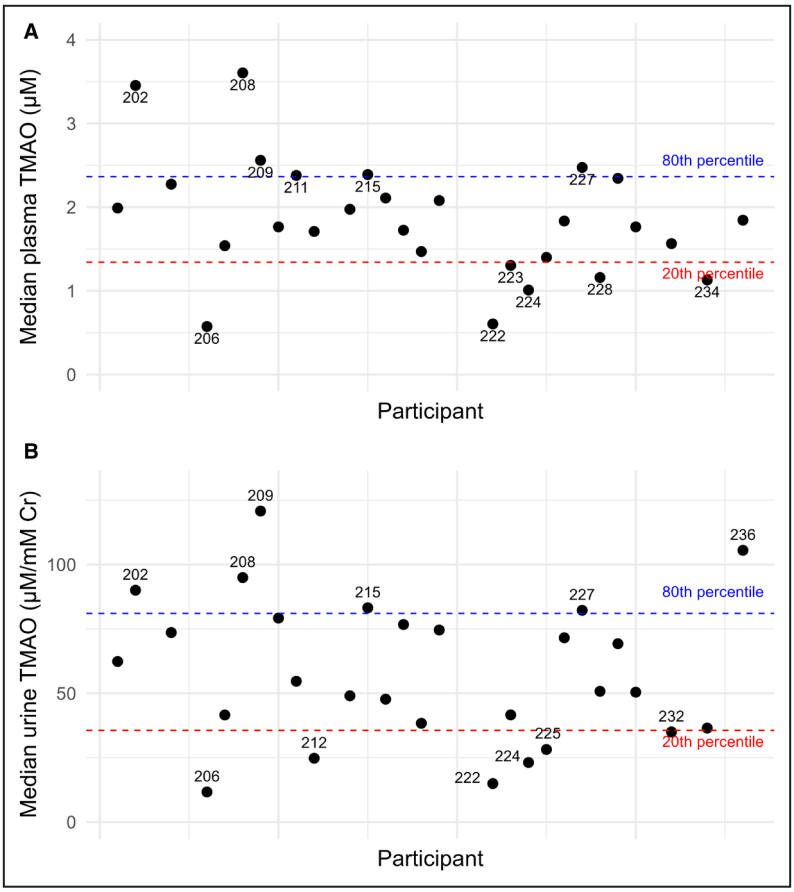

Figure 4. Individual participant median TMAO concentrations across all 4 diets were separated into high producers and low producers and visualized. **A**, Median plasma TMAO values are shown, and dotted lines indicate cutoffs for the 80th percentile (blue) and the 20th percentile (red). Participants above the 80th percentile or below the 20th percentile are labeled. **B**, Median urine TMAO values are shown, and dotted lines indicate cutoffs for the 80th percentile (blue) and the 20th percentile (red). Participants above the 80th percentile or below the 20th percentile are labeled. Cr indicates creatinine; and TMAO, trimethylamine *N*-oxide. Statistical analysis was performed in R (v 4.4.0).

analysis, trimethylamine dehydrogenase and TMAO reductase (cytochrome c), did not predict either plasma or urinary TMAO (P>0.05).

DISCUSSION

Our results demonstrate that consumption of a MED dietary pattern with 71 g/day/2000 kcal of lean beef lowered plasma and urinary concentrations of TMAO compared with a typical American dietary pattern containing an equal amount of beef in healthy adults. Importantly, the inclusion of lean beef in a MED dietary pattern did not dose-dependently (14, 71, or 156 g/day/2000 kcal) increase plasma or urinary TMAO concentrations. We also observed higher gut microbiota diversity with the MED diets (14 and 71 g/day beef) compared with the AAD with 71 g/day of beef. Surprisingly, gut microbiota diversity was higher in individuals with the greatest TMAO concentrations, suggesting a complex relationship between diet, microbiota, and TMAO.

Plasma and urinary TMAO were lower following the MED diets with the lower amounts of beef (14 and 71 g/day) compared with the AAD, despite similar dietary TMAO precursor composition. However, average plasma TMAO concentrations were low across all diets, staying below the clinical cut point for increased CVD risk (6.20 μM).[31] Discordance in the literature on beef intake and TMAO concentrations[19] may be partially explained by the dietary pattern beef is incorporated into. The lack of a TMAO difference between the MED diets with 14, 71, and 156 g/day of lean beef suggests that a MED diet mitigates TMAO production, even with higher lean beef intake. Similar results were observed in a 10-day controlled-feeding study where no difference in

circulating TMAO was detected following a healthy diet based on the Dietary Guidelines for Americans with 156 g/day of pork (40.3 mg/day L-carnitine) compared with the same diet with chicken (5.5 mg/day L-carnitine).[32] In contrast, results from a crossover controlled-feeding study using 2 MED diets with 28 and 71 g/day of lean beef showed higher plasma TMAO after 5 weeks on the higher beef diet.[10] However, differences in the study population and baseline TMAO concentrations between the studies may explain these findings. In our study, the median baseline plasma TMAO concentration was 1.36 μM, whereas Krishnan et al. reported mean baseline values of 5.2 and 4.4 μM for the 2 diet periods. End point plasma TMAO concentrations also differed greatly, with model-derived means of 1.58, 1.39, and 1.78 μM across our 3 MED diets, compared with means of 3.1 and 5.0 μM following their 2 MED diets. These studies highlight the importance of evaluating both dietary context and population when assessing how red meat consumption influences TMAO concentrations. Distinguishing the effects of red meat intake from overall diet quality is needed because dietary patterns rich in plant-based foods and fiber may alter gut microbiota composition in ways that attenuate TMAO production, even in the presence of higher meat intake.

Gut microbiota diversity was higher after the MED0.5 and the MED2.5 diets compared with the AAD, indicating that including up to 2.5 oz/day of lean beef in a Mediterranean-style diet does not diminish improvements in microbial diversity. Notably, no differences in microbial diversity indices were observed among the 3 MED diets, despite lean beef intake varying from 14 g to 156 g/day. This result aligns with a previous crossover study in women that showed that consuming a healthy lacto-ovo vegetarian diet with and without 3 oz/day of lean red meat did not have an impact on alpha or beta diversity.[33] Differences in microbial diversity between the AAD and MED diets may reflect changes in trimethylamine metabolic pathways. Higher plasma L-carnitine after the MED2.5 and MED5.5 diets compared with the AAD suggests greater use of dietary carnitine for trimethylamine production in the AAD, supported by higher plasma TMAO after the AAD. Furthermore, the increased fecal excretion of choline observed in the AAD in comparison to the MED2.5 diet indicates that less was being used by the gut microbiota for trimethylamine production and instead excreted. This effect may be partially driven by the higher fiber content in the MED diets (26 g for MED0.5 and MED2.5, 23 g for MED5.5, and 20 g for AAD), which may inhibit trimethylamine formation through short-chain fatty acid production and a subsequent pH increase.[34] However, evidence remains insufficient to confirm this fiber-mediated effect within the context of a healthy dietary pattern. More research is needed to elucidate fiber's role in gut microbiota modulation and TMAO metabolism within a MED diet.

The hypothesis that diet-induced shifts in the gut microbiota and their function alter trimethylamine production aligns with evidence that omnivores exhibit higher TMAO concentrations compared with vegetarians or vegans, both while fasting and after consuming an L-carnitine challenge.[14] Increased consumption of plant-based foods may reduce TMAO production from carnitine by shifting gut microbiome function, potentially explaining the lower TMAO concentrations observed in our study. Because an individual's baseline microbiota primarily dictates the composition and the function even after a dietary intervention,[35] individuals who habitually consume healthier dietary patterns may have different trimethylamine production from red meat. This is supported by findings from an 8-week randomized crossover study examining the effect of animal meat compared with plant-based alternative meats on TMAO concentration.[36] Crimarco et al. reported carryover effects such that participants who consumed the plant-based meat first had lower TMAO concentrations when later consuming animal meat than those who consumed animal meat first. Although no order effects were seen for gut microbiota abundances, changes in the broader communities of trimethylamine producers or gene expression within these taxa may have occurred. These findings highlight the need for a deeper understanding of how habitual dietary patterns modulate the conversion of TMAO precursors like carnitine to trimethylamine, in order to better predict diet-specific TMAO responses.

Study diets explained a small portion of the variability in gut microbiota (2.2%) and metabolomes (2.7%–4.7%), whereas individual identity accounted for the majority of variance in gut microbiota (71.3%), and plasma (61.2%), urine (60%), and fecal (54.8%) metabolomes. In alignment, findings from a controlled-feeding study examining the impact of a homogenous diet on interpersonal variation[37] showed that diet accounted for 1% to 2% of the variance in gut microbiome composition and metabolomes, whereas host identity explained 78% of microbiome and 23% to 47% of metabolome variability.[38] For both the gut microbiota and metabolomes, diet group samples tended to cluster closer to each other than to baseline samples when visualizing samples from multilevel PLS-DA models, highlighting how these carefully formulated study diets differ from the habitual diet of participants. Notably, no reduction in interindividual variation was observed from baseline in our study, suggesting that host-specific factors remain the dominant drivers of variation in microbiota and metabolomes. Further research is needed to identify the primary mechanisms driving interindividual variation in microbiota composition and metabolism.

There is considerable variation in TMAO concentrations between individuals, even when consuming the same diet or dietary TMAO precursors.[14,17,39–41] We observed wide variation in TMAO concentrations within each diet period and identified distinct high- and low-TMAO producers and excreters when averaging each participant's values across diet periods. Notably, high producers/excreters across all 4 diet periods exhibited greater microbial diversity on average, despite the unfavorable phenotype. This suggests that microbial metabolic activity, rather than diversity per se, may be more important in predicting TMAO metabolism. A longitudinal cohort study in men found that the association between red meat consumption and plasma TMAO concentrations was influenced by the presence of *Eubacterium balli*, *Roseburia hominis*, and *Alistipes shahii*, and the absence of *Eubacterium bioforme*[42]; however, none of these taxa were altered in our study. Although several studies have identified microbial families, genera, and species involved in trimethylamine metabolism,[18,43] the specific microbes contributing to TMAO production appear to vary widely between individuals. A crossover study using diets with different protein sources further demonstrated that neither taxonomic groups nor bacterial gene presence in fecal samples reliably predicted postdiet TMAO concentrations.[44] Although shifts in microbiota composition were observed by diet in our study, PICRUSt2 functional prediction analysis did not reveal diet effects on predicted microbial functional pathways related to trimethylamine metabolism. These findings suggest that dietary interventions may significantly influence TMAO metabolism through changes in microbial function, rather than shifts in taxonomy or predicted functional potential.[44] The lack of diet-related shifts in predicted functional pathways suggests that predictive analysis may have limitations in capturing true functional changes in the microbiota, which are best captured by direct measures of microbial activity, such as metatranscriptomics or proteomics.

This study's strengths include its crossover controlled-feeding design, where participants were provided all foods and beverages for the course of the diet periods and served as their own controls, reducing interindividual variability. It also used comprehensive metabolomics analyses across feces, plasma, and urine, along with gut microbiota profiling. However, there are some limitations. TMAO precursor amounts were not measured in the test diets, and some testing days occurred after fish consumption, though a sensitivity analysis showed minimal impact on results. Participants were metabolically healthy adults, limiting generalizability to other populations. The fiber amount in the AAD (20 g/2000 kcal) is higher than the average American fiber intake of ~16 g/2000 kcal,[45] which limits the ability to assess the true impact of MED diets compared with a typical American diet. Alcohol consumption was limited to ≤2 drinks per week and restricted to >48 hours before testing, but the exact amount consumed was not recorded so it could not be accounted for in the analyses. Alcohol intake is known to influence gut microbiota.[46] Microbiota were assessed using 16S rRNA gene sequencing, which provides limited taxonomic resolution. The study was not powered to detect diet differences for microbiota or metabolomics and lacked bacterial mRNA data to assess microbial function. Additionally, the study was not powered to detect associations between changes in microbiota composition and TMAO shifts across diet periods because participants had low baseline TMAO concentrations. Finally, variations in probiotic intake because of yogurt differences between the diets may have influenced microbiota composition, although a sensitivity analysis showed no impact on diversity.

Conclusions

In conclusion, consumption of a healthy MED dietary pattern with 71 g/day of lean beef lowered plasma and urine TMAO when compared with a typical American dietary pattern containing an equal amount of beef in healthy adults. Inclusion of lean beef in a healthy MED dietary pattern did not dose-dependently (14, 71, or 156 g/day) affect plasma or urinary TMAO. These findings extend to gut microbiota as well, showing that the inclusion of up to 156 g/day of lean beef in the MED dietary pattern maintains microbiota richness, evenness, and community structure. These findings underscore the role of healthy dietary patterns in influencing gut microbiota-mediated processes and demonstrate that the inclusion of up to 156 g/day of lean beef in a MED diet does not affect the novel CVD risk factor TMAO in a healthy population.

ARTICLE INFORMATION

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Affiliations

Department of Nutritional Sciences (Z.S.D., J.A.F., P.M.K., K.S.P.), Department of Biochemistry and Molecular Biology (J.Z., J.E.B.) and Department of Veterinary and Biomedical Sciences (F.H., A.D.P.), The Pennsylvania State University, University Park, PA; The Huck Institutes of the Life Sciences, The Pennsylvania State University, University Park, PA (S.K.); and One Health Microbiome Center, Huck Life Sciences Institute, The Pennsylvania State University, University Park, PA (J.E.B.).

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The authors' responsibilities were as follows: Penny M. Kris-Etherton and Jennifer A. Fleming: designed and conducted the parent study; Kristina S. Petersen and Zachary S. DiMattia: designed the research; Jingcheng Zhao, Fuhua Hao, Sergei Koshkin, and Zachary S. DiMattia: conducted the

laboratory analyses; Zachary S. DiMattia and Jingcheng Zhao: analyzed and interpreted the data; Zachary S. DiMattia: drafted the article; all authors: critically reviewed the article; Kristina S. Petersen: had primary responsibility for final content; and all authors: took responsibility for the final content and read and approved the final article.

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Supplemental Material

Tables S1–S7
Figure S1–S6

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