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RESEARCH ARTICLE

Trimethylamine-*N*-oxide (TMAO) response to animal source foods varies among healthy young men and is influenced by their gut microbiota composition: A randomized controlled trial

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Scope: Trimethylamine-*N*-oxide (TMAO), a metabolite linked to the gut microbiota, is associated with excess risk of heart disease. We hypothesized that (i) TMAO response to animal source foods would vary among healthy men and (ii) this response would be modified by their gut microbiome.

Methods and results: A crossover feeding trial in healthy young men ($n = 40$) was conducted with meals containing TMAO (fish), its dietary precursors, choline (eggs) and carnitine (beef), and a fruit control. Fish yielded higher circulating and urinary concentrations of TMAO (46–62 times; $p < 0.0001$), trimethylamine (8–14 times; $p < 0.0001$), and dimethylamine (4-6-times; $P<0.0001$) than eggs, beef, or the fruit control. Circulating TMAO concentrations were increased within 15 min of fish consumption, suggesting that dietary TMAO can be absorbed without processing by gut microbes. Analysis of 16S rRNA genes indicated that high-TMAO producers (≥20% increase in urinary TMAO in response to eggs and beef) had more Firmicutes than Bacteroidetes ($p = 0.04$) and less gut microbiota diversity ($p = 0.03$).

Conclusion: Consumption of fish yielded substantially greater increases in circulating TMAO than eggs or beef. The higher Firmicutes to Bacteroidetes enrichment among men exhibiting a greater response to dietary TMAO precursor intake indicates that TMAO production is a function of individual differences in the gut microbiome.

Keywords:
Dietary precursor intake / Gut microbiota / Human / Metabolism / Trimethylamine-*N*-oxide

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1 Introduction

Trimethylamine-*N*-oxide (TMAO) is a naturally occurring small organic dietary compound that is abundant in fish, or can be generated from other nutrients including choline (abundant in eggs) and carnitine (abundant in beef). Upon consumption of foods containing TMAO or its dietary precursors (choline and carnitine), it is proposed that gut bacteria generate trimethylamine (TMA; volatile, fish odor compound) [1] and to a lesser extent, dimethylamine (DMA) with subsequent absorption via enterohepatic circulation [2]. The majority of TMA is enzymatically converted to the odorless TMAO metabolite in a reversible reaction catalyzed by vitamin B2 dependent flavin-containing monooxygenase 3 (FMO3) in the liver. Loss-of-function mutations in this

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Abbreviations: **ANOVA**, analysis of variance; **Cr**, creatinine; **DMA**, dimethylamine; **EBA**, ethyl bromoacetate; **FMO3**, flavin-containing monooxygenase 3; **HMRU**, Human Metabolic Research Unit; **MA**, methylamine; **ND**, not detectable; **OTU**, operational taxonomic unit; **PCoA**, principal coordinates analysis; **TMA**, trimethylamine; **TMAO**, trimethylamine-*N*-oxide

Colour online: See the article online to view Fig. 1 in colour.

enzyme give rise to the rare genetic disorder known as trimethylaminuria or "the fish odor syndrome" [3]. Alternatively, TMA can be demethylated to DMA and methylamine (MA).

The significance of TMAO in physiologic processes received early attention due to its function as an osmolyte [4]. In addition, farmers recognized the benefits of adding TMAO to animal feed for the purposes of improving growth, carcass quality, and nutrient digestibility [5]. More recently, TMAO has emerged as a predictive risk factor for heart disease in cardiac patients [6, 7] and colorectal cancer among postmenopausal women [8]. However, very little is known about the effects of animal source foods on TMAO generation, absorption, and elimination in healthy adults. Furthermore, although there is an apparent role of the gut microbes in TMAO production, the gut microbiota composition in relation to TMAO production in humans has not been determined.

Therefore, we aimed to test the hypotheses that (i) TMAO response to animal source foods would vary among healthy men and (ii) this response would be modified by their gut microbiota composition. To achieve these aims, we conducted a crossover feeding trial whereby healthy young men consumed study meals of fish, eggs, meat, and a fruit control in random order with 1-week washout periods. TMAO biomarker response to the study meals was quantified in plasma and urine, while gut microbiota composition was assessed in feces. Because the *FMO3* G472A genetic variant may adversely influence TMA conversion to TMAO [9], men were genotyped for this polymorphism, which was considered as a covariate in the statistical models.

2 Methods and materials

2.1 Participants

Healthy men ($n = 40$) aged 21–50 years with a BMI of 20–29.9 kg/m^2 were recruited by flyers posted around the Cornell University's Ithaca campus and surrounding area from May to June 2014. A sample size of $n = 40$ was determined from a power analysis of a within-subject design to detect a 10% difference of plasma TMAO concentration at $\alpha < 0.05$ and $\beta = 0.8$. Participants were screened by the use of a blood chemistry profile, cell count, and health history questionnaire. The exclusion criteria were men of age >50 years, BMI $\geq$30 kg/m^2, women, vegetarians, smokers, and individuals with gastrointestinal diseases or complaints, chronic illnesses or other metabolic diseases (including trimethylaminuria), abnormal blood chemistry values indicative of organ dysfunctions, and those taking nutritional supplements, antibiotics or probiotics within 2 months of recruitment. Written informed consent was obtained from all participants, and the protocol was approved by the Institutional Review Board for Human Study Participants at Cornell University (Protocol ID#: 1403004534). This trial was registered at clinicaltrials.gov as NCT02558673.

2.2 Study design

A randomized, controlled crossover design with four arms composed of study meals representing animal sources of TMAO and a fruit control was used in this study. Figure 1A depicts the participant flow throughout the study and the study design. The study meals were (i) eggs (three whole hard boiled; Wegmans), (ii) beef (6 ounces Philly-Gourmet Beef Patties, 100% Pure; Tops), (iii) fish (6 ounces cod fillet; Tops)], and (iv) the fruit control (two single-serve packages of Mott's natural applesauce; Tops), and were administered in commonly consumed servings. The order of study meals for each participant was assigned by a study investigator using random number generator (random.org), and each meal was administered in a single day separated by a 1-week washout period. All meals were prepared in the Human Metabolic Research Unit (HMRU) kitchen at Cornell University on the morning of testing.

2.3 Protocol

The study procedure is illustrated in Fig. 1B. After an overnight fast (10 h), participants arrived at the HMRU between 0700–1000 h for each of the four visits. The day prior to the study session, participants were advised to avoid consumption of grapefruit juice and indole-containing vegetables (i.e., broccoli, Brussel sprouts, cabbage, cauliflower, kale, and bok choy) as these foods can decrease FMO3 enzyme activity and alter TMAO metabolism [2]. Participants were also asked to maintain their normal routine, including exercise, and eat a similar meal the night before each of the four sessions.

At the beginning of each session, participants completed a 24-h dietary recall to assess compliance to the grapefruit juice and indole food restriction. If participants reported consumption of these foods, or significant deviations from their normal routine, the session was rescheduled. A baseline blood sample was obtained by a phlebotomist using a standard venipuncture procedure, and participants collected their baseline urine sample.

Participants then consumed a randomly allocated study meal with one cup of water within a 15-min period. Following the consumption of the study meal, serial blood samples were obtained at 15 and 30 min, and 1, 2, 4, and 6 h, and each participant collected their urine samples into bottles throughout the 6-h study period, which were subsequently pooled. At 4.5 h, participants were provided a fixed snack (i.e., applesauce) and water. Throughout each study session, participants refrained from eating and drinking foods and beverages (other than water) outside those provided by study personnel.

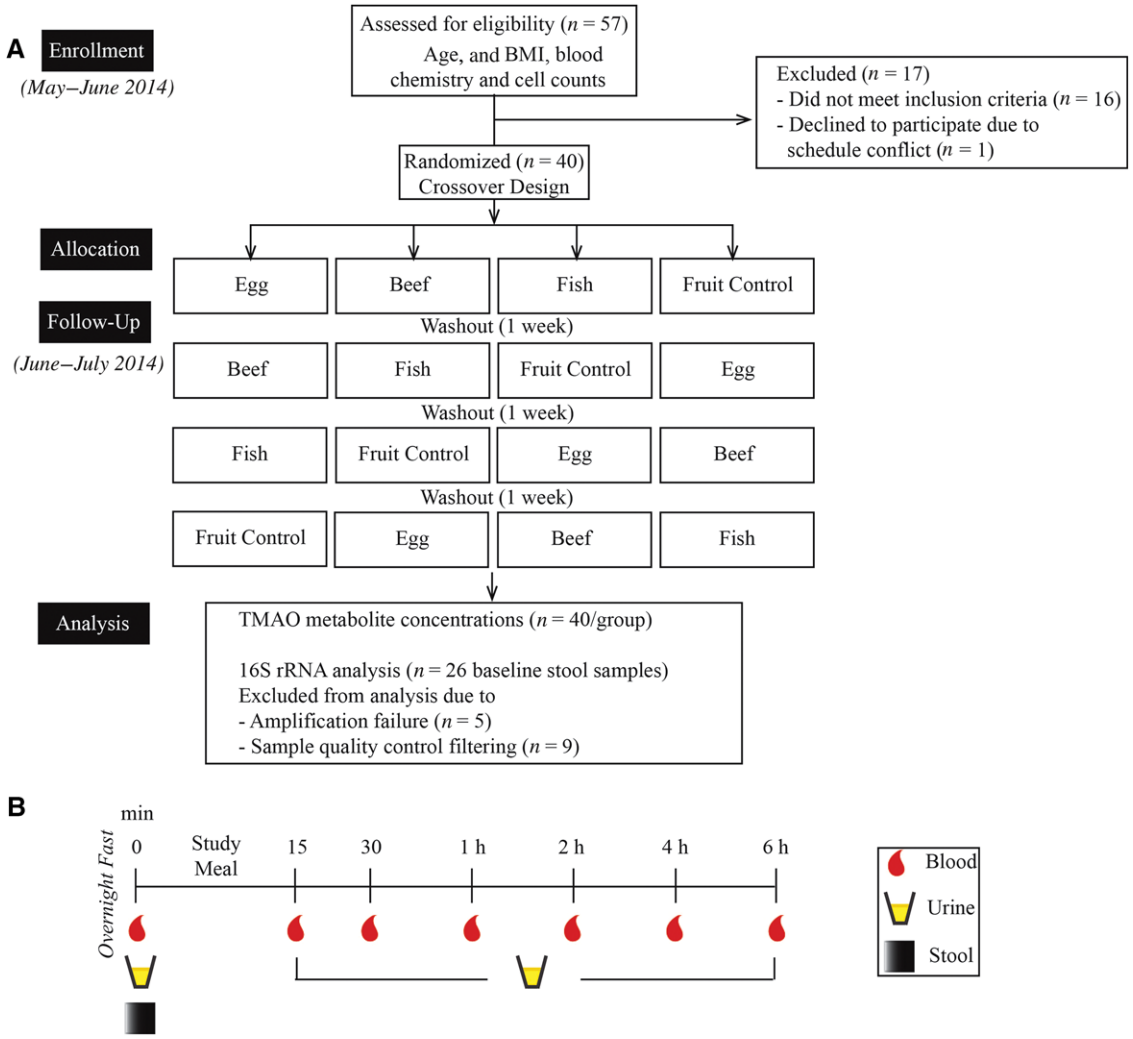

Figure 1. A schematic of the participant flow throughout the study and the study design (A) and the study protocol (B).

2.4 Sample collection

At the screening visit, fasting serum and whole blood were obtained for blood chemistry profiles and complete cell counts, respectively, using a previously described method [10]. During the feeding trials, plasma and buffy coat were obtained for metabolite measurements [11, 12] and *FMO3* G472A (rs 2266782) genotyping.

Urine was self-collected in wide-mouth specimen containers (120 mL; Thermo Scientific) at study baseline, and in wide-mouth polyethylene bottles (1 L; Nalgene) during the 6-h period, and kept on ice. Urine volumes were recorded separately for study baseline and the 6-h study period. In addition, prior to consumption of the fruit control, participants collected stool (complete bowel movement) in a bag provided by study personnel and delivered to the HMRU in a thermoinsulated bag with ice packs. All samples were deidentified, distributed among several storage vials, and stored at –80°C for further analyses.

2.5 Analytical methodology

2.5.1 Blood chemistry profiles and complete cell counts

HDL, LDL, lipids, protein, creatinine (Cr), bilirubin, alkaline phosphatase, lactate dehydrogenase, aspartate aminotransferase, alanine aminotransferase, gamma-glutamyl transpeptidase and blood urea nitrogen concentrations were measured in serum collected at screening by the Dimension Xpand chemistry analyzer (Siemens Healthcare Diagnostic).

White blood cells, lymphocytes, monocytes, granulocytes, and red blood cells were measured in whole blood collected

at screening by the Act diff 2 hematology analyzer (Beckman Coulter).

2.5.2 TMAO metabolite measurements

TMAO, TMA, DMA, and MA were measured in plasma, urine, and study meals by LC-MS/MS as described by Johnson *et al.* [13] with modifications to measure all of the metabolites in a single run. Samples were run in batches with each batch containing all time points and study meals. Study meal total choline (sum of free choline, glycerophosphocholine, phosphocholine, phosphatidylcholine, and sphingomyelin), betaine, and carnitine contents were quantified by the methods of Koc *et al.* [14] and Holm *et al.* [15] with modifications [16]. All meals were quantified in a single batch.

2.5.2.1 Sample preparation

For plasma metabolites, 100 μL plasma was mixed with 200 μL ACN and 10 μL internal standard, and centrifuged at 20 817 × g for 5 min at room temperature. Stock solutions for the internal standard were prepared by mixing d3-MA hydrochloride (Sigma-Aldrich), d6-DMA hydrochloride (Sigma-Aldrich), *NG*-methyl-L-arginine acetate (Sigma-Aldrich), $^{13}C_3$-TMA hydrochloride (Isotec), d3-betaine ammonium chloride (CDN Isotopes), L-carnitine-d3 hydrochloride (CDN Isotopes), d13-choline chloride (CDN Isotopes), $^{13}C_3$-TMAO (Toronto Research Chemicals Inc), and methyl-^{13}C L-methionine (Cambridge Isotope Laboratories) in methanol:water (1:1). The supernatant was transferred to a vial with a disposable glass insert and 2 μL concentrated NH_4OH and 30 μL ethyl bromoacetate (EBA; 20 mg/mL in ACN) were added. After incubation at room temperature for 45 min, the solution was diluted 1:1 with a mixture of water:ACN:CH_2O_2 (1:1:0.0005) prior to injection into the LC-MS/MS system. The calibration points were prepared from stock solutions to yield the following final concentrations (all in nmol/mL): TMAO (0.04–400), TMA (0.002–0.3), DMA and choline (0.2–30), betaine (0.4–200), and carnitine (0.2–200).

For urine metabolites, 50 μL urine was mixed with 10 μL internal standard, 2 μL concentrated NH_4OH, and 60 μL EBA (20 mg/mL in ACN). After incubation at room temperature for 45 min, the solution was diluted 7:1 with a mixture of water:ACN:CH_2O_2 (1:1:0.0005) and injected into the LC-MS/MS system. The calibration points were prepared from stock solutions to yield the following final concentrations (all in nmol/mL): TMAO and DMA (40–8000); TMA (0.1–20); MA (2–400); and choline, betaine, and carnitine (2-400). Metabolite concentrations in urine were adjusted for Cr, which was measured using the Dimension Xpand chemistry analyzer (Siemens Healthcare Diagnostic) in the Human Nutritional Chemistry Service Laboratory at Cornell University.

For food, 40 mg food homogenate (study meal homogenized in 0.1 M sterilized potassium phosphate buffer) was mixed with 150 μL of 0.1% CH_2O_2 in ACN and 10 μL of internal standard. The mix was centrifuged at 20 817 × *g* for 10 min at 4°C followed by transfer of the supernatant into a vial with a disposable glass insert. Concentrated NH_4OH (2 μL) and 30 μL EBA were added to the supernatant and incubated at room temperature for 45 min. The solution was then diluted 1:1 with a mixture of water:ACN:CH_2O_2 (1:1:0.0005) prior to injection into the LC-MS/MS system. The calibration points were prepared from stock solutions to yield the following final concentrations (all in nmol/extract): TMAO, betaine and carnitine (0.3–80), TMA, and DMA (0.15–40), MA (0.75–200), and choline (1.5–400).

2.5.2.2 LC-MS/MS analyses

Metabolites in urine and study meals were measured using the Accela HPLC system TSQ Quantum Access mass spectrometer (ThermoElectron Corp), while those in plasma were measured using the Surveyor HPLC system TSQ Quantum Ultra mass spectrometer. The injection volume was 10 μL, and the column and autosampler temperatures were at 25 and 5°C, respectively. Metabolites were separated on a Prevail silica column (150 × 2.1 mm, 5 μm; Grace) with a matching guard column (7.5 × 2.1 mm, 5 μm). The mobile phase consisted of ACN and 0.1% formic acid in 0.15 mmol/L ammonium acetate in water (CH_2O_2-NH_4OAc) with the following gradient: 81% ACN, 19% CH_2O_2-NH_4OAc at 0 min with a flow rate of 200 μL/min, 81% ACN, 19% CH_2O_2-NH_4OAc at 8 min with a flow rate of 500 μL/min, then linear gradient to 65% ACN, 35% CH_2O_2-NH_4OAc at 10 min with a flow rate of 500 μL/min, 65% ACN, 35% CH_2O_2-NH_4OAc at 13 min with a flow rate of 400 μL/min, then from 13 to 15 min linear gradient back to 81% ACN, 19% CH_2O_2-NH_4OAc, and from 15 to 20 min the column was equilibrated to original conditions with a flow rate of 500 μL/min.

ESI in the positive ion mode and multiple reaction monitoring were used to detect the following transitions: *m/z* 76.3 → 58.4 for TMAO and *m/z* 162.1 → 60.1 for carnitine. TMA, DMA, and MA were derivatized with EBA and the transitions were as follows: *m/z* 146.1 → 118.1 for TMA-EBA, *m/z* 132.1 → 58.1 for DMA-EBA, and *m/z* 118.1 → 44.3 for MA-EBA.

Metabolite and food concentrations were determined relative to standard curves, which were prepared by mixing various amounts of TMAO, TMA, DMA, and MA with water. The intraassay CV for plasma and urine was 2% and 1% for TMAO, 17% and 4% for TMA, 2% and 2% for DMA, and ND and 8% for MA, whereas the interassay CV for plasma and urine was 6% and 5% for TMAO, 38% and 8% for TMA, 6% and 6% for DMA, and ND and 49% for MA based on in-house control duplicates ($n = 3$ with differing TMAO concentrations for plasma; $n = 2$ with differing TMAO concentrations for urine). The relatively low precision for TMA and MA represents the volatility and/or low abundance of these compounds.

2.5.3 Genotyping

DNA was extracted according to the manufacturer's protocol (DNeasy Tissue kit; Qiagen) from buffy coat. The *FMO3* G472A (rs 2266782) variant [9] was determined using a commercially available fluorescent Taqman probe kit (ThermoFisher Scientific) on the LightCycler® 480 real-time RT-PCR instrument (Roche) as previously described [17].

2.5.4 16S rRNA gene sequencing

Genomic DNA was extracted from ~100 mg of stool using the PowerSoil High-Throughput DNA Isolation Kit (MoBio Laboratories) with bead beating according to the manufacturer's protocol. Bacterial 16S rRNA genes were PCR amplified for the V4 hypervariable region using the 515F and 806R primers as previously described [18] and sequences found at the Earth Microbiome Project (http://www.earthmicrobiome.org/emp-standard-protocols/16s/). PCR reactions consisted of Hot Master PCR mix (Five Prime), 10–100 ng DNA template and 10 μM of each primer, with initial denaturation at 94°C for 3 min followed by 25 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 60 s, extension at 72°C for 90 s, and final extension at 72°C for 10 min. Duplicate PCR reactions were performed for each sample and were combined and purified using magnetic beads (Mag-Bind EZPure, Omega Bio-Tek). Purified PCR amplicons were quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen). Amplicons were then combined into a single tube with a final concentration of 11 ng/μL. Of the 40 stool samples, five samples failed to amplify, thus 35 samples of DNA were sequenced paired-end on the Illumina MiSeq platform at Cornell Biotechnology Resource Center Genomics Facility.

2.6 16S rRNA gene sequence analyses

Matching paired-end sequences were merged using the fastq-join command and demultiplexed in the open-source software package Quantitative Insights Into Microbial Ecology version 1.8.0 [19]. Quality filtering removed sequences containing low-quality reads (Phred quality score ≤25), ambiguous bases, primer mismatches, uncorrectable barcodes, and lengths <200 bp. Of the 35 sequenced samples, nine samples did not meet the quality control filtering, therefore 26 samples were used for the gut microbiome analyses. De novo operational taxonomic unit (OTU) picking was performed using the UCLUST algorithm. Representative OTU sequences were aligned using PyNAST with 80% identity and were taxonomically classified using Ribosomal Database Project (RDP) classifier at 97% identity. A phylogenetic tree was built using FastTree. Rarefaction was performed at 60,000 sequences per sample to calculate α-diversity (within sample diversity) using Faith's phylogenetic diversity, Chao 1 and Observed Species, as well as jack-knifed β-diversity (between sample diversity) using the unweighted UniFrac distance matrices for principal coordinates analysis. A heatmap was created from the log abundance of all genera using the R package.

2.7 Statistical analyses

Statistical analyses were conducted in SAS (Version 9.3, SAS Institute). Two-way repeated measures analysis of variance (ANOVA) using the PROC MIXED model procedure determined the effect of study meal, time, and study meal-by-time interaction on metabolite concentrations in plasma and urine. The covariates, age, BMI, study session order, and genotype (*FMO3* G472A), which did not reach a significance of $p \leq 0.05$, were removed from the model in a stepwise manner. A significant interaction effect was followed by one-way repeated measures ANOVA and Tukey–Kramer post hoc test. Participants were categorized as high-TMAO producers or low-TMAO producers based on the median excretion of TMAO (20%) following the consumption of the egg and beef meals. After sample sequencing and data quality filtering, the gut microbiota composition was compared between high-TMAO producers ($n = 11$) and low-TMAO producers ($n = 15$) using false discovery rate corrected ANOVA and nonparametric anosim statistical method within Quantitative Insights Into Microbial Ecology. Unpaired *t*-test was used to assess differences in the baseline characteristics of the TMAO response groups. Significant differences were reported at a False Discovery Rate adjusted $p < 0.05$. All data are expressed as means ± SEM.

3 Results

3.1 Participant characteristics and baseline metabolite concentrations

Forty healthy men had a mean age of 27.8 ± 1.0 years, BMI of 24.2 ± 0.4 kg/m^2, and serum blood chemistry and blood cell counts within the normal range (Table 1). Thirty-five percent of the participants were homozygous wild-type GG genotype for *FMO3* G472A, 55% were heterozygous GA, and 10% were homozygous variant AA, which is consistent with the distribution observed in the general population [20]. TMAO and its derivatives did not differ across study meals.

3.2 Study meal TMAO content (see Table 2)

The fish meal contained 650 times more TMAO, 200 times more TMA, and 1600 times more DMA compared to the egg

Table 1. Participant characteristics and baseline measures ($n = 40$)

Participant characteristics		
Age	years	27.8 ± 1.0
BMI	kg/m^2	24.2 ± 0.4
Genotype	GG %	35
	GA %	55
	AA %	10
Blood chemistry concentrations (all serum)		
HDL	mg/dL	55 ± 2
LDL	mg/dL	117 ± 5
Cholesterol	mg/dL	170 ± 6
Triglycerides	mg/dL	67 ± 5
Total bilirubin	mg/dL	0.8 ± 0.0
Direct bilirubin	mg/dL	0.2 ± 0.0
ALP	U/L	75 ± 3
Cr	mg/dL	1.1 ± 0.0
CK	U/L	156 ± 10
LDH	U/L	157 ± 3
Amylase	U/L	57 ± 3
Lipase	U/L	157 ± 8
AST	U/L	21 ± 1
ALT	U/L	33 ± 2
GGT	U/L	32 ± 2
Total protein	g/dL	7.6 ± 0.1
Albumin	g/dL	4.3 ± 0.0
BUN	mg/dL	15 ± 1
Cell counts		
WBC	$\times 10^3/\mu$L	5.6 ± 0.2
Lymphocytes	$\times 10^3/\mu$L	1.9 ± 0.1
Monocytes	$\times 10^3/\mu$L	0.2 ± 0.0
Granulocytes	$\times 10^3/\mu$L	3.5 ± 0.2
RBC	$\times 10^6/\mu$L	5.2 ± 0.1
Plasma methylamine metabolite concentrations		
TMAO	nmol/mL	3.3 ± 0.2
TMA	pmol/mL	18.1 ± 0.8
DMA	nmol/mL	1.9 ± 0.0
MA	nmol/mL	ND
Urinary methylamine excretion		
TMAO	nmol/mmol Cr	26.6 ± 1.0
TMA	nmol/mmol Cr	0.1 ± 0.0
DMA	nmol/mmol Cr	27.0 ± 0.3
MA	nmol/mmol Cr	4.2 ± 0.4

Baseline plasma and urine concentrations (averaged across all four sessions) of TMAO and its derivatives TMA, DMA, and MA. Urinary concentrations were adjusted by urinary Cr. Values are mean ± SEM, n = 40.
ALP, alkaline phosphatase; Cr, creatinine; CK, creatine kinase; LDH, lactate dehydrogenase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transpeptidase; BUN, blood urea nitrogen; WBC, white blood cell; RBC, red blood cell; TMAO, trimethylamine-*N*-oxide; TMA, trimethylamine; DMA, dimethylamine; MA, methylamine; ND, not detectable.

and beef meals ($p < 0.0001$), whereas the fruit did not show any detectable levels of these metabolites (Table 2). Food MA did not differ among the study meals. Total choline concentration was 125 times higher in eggs and 38 times higher in beef and fish ($p < 0.0001$) compared to the fruit control. Food betaine content was 27 times higher in beef and fish compared to egg and fruit meals ($p < 0.0001$). Carnitine was highest ($p < 0.0001$) in beef followed by fish and then eggs and not detected in the fruit control.

3.3 TMAO biomarker response to the study meals

Study meal and time interacted ($p < 0.0001$) to influence plasma concentrations of TMAO and its derivatives (Fig. 2). As compared to egg, beef, and fruit control, fish consumption yielded plasma concentrations that were 48–62 times higher for TMAO ($p < 0.0001$), 8–14 times higher for TMA ($p < 0.0001$), and 4–5 times higher for DMA ($p < 0.0001$), all of which peaked at 2 h and remained elevated

Table 2. Food concentrations of trimethylamine-*N*-oxide (TMAO) and its derivatives trimethylamine (TMA), dimethylamine (DMA), and methylamine (MA) as well as total choline (sum of free choline, glycerophosphocholine, phosphocholine, phosphatidylcholine, and sphingomyelin), betaine, and carnitine in egg, beef, fish, and fruit study meals

Food content (mg)	Fruit	Egg	Beef	Fish	*p* value
TMAO	–	0.8 ± 0.1[a]	0.9 ± 0.1[a]	528.9 ± 9.4[b]	<0.0001
TMA	–	0.0 ± 0.0[a]	0.1 ± 0.0[a]	5.1 ± 0.7[b]	<0.0001
DMA	–	0.0 ± 0.0[a]	0.0 ± 0.0[a]	58.5 ± 0.2[b]	<0.0001
MA	–	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	NS
Total choline	3.8 ± 0.1[a]	478.8 ± 15.7[c]	132.3 ± 7.0[b]	161.2 ± 0.6[b]	<0.0001
Carnitine	–	0.0 ± 0.0[a]	71.0 ± 3.0[c]	7.5 ± 0.3[b]	<0.0001
Betaine	0.0 ± 0.0[a]	0.9 ± 0.0[a]	12.7 ± 0.3[b]	12.4 ± 0.2[b]	<0.0001

Values within rows with different letter superscripts are significantly different by one-way ANOVA, Tukey–Kramer post hoc test. Values are mean ± SEM, each meal with five replicates.
NS, not significant; ND, not detectable.

until the end of the 6-h study period. MA concentrations were not detectable in plasma.

Similar to plasma levels, study meal interacted with time ($p < 0.005$) to influence urinary TMAO and its derivative concentrations (Table 3). Fish consumption resulted in urinary excretions that were 46–51 times higher for TMAO ($p < 0.0001$), 9–12 times higher for TMA ($p < 0.0001$), and six times higher for DMA ($p < 0.0001$) as compared to egg, beef, and fruit control during the 6-h study period ($p < 0.0001$).

Because of the substantial TMAO response following consumption of the fish study meal, we were unable to detect differences in TMAO response among the egg, beef, and fruit study meals. Thus, we assessed TMAO response for eggs, beef, and fruit separately from fish, and compared the response to study baseline (Fig. 3). A greater increase in plasma TMAO at each time point was detected for eggs and beef compared to the fruit control ($p < 0.05$). Likewise, the change in urinary TMAO excretion across the 6-h study period was greater following the consumption of eggs and beef compared to the fruit control ($p = 0.03$).

Notably, the individual variations in urinary TMAO response after the consumption of eggs and beef ranged from –30% to 270% (Fig. 4). We therefore stratified our participants into high-TMAO producers ($n = 11$; those with ≥20% increase in urinary TMAO in response to eggs and beef) versus low-TMAO producers ($n = 15$; those with <20% increase in urinary TMAO in response to eggs and beef) for the microbiome analyses (see Section 3.4). We also assessed for differences in the baseline characteristics of the TMAO response groups but none were detected (see Supporting Information Table 1).

3.4 Gut microbiota composition

To address whether microbiota composition was a determinant of TMAO response, we obtained a one-time baseline stool sample from each participant. Of the 40 stool samples, 26 samples were used in the analyses after amplification and quality control yielding 6,770,441 high-quality gene sequences with average sequences lengths (mean ± standard deviation) of 254 ± 10. High-TMAO producers had lower α-diversity (within-individual) measure than low-TMAO producers ($p = 0.03$ with 999 permutations by Monte Carlo method; Fig. 5). Principal coordinates analysis of the unweighted UniFrac distances and relative abundance of

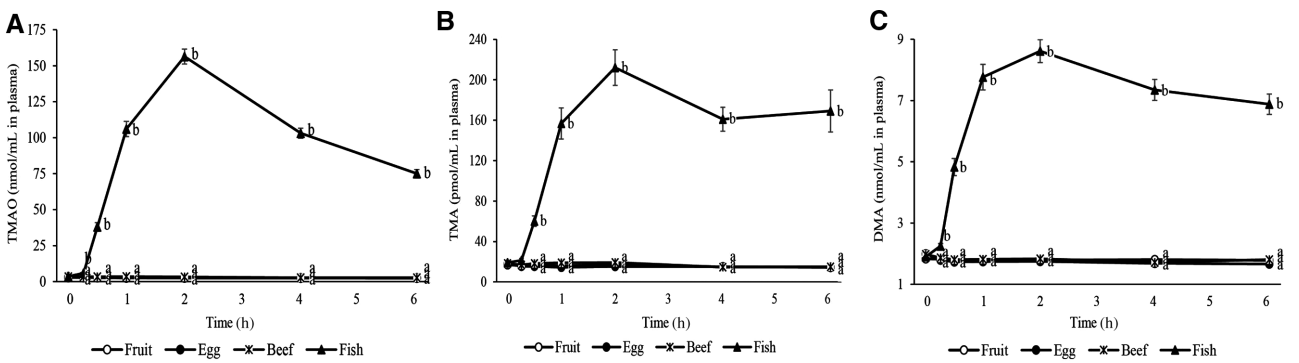

Figure 2. Effects of the study meals on plasma concentrations of trimethylamine-*N*-oxide (TMAO) (A), trimethylamine (TMA) (B), and dimethylamine (DMA) (C) across the 6-h study period. Different letter superscripts show a significant effect of study meal at each time point (one-way ANOVA, Tukey–Kramer post hoc test). Values are mean ± SEM, $n = 40$ per study meal.

Table 3. Effects of the study meals on urinary concentrations of trimethylamine-*N*-oxide (TMAO) and its derivatives trimethylamine (TMA), dimethylamine (DMA), and methylamine (MA) adjusted for creatinine (Cr) at study baseline (0 min) and across the 6-h study period

(nmol/mmol Cr in urine)	Time	Fruit	Egg	Beef	Fish	*p* value
TMAO	0 min	28.4 ± 2.1	24.5 ± 1.9	26.0 ± 2.5	27.9 ± 1.8	NS
	Study (0–6 h)	29.0 ± 1.8[a]	32.2 ± 2.7[a]	31.9 ± 3.0[a]	1486.9 ± 57.4[b]	<0.0001
TMA	0 min	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	NS
	Study (0–6 h)	0.2 ± 0.1[a]	0.1 ± 0.0[a]	0.1 ± 0.0[a]	1.7 ± 0.6[b]	<0.0001
DMA	0 min	27 ± 1	27 ± 1	27 ± 1	27 ± 1	NS
	Study (0–6 h)	30 ± 1[a]	31 ± 1[a]	28 ± 1[a]	180 ± 9[b]	<0.0001
MA	0 min	3.8 ± 0.5	3.3 ± 0.4	5.5 ± 1.2	4.0 ± 0.4	NS
	Study (0–6 h)	4.6 ± 0.4	6.2 ± 0.8	5.9 ± 0.7	6.2 ± 0.6	NS

Two-way ANOVA showed a significant study meal-by-time interaction ($p < 0.005$). One-way ANOVA, Tukey–Kramer post hoc test showed a significant effect of diet for pooled study urine across 6-h indicated by different letter superscripts. Values are mean ± SEM, $n = 40$ per study meal.
NS, not significant.

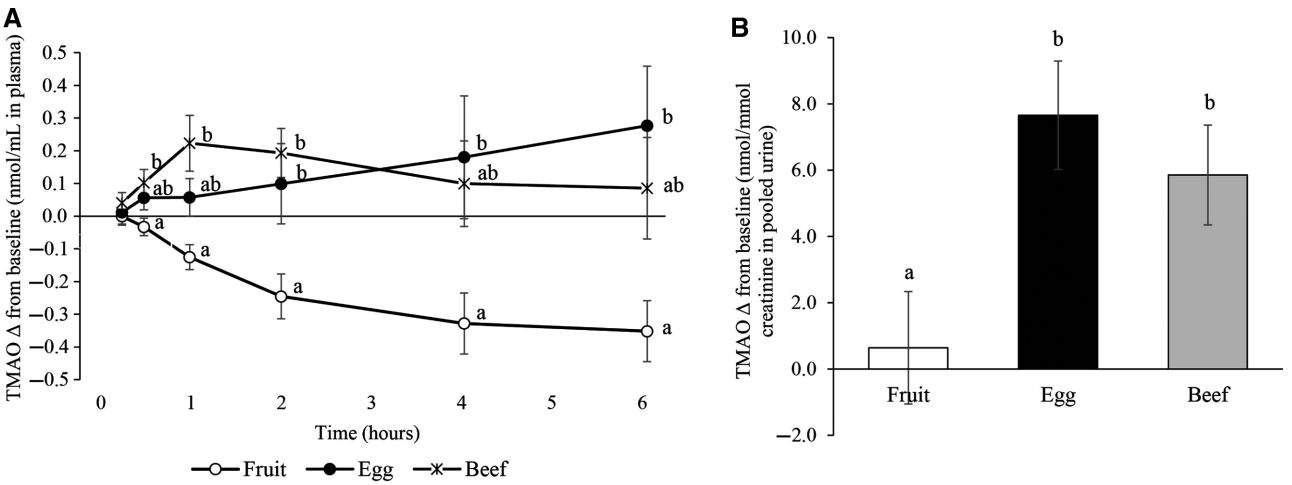

Figure 3. Effects of the study meals (eggs, beef, and fruit only) on trimethylamine-*N*-oxide (TMAO) concentrations in serially collected plasma across time (A) and in pooled urine (B). Data are expressed as changes from study baseline (0 min). Different letter superscripts show a significant effect of study meal (one-way ANOVA, Tukey–Kramer post hoc test). Values are mean ± SEM, $n = 40$ per study meal.

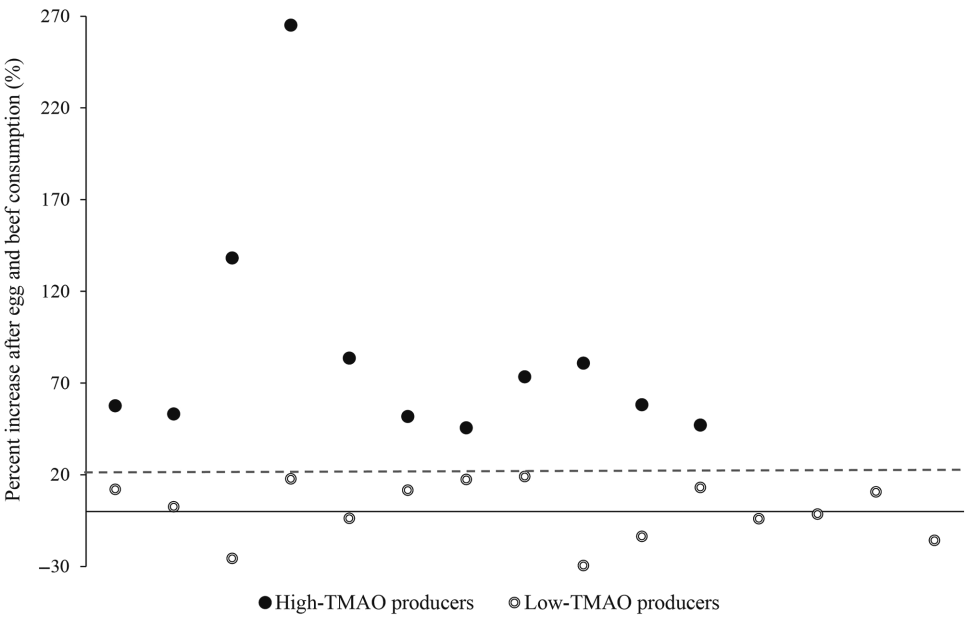

Figure 4. Percent variation of urinary trimethylamine-*N*-oxide (TMAO) concentration after the consumption of egg and beef study meals. The urinary TMAO response was averaged between egg and beef consumption. The variation ranged from –30 to 270% among individuals. The participants were stratified into high-TMAO producers ($n = 11$) shown as black circles and defined as having ≥20% increase in urinary TMAO concentrations after egg and beef consumption, versus low-TMAO producers ($n = 15$) in white circles and defined as having <20% increase in urinary TMAO concentrations.

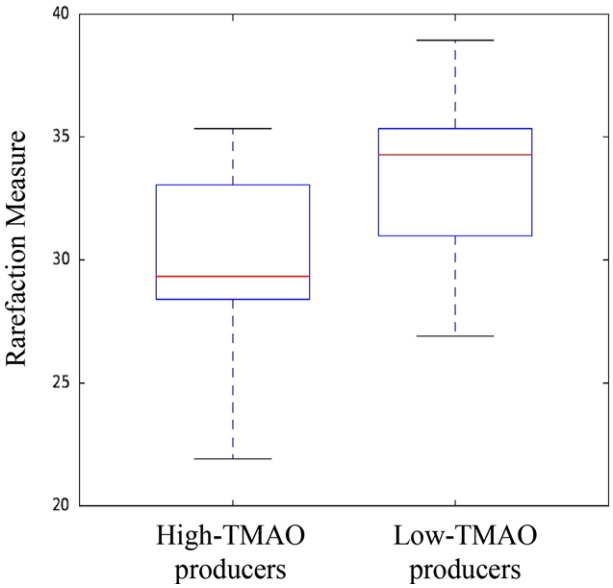

Figure 5. Within-individual differences shown as box-plots (mean ± standard deviation) for the 16S rRNA sequence data from baseline stool samples of healthy male participants ($n = 26$). Alpha diversity metrics were computed for rarefied operational taxonomic unit (OTU) at maximum sampling depth of 60,000 sequences per sample. High trimethylamine-*N*-oxide (TMAO) producers ($n = 11$) were defined as having ≥20% increase in urinary TMAO concentrations after egg and beef consumption, whereas low-TMAO producers ($n = 15$) were defined as having <20% increase in urinary TMAO concentrations. $p = 0.03$ with 999 permutations by Monte Carlo method.

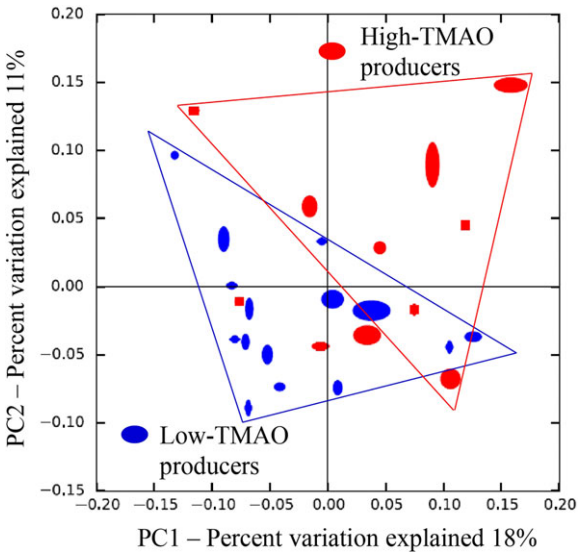

Figure 6. Principal coordinates analysis (PCoA) of the unweighted UniFrac distances for the 16S rRNA sequence data from baseline stool samples of healthy male participants ($n = 26$). Beta diversity metrics (between-individual differences) were jackknifed by repeatedly resampling at 60 000 sequences per sample. Axes show the percent variation explained by the principal components (PCs). High-trimethylamine-*N*-oxide (TMAO) producers ($n = 11$) were defined as having ≥20% increase in urinary TMAO concentrations after egg and beef consumption, whereas low-TMAO producers ($n = 15$) were defined as having <20% increase in urinary TMAO concentrations. The size of the symbols indicates operational taxonomic unit (OTU) clustering in the microbiomes with red points representing those from high-TMAO producers and the blue points from low-TMAO producers. A distinct grouping of the gut microbiota profiles is indicated by the red triangle outline for high-TMAO producers versus the blue triangle outline for low-TMAO producers. $p = 0.04$, $R^2 = 0.11$ with 999 permutations by the anosim method.

the bacteria showed distinct bacterial profiles between low-TMAO producers and high-TMAO producers ($p = 0.04$, $R^2 = 0.11$ with 999 permutations by the anosim method; Fig. 6).

We then visualized gut microbiota differences between high-TMAO producers and low-TMAO producers using a heatmap (Fig. 7). A table of representative OTUs is included as Supporting Information Table 2. High-TMAO producers had 58.1% Firmicutes to 32.6% Bacteroidetes (~2:1 Firmicutes:Bacteroidetes), whereas low-TMAO producers had 47.7% Firmicutes to 47.2% Bacteroidetes (1:1 Firmicutes:Bacteroidetes). High-TMAO producers were represented by Clostridiales within the Firmicutes phylum of which were Clostridiaceae, Lachnospiraceae, and Veillonellaceae. Low-TMAO producers were represented by Bacteroidales within the Bacteroidetes phylum of which were Bacteroidaceae and Prevotellaceae. The Archaea phylum was represented in the low-TMAO producers but absent in the high-TMAO producers.

4 Discussion

This study sought to advance understanding of the effects of animal source foods on TMAO production, and to determine whether this response was influenced by an individual's gut microbiome. Three main findings emerged: (i) fish consumption yielded several-fold higher quantities of TMAO metabolites than either eggs or beef, (ii) dietary TMAO can be absorbed intact without processing by gut microbes, and (iii) high-TMAO producers (≥20% increase in urinary TMAO in response to eggs and beef) had more Firmicutes than Bacteroidetes and a less diverse gut microbiome.

4.1 Fish consumption yields the highest concentrations of plasma and urinary TMAO concentrations compared to all other study meals

Fish consumption yielded ~50 times higher circulating concentrations of TMAO than either eggs or beef, which is consistent with previous reports of substantially higher urinary TMAO concentrations [21, 22] following the consumption of fish compared to meat, dairy, fruits, vegetables, or grain. Notably, plasma TMAO was elevated within 15 min

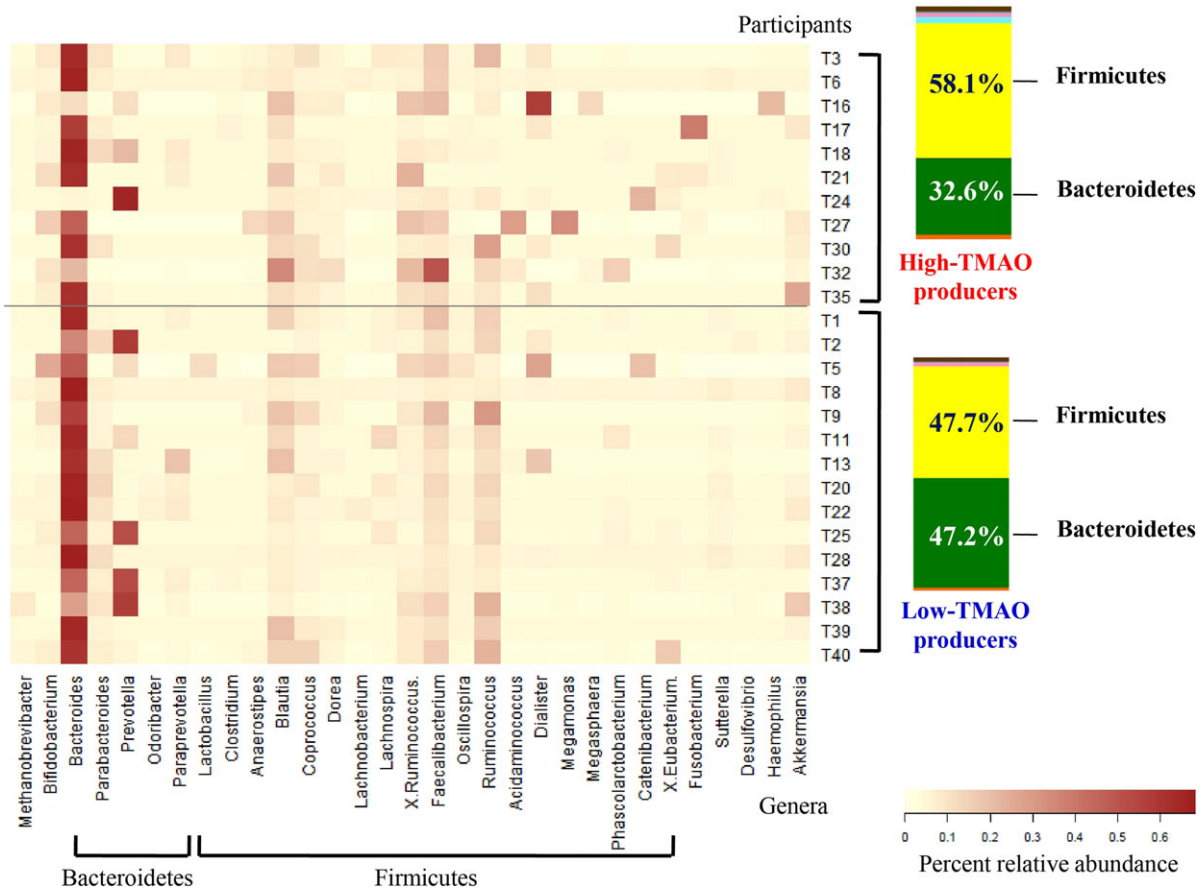

Figure 7. Heatmap of relative operational taxonomic unit (OTU) abundances for the 16S rRNA sequence data from baseline stool samples of male participants ($n = 26$) to differentiate between high-trimethylamine-*N*-oxide (TMAO) producers ($n = 11$; those having ≥20% increase in urinary TMAO concentrations after egg and beef consumption) versus low-TMAO producers ($n = 15$; those having <20% increase in urinary TMAO concentrations). The taxonomic assignment of each OTU represents the gut microbiota at the genus level, which is grouped into the phylum level as indicated with black square brackets. The color key corresponds to percent relative abundance of the gut microbiota of OTU at each expression level.

of fish consumption indicating that the absorption of dietary TMAO may occur without processing to TMA by the gut microbes as previously proposed [23]. The sustained elevation in circulating TMAO in response to fish consumption, despite substantial urinary excretion, suggests that a portion of the TMAO is retained by the body possibly due to its functions as an osmolyte [4] and protein stabilizer [24]. Other derivatives influenced by diet, albeit to a lesser extent than TMAO, included TMA and DMA, both of which showed higher levels in response to fish versus eggs and beef.

In light of the recent proposed role of TMAO as a causative agent for cardiovascular disease [7, 23], and a recent report of higher serum TMAO concentrations and accelerated aortic plaque formation in apoE null mice with increased fish intake [25], some researchers have advocated for the restriction of animal source foods that raise circulating TMAO concentrations [7]. However, these animal source foods are enriched in nutrients that are required for optimal health [26], and fish consumption is well known for its cardioprotective attributes in humans [27]. Moreover, in more recent studies, circulating TMAO and carnitine concentrations were inversely associated or showed no association with the development of chronic diseases [28, 29]. Thus, caution is warranted when proposing dietary recommendations that restrict the intakes of animal source foods because of their TMAO-raising characteristics.

4.2 TMAO response to dietary precursors may be a biomarker of the gut microbiota composition

Gut microbes are known to participate in modulating TMAO response to its dietary precursors and potentially chronic disease susceptibility [6, 23, 30]. However, TMAO response is highly variable and it is unclear whether this variation arises from individual differences in gut microbiota composition. To address this question, we used urinary TMAO excretion to stratify our participants into "high" (≥20% increase) or

"low" (<20% increase) TMAO producers, and examined differences in the gut microbiota composition between the two groups.

Although differences in the gut microbiota were subtle at the individual OTU level, high-TMAO producers were characterized by enriched ratios of Firmicutes to Bacteroidetes compared to low-TMAO producers (Fig. 4), which is consistent with previous reports that TMAO production potential is detected in Firmicutes but absent in Bacteroidetes [31]. Furthermore, low-TMAO producers had more archaea *Methanobrevibacter*, which has been proposed to deplete host TMA levels [32]. Lower α- diversity (within-individual difference) was also observed among high-TMAO producers indicating that TMAO production may be driven by a select set of bacteria (e.g., Firmicutes).

Distinct gut microbiota profiles between high-TMAO compared to low-TMAO producers in response to its dietary precursors indicate that circulating TMAO may be a biomarker of the gut microbiota. As such, higher circulating concentrations of TMAO in a disease versus nondisease state may reflect differences in gut microbe composition, rather than indicating a causative role of TMAO in the disease process. A greater ratio of Firmicutes to Bacteroidetes has previously been associated with increased risk of obesity and metabolic syndrome [33], and this gut microbiota characterization may also be linked to atherosclerosis-associated dysbiosis. The significance of these findings is that circulating TMAO may be used to identify individuals with microbiota profiles that increase disease susceptibility, and thereby inform the development of dietary and pharmaceutical strategies aimed at increasing gut microbiota diversity and restoring the symbiotic relationship between the gut microbes and their host.

4.3 Study limitations and future directions

This was a short-term feeding study that focused on advancing current understanding of TMAO metabolism in healthy male adults. As such, the findings may not reflect long-term effects of the diet and may not be generalizable to other segments of the population including women and those with health conditions. In addition, predisease clinical endpoints, such as markers of inflammation, were not evaluated. More studies are needed to address these important issues and evaluate the clinical utility of lowering circulating concentrations of TMAO as a means to improve human health.

5 Conclusion

Consumption of fish, which is high in TMAO, yielded substantially greater increases in circulating TMAO than eggs or beef, which contain high amounts of dietary TMAO precursors. The rapid rise in circulating TMAO in response to fish consumption demonstrated that the absorption of intact dietary TMAO occurs in a manner that is independent of the gut microbes. The higher Firmicutes to Bacteroidetes microbial enrichment among men exhibiting greater response to dietary TMAO precursor intake indicates that circulating TMAO can reflect a person's microbiota, which may in turn modulate their risk of disease.

C. E. C. contributed to the study design, collected the data, conducted the statistical analyses, interpreted the data, and prepared the manuscript. S. T. contributed to the study design, collected the data and assisted in the statistical analyses and data interpretation. O. V. M., E. B., and J. Y. provided technical assistance and contributed to the data collection. N. F. T. assisted in the data collection and study meal metabolite measurements. J. L. S. provided technical assistance for the 16S rRNA gene sequencing. M. A. C. conceived the study, contributed to the data interpretation. and critically reviewed the manuscript. All authors read and approved the final manuscript.

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