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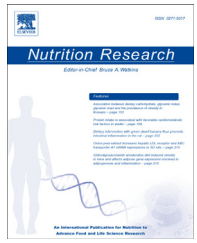

In older women, a high-protein diet including animal-sourced foods did not impact serum levels and urinary excretion of trimethylamine-N-oxide

Wendy J. Dahl[a,*], Wei-Lun Hung[c], Amanda L. Ford[a], Joon Hyuk Suh[a], Jerémié Auger[b], Varuni Nagulesapillai[b], Yu Wang[a]

[a] Department of Food Science and Human Nutrition, University of Florida, 359 Newell Dr, Gainesville, FL 32611, USA
[b] Rosell Institute for Microbiome and Probiotics, 6100 Royalmount, Montreal, Quebec,, Canada H4P 2R2
[c] School of Food Safety, Taipei Medical University, 250 Wu-Hsing St, Taipei, 11031, Taiwan

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ABSTRACT

Diets including red meat and other animal-sourced foods may increase proteolytic fermentation and microbial-generated trimethylamine (TMA) and, subsequently, trimethylamine-N-oxide (TMAO), a metabolite associated with increased risk of cardiovascular disease and dementia. It was hypothesized that compared to usual dietary intake, a maintenance-energy high-protein diet (HPD) would increase products of proteolytic fermentation, whereas adjunctive prebiotic, probiotic, and synbiotic supplementation may mitigate these effects. An exploratory aim was to determine the association of the relative abundance of the TMA-generating taxon, *Emergencia timonensis*, with serum and urinary TMAO. At 5 time points (usual dietary intake, HPD diet, HPD + prebiotic, HPD + probiotic, and HPD + synbiotic), urinary (24-hour) and serum metabolites and fecal microbiota profile of healthy older women (n = 20) were measured by liquid chromatography–tandem mass spectrometry and 16S rRNA gene amplicon sequencing analyses, respectively. The HPD induced increases in serum levels of L-carnitine, indoxyl sulfate, and phenylacetylglutamine but not TMAO or *p*-cresyl sulfate. Urinary excretion of L-carnitine, indoxyl sulfate, phenylacetylglutamine, and TMA increased with the HPD but not TMAO or *p-cresyl sulfate*. Most participants had undetectable levels of *E. timonensis* at baseline and only 50% during the HPD interventions, suggesting other taxa are responsible for the microbial generation of TMA in these individuals. An HPD diet with or without a prebiotic, probiotic, or synbiotic elicited an increase in products of proteolytic fermentation. The urinary L-carnitine response suggests that the additional dietary L-carnitine provided was primarily bioavailable, providing little substrate for microbial conversion to TMA and subsequent TMAO formation.

Abbreviations: ALT, alanine aminotransferase; ASV, amplicon sequence variant; CKD, chronic kidney disease; FMO3, flavin-containing mono-oxygenase 3; HPD, high-protein diet; IS, indoxyl sulfate; LC, liquid chromatography; MS, mass spectrometry; PAG, phenylacetylglutamine; PCS, *p*-cresyl sulfate; QC, quality control; TMA, trimethylamine; TMAO, trimethylamine-N-oxide.

* *Corresponding author:* Tel.: +1 352 294 3707.
E-mail address: wdahl@ufl.edu (W.J. Dahl).

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1. Introduction

In the United States, protein intake is increasing, and although dietary guidance recommendations are to reduce red meat, intake is not decreasing [1]. For older adults, muscle strength and functionality benefits are seen with protein intakes exceeding the RDA by 50% or more [2]. However, higher protein intakes, particularly from meat, have been shown to increase proteolytic fermentation [3], which may have deleterious effects [4]. Unrestricted omnivore diets increase urinary excretion of *p*-cresyl sulfate (PCS) and indoxyl sulfate (IS), microbial-generated from tryptophan and tyrosine, respectively, compared to a vegetarian diet [5]. This difference is thought to be due to the lower protein intake of a vegetarian diet but also higher microbial-available carbohydrate, specifically fiber and resistant starch, promoting saccharolytic vs proteolytic fermentation [5]. PCS and IS are considered uremic molecules in chronic kidney disease (CKD) [6]; however, their health implications in the non-CKD population, particularly their contribution to cardiovascular risk, remain uncertain [7,8]. In contrast, as IS modulates mucosal barrier function, lower urinary excretion may indicate dysbiosis [9]. Furthermore, serum levels of phenylacetylglutamine (PAG) from the microbial conversion of phenylalanine may be an independent risk factor for cardiovascular disease and mortality [10]. Although markers of proteolytic fermentation, the health implications of PCS, IS, and PAG in the non-CKD population remain unclear.

In addition to products of anaerobic microbial degradation of aromatic amino acids, higher-protein diets including red meats provide more L-carnitine, which, if not absorbed, undergoes a multistep transformation by the gut microbiota to produce trimethylamine (TMA) [11]. Following intestinal uptake, TMA is rapidly oxidized by flavin-containing monooxygenase 3 (FMO3) into trimethylamine-*N*-oxide (TMAO), a metabolite that has been associated with cardiovascular [12,13], hypertension [14], and Alzheimer disease risk [15]. Although TMAO generation is affected by FMO3 expression [16], the considerable intraindividual variation suggesting other factors such as the composition of microbiota [17], in addition to dietary intake [11], may influence its production. The relationships among microbiota composition, L-carnitine, and its microbial-generated metabolite require further elucidation. Herein, the impacts of a controlled, high-protein diet in older women on serum and urinary L-carnitine, TMA, and TMAO, and markers of proteolytic fermentation are examined. It was hypothesized that the HPD, including animal-sourced foods, would increase proteolytic activity in comparison to usual dietary intake and that prebiotic, probiotic, and synbiotic supplementation may mitigate the proteolytic response through their potential influence on stool form [18,19] which is associated with proteolytic fermentation [20], microbiota composition [21], or enhanced saccharolytic fermentation [22,23]. An exploratory aim was to determine the association of the relative abundance of *Emergencia timonensi*, a species implicated as the rate-limiting step of the conversion of L-carnitine to TMA [11], with TMAO, hypothesizing that abundance would be positively associated with serum and urinary TMAO. Older women were targeted because higher-protein dietary patterns are recommended to prevent loss of lean body mass—women being at higher risk [2]. This is a report of secondary outcomes of a trial examining the effect of the high-protein diet on microbiota profile, probiotic strain recovery, and wellness in older women [24].

2. Methods and materials

2.1. Study design

As previously reported, an 18-week, double-blind, placebo-controlled, crossover trial with four 2-week controlled high-protein diet (HPD) periods was carried out in Florida, USA, from May 2015 to December 2015 [24]. In brief, following a 2-week baseline of usual dietary intake (0.9±0.2 g/kg body weight from protein), participants were randomized by Latin-square design to the following: HPD, HPD + probiotic, HPD + prebiotic, and HPD + synbiotic, separated by 2-week washouts. The probiotic formulation contained *Bifidobacterium bifidum* HA-132 (1.54 billion), *B breve* HA-129 (4.62 billion), *B longum* HA-135 (4.62 billion), *Lactobacillus acidophilus* HA-122 (4.62 billion), and *L plantarum* HA-119 (4.62 billion) (Lallemand, Montreal, Quebec, Canada), and 5.6 g of inulin (Sensus FRUTAFIT IQ, Lawrenceville, NJ, USA) was provided as the prebiotic. During the synbiotic period, both the probiotic formulation and the inulin were provided; placebos were previously described [24]. Fasting blood, 24-hour urine, and stools were collected at the end of baseline and each HPD period. The Institutional Review Board (IRB-01 protocol #2014-00955) at the University of Florida approved this study, and it was performed in accordance with the Declaration of Helsinki. All participants provided written informed consent. The study is registered at clinicaltrials.gov NCT#02445560.

2.2. Participants

Healthy women, 65 years and older with a body mass index of less than 30, were recruited for the study. Vegetarians were excluded, and inclusion criteria required a habitual diet of <15% dietary protein and <20 g/d dietary fiber. In brief, individuals were excluded if they had a current immune-modulating disease, diabetes, chronic kidney disease, food allergies/dietary restrictions, or gastrointestinal disease; were taking medications for diarrhea/constipation; were current smokers; had alcoholic beverage intake of >1 drink per day; had recent antibiotic therapy; or planned to lose/gain weight.

2.3. High-protein diet

As reported previously [24], the HPD provided, on average, 29% of energy from protein (1.5-2.2 g/kg/d), 26% fat, and 45% carbohydrate. Individual energy levels provided were set following a 7-day assessment of participants' energy expenditures using SenseWear Pro 2 armbands (Body Media Inc, Pittsburgh, PA, USA). Fiber was provided at 15.5 g/d, a level matched to the mean usual intake of the participants during

baseline (15.1 g/d), in an attempt to control for the potential confounding effects of fiber intake. The 4-day cycle menu included precut frozen meat, fresh and single-serving packaged fruit and vegetables/salads, frozen meals, protein beverages, breakfast items, and snacks and provided all the dietary intake for the participants during the 2-week HPD intervention periods with the exception of non-energy-providing beverages (eg, coffee, tea, water) which were unrestricted. Beef was included in 3 of 4 of the evening meals, and other sources of animal-sourced protein included chicken, pork, fish (salmon), and eggs, as well as various plant sources of protein, thus providing dietary sources of L-carnitine and other potential substrates (eg, choline) for the microbial generation of TMA. A sample menu has been previously published [24].

3. PCS, IS, and PAG analyses

Urine collections (24 hours) were made using containers (Fisherbrand Low Form 24-Hour Urine Collection Containers, Pittsburgh, PA, USA; 3.5 L) and apparatus (Dover Commode Specimen Collector 800 mL, Dover, OH, USA) provided and were refrigerated during the collection period. Aliquots were stored at –80°C until analyses. Solvents were sourced from Fisher Scientific (Waltham, MA, USA), PCS and IS from Alsachim (Illkirch-Graffenstaden, France), PAG from LGC (Middlesex, UK), *p*-toluene sulfonic acid sodium salt from Sigma Aldrich (St. Louis, MO, USA), and liquid chromatography (LC)–mass spectrometry (MS)-grade formic acid from Fisher Scientific.

The method of quantification of metabolites was adapted from Cuoghi et al [25]. Urine samples (20 μL) were mixed with 5 μL *p*-toluene sulfonic acid (200 μg/mL, as internal standard) and followed by addition of 975 μL acetonitrile for protein precipitation. After shaking for 2 minutes, the mixture was centrifuged at 20,000*g* at 4°C for 5 minutes. After centrifugation, 500 μL supernatant was diluted with 500 μL 0.5% formic acid aqueous solution and then filtered using 0.22-μm nylon filter prior to LC-MS analysis. PCS, IS, and PAG in urine were quantified using the following methods. A Thermo Ultimate 3000 HPLC was equipped with a Thermo Quantiva triple quadrupole electrospray ionization tandem mass spectrometer (Thermo, Waltham, MA, USA). Chromatographic separations were performed using an Acquity BEH C18 column (1.7 μm, 2.1 × 50 mm) (Waters, Milford, MA, USA) with a mobile phase consisting of 0.1% formic acid aqueous solution (A) and 0.1% formic acid in acetonitrile (B). The gradient program was set as follows: 0-3 minutes, 50% B, 3-3.5 minutes, ramped to 95% B; 3.5-6 minutes, 95% B; 6-6.1 minutes, 50% B; 6.1-9 minutes, 50% B. The flow rate was set at 0.2 mL/min. The column temperature was maintained at 25°C. The injection volume was 10 μL. Multiple reaction monitoring in negative mode was used for quantification at *m/z* 171 □ 80 for *p*-toluene sulfonic acid; *m/z* 187 □ 107 for *p*-cresol sulfate, and *m/z* 212 □ 80 for indoxyl sulfate, while *m/z* 265 □ 130 was used for PAG in positive mode. The spray voltage in positive and negative modes was set at 3500 and 2500 V, respectively. Other MS parameters were as follows: sheath gas, 45 Arb; aux gas, 15 Arb, sweep gas, 1 Arb; CID gas, 0.002 mm Hg, ion transfer tube temperature: 325°C, vaporizer temperature: 300°C.

3.1. L-Carnitine, TMA, and TMAO analyses

The standards, L-carnitine, TMA hydrochloride, and TMAO, were purchased from Sigma-Aldrich (St. Louis, MO, USA). For internal standards, trimethylamine-d_9 hydrochloride was obtained from Toronto Research Chemicals Inc. (North York, Ontario, Canada), and L-carnitine-methyl-d_3 hydrochloride and trimethylamine-d_9-*N*-oxide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Synthetic urine for blank sample was provided by Spectrum Labs (Cincinnati, OH, USA). Acetonitrile, water, ammonium formate, and formic acid were of LC-MS grade and were purchased from Fisher Scientific (Fair Lawn, NJ, USA).

Forty microliters of serum (or 20 μL of urine) was aliquoted into 1.5-mL Eppendorf tube and mixed with acetonitrile containing 3 internal standards to a total volume of 200 μL. The sample was vigorously vortexed for 5 minutes and centrifuged at 20,000*g* for 10 minutes. The supernatant was transferred into a vial, and 2 μL of this solution was injected into LC–tandem MS system. LC–tandem MS analyses were carried out using an Ultimate 3000 LC system coupled to a TSQ Quantiva triple quadrupole MS (Thermo Fisher Scientific, San Jose, CA, USA) using hydrophilic interaction chromatography. The analytes were separated using a Thermo Scientific Accucore hydrophilic interaction chromatography column (2.1 × 100 mm, particle size 2.6 μm) at a column temperature 30°C using a gradient elution with acetonitrile (eluent A) and 15 mmol/L ammonium formate (pH 3.5) (eluent B). The gradient was as follows: 0-4 minutes 10%-40% B and 4-6 minutes 40% B. The column was re-equilibrated in 4 minutes using the initial composition of mobile phase. The column temperature was 30°C, and the flow rate was 0.4 mL/min. Injection volume was set at 2 μL. The mass spectrometer was equipped with an electrospray ionization interface operating in the positive ionization mode. The electrospray ionization parameters were as follows: spray voltage, 2500 V; ion transfer tube temperature, 350°C; vaporizer temperature, 350°C; sheath gas, 45 Arb; aux gas, 15 Arb; and sweep gas, 1 Arb. The MS/MS detection was operated using selective reaction monitoring mode. Dwell time was 100 milliseconds, and CID gas was set at 0.002 mm Hg. MS/MS parameters for each analyte were optimized using flow injection analysis of individual standards. Xcalibur software (Ver. 3.0, Thermo, Waltham, MA, USA) was used for data processing and instrument control. Samples were analyzed in accordance with the validated method. Calibration and quality control (QC) samples were prepared freshly in every batch. Each batch included a calibration curve, a double blank (blank with no analyte and internal standard), a blank (blank with internal standard), and triplicate QC samples at 2 concentrations within the calibration range. The samples were interspersed with calibration standards and QC samples within the batch. The precision was expressed as the relative standard deviation, and the accuracy was expressed as the percentage value of observed analyte concentration to true concentration. The batch reliability was evaluated by analyzing QC samples with a precision of <15% (relative standard deviation) and an

accuracy between 85% and 115%. The analytes in this study were endogenous compounds present in blank urine and serum. Calibration standards for urine were therefore prepared using synthetic blank urine; however, calibration standards for serum were prepared using mobile phase described elsewhere [26-28] because blank serum was not available commercially. This was compensated by using deuterium internal standards and assessing recovery and matrix effect for serum. The recovery was investigated by spiking pooled serum and urine samples with 3 different concentrations of the analytes and was calculated by measuring the amount of analyte added as compared to known amounts of the pure standard. Matrix effect was determined by comparing the response of postextraction samples with those of corresponding pure standard. Recovery of the established method was greater than 90% for all analytes, and matrix effect was 97%-104%.

3.2. *Fecal E timonensis abundance*

Single stools were collected at baseline and during the last 3 days of each HPD intervention, put on ice, aliquoted, and stored at –80°C within 6 hours of defecation. Details of the genomic DNA extraction, amplification, sequencing, and general comparisons have been reported elsewhere [24]. In brief, the QIAamp Fast DNA Stool Mini Kit (Qiagen), Valencia, CA, USA was used for genomic DNA extraction as per manufacturer's protocol, with minor modifications; DNA concentrations were determined using a Nanodrop Spectrophotometer (ND-1000; V3.8.1 program, Waltham, MA, USA). DNA was amplified using primers from the V4 hypervariable region of the 16S rRNA gene and tagged. The bacterial 16S rRNA gene libraries were prepared using the Illumina's "16S Metagenomic Sequencing Library Preparation" guide (Part # 15044223 Rev. B) (Illumina Canada Inc., Vancouver, BC, Canada). The quality filter software of QIIME2 (Quantitative Insight Into Microbial Ecology–2) was used to process the demultiplexed amplicons [29]. The imported data set was inspected, the reads were trimmed at 240 base pairs, and the quality-filter *q*-score using the default parameters was used for quality control. The reads were then clustered into amplicon sequence variants (ASVs) with the denoising algorithm Deblur of the QIIME2 suite [29]. The feature classifier was used to attribute the ASVs to the closest known taxa using QIIME2's sk-learn machine learning classification module [29-34]. The taxonomy file (linking ASV sequences to known taxonomic groups) was trained on a 99% clustered GreenGenes database [35] to which the rRNA sequences of *E timonensis* were added. The tables of known taxa and relative abundance were collapsed at the genus level and exported from the QIIME2 framework for further analysis.

3.3. *Statistical analyses*

Urine and serum metabolites were analyzed per protocol (n = 20). Unless noted otherwise, data are presented as mean ± SE. α was set at .05. A repeated-measures linear mixed model was used analyze the data, comparing the 5 treatments, baseline and the 4 HPD periods (model 1), and the 4 HPD periods (model 2), where subject was treated as random, and an autoregressive correlation structure was used account for the repeated measures. Sequence and period were treated as fixed effects, and a post hoc linear contrast was used to test for overall differences from baseline for all treatment groups. All responses were log transformed to meet the model assumptions of normality and constant variance.

4. Results

4.1. *Participants*

Participants with complete blood, urine, and stool collections were older women (n = 20, 72.4 ± 5.0 years) with a body mass index of 25.8 ± 3.1 (mean ± SD) and a range from 20.0 to 29.9. The per-protocol study flow diagram is presented in Fig. 1 [24]. The mean urine urea nitrogen at baseline (usual diet) was 8.8 ± 2.6 g/24 h and 13.9 ± 3.2 g/24 h ($P < .001$) during the high-protein interventions [24], representing an estimated 32-g/d average increase in protein, assuming a 16% nitrogen content of the protein provided. Participants exhibited normal fasting glucose (<100 mg/dL) with the exception of 1 participant at 103 ± 5 mg/dL. Estimated glomerular filtration rate averaged 84 ± 9 mL/min/1.73 m^2 using the 2009 CKD-EPI creatinine equation of the CKD Epidemiology Collaboration [36] and 83 ± 11 mL/min/1.73 m^2 using the Modification of Diet in Renal Disease Study equation [37] with no participants exhibiting an estimated glomerular filtration rate below 60 mL/min/1.73 m^2. Liver function tests, alkaline phosphatase, aspartate aminotransferase, and alanine aminotransferase (ALT) were reported as normal throughout the study with the exceptions of 2 elevations, not considered clinically significant, during the first HPD period, specifically ALT in 1 subject and ALT and aspartate aminotransferase in 1 subject. Additional details of metabolic parameters of participants are reported elsewhere [24].

5. PCS, IS, and PAG analyses

No differences were seen for urinary PCS, but increases were seen for IS and PAG in all HPD periods compared to baseline (Table 1). Similarly, no change was seen for serum level of PCS, but IS and PAG were increased in the HPD periods compared to baseline (Table 1). When only the 4 HPD periods were compared, no significant differences were seen (Table 1).

5.1. *L-Carnitine, TMA, and TMAO analyses*

There was a significant period effect for total 24-hour urinary excretion of L-carnitine and TMA with baseline (usual diet) differing from all HPD periods, but no change in TMAO (Table 1). When only the 4 HPD periods were compared, there were no differences in urinary L-carnitine, TMA, or TMAO. Serum levels of L-carnitine were slightly but significantly increased in all HPDs compared to baseline, but levels did not differ between HPD periods (Table 1). Serum levels of TMAO did not differ from baseline or between HPD treatments.

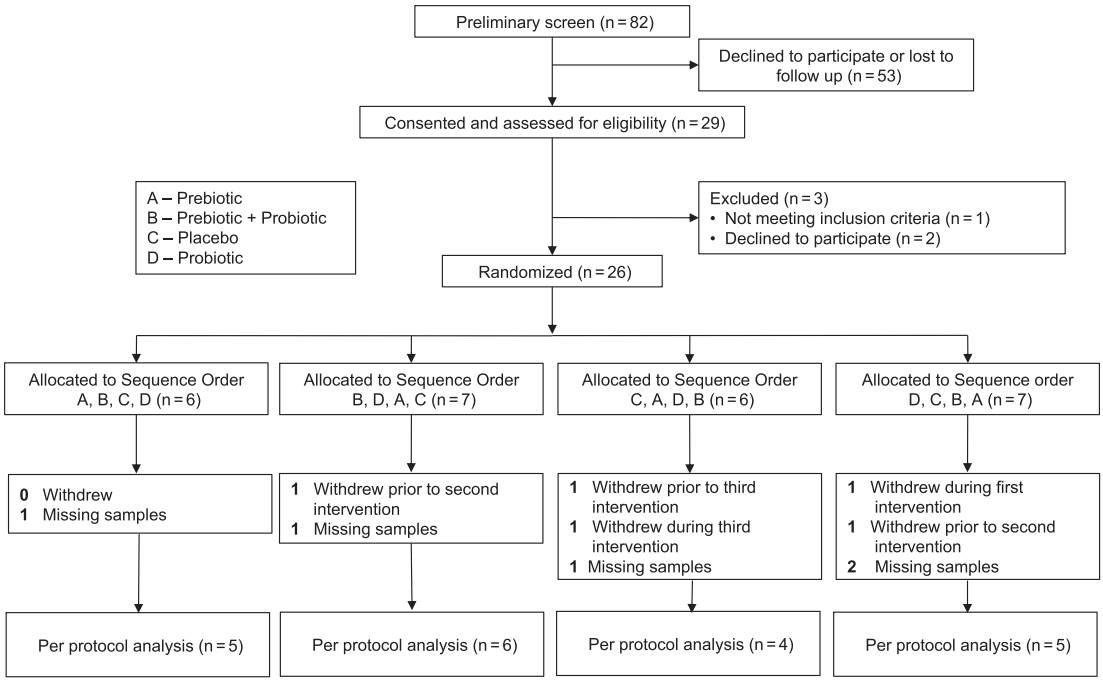

Fig. 1 – Participant recruitment and study flow diagram (per protocol).

5.2. *Fecal E timonensis abundance*

Relative abundance of *E timonensis* is presented in Fig. 2. Of the 20 participants, *E timonensis* was not detectable at baseline in 16 (80%). Following exposure to the HPD, *E timonensis* was detectable in at least 1 of the HPD periods in an additional 6 participants, whereas 10 (50%) of participants exhibited no detectable levels of *E timonensis* at any time point.

6. Discussion

Markers of protein fermentation include the metabolites of aromatic amino acids [38]. The results of the present study demonstrate that providing a high-protein diet significantly increased the metabolite levels reflective of proteolytic fermentation, and thus, we accept our primary hypothesis. The increases in serum and urinary IS and PAG suggest an increase in proteolytic fermentation with the high-protein diet, an expected outcome particularly following a diet containing meat [3]. The potential health effects of IS and PAG in the non-CKD population have not been well described. However, as PAG has been implicated in cardiovascular disease risk in individuals with CKD [10], this metabolite from proteolytic fermentation, specifically from red meat [39], and its relationship with chronic disease require examination. We did not see an increase in urinary PCS, a common indicator of proteolytic fermentation, which has been previously shown to increase in healthy subjects fed a high-protein vs a low-protein diet [40]. The results of the present study provide no evidence to support the efficacy of the prebiotic, probiotic, or synbiotic supplement in mitigating serum or urinary levels of these compounds, and thus, we reject our secondary hypothesis. This is in contrast to a study showing that the probiotics *L casei* Shirota and *B breve*, administered with oligofructose and inulin, resulted in a significant decrease in *p*-cresol excretion in healthy adults [41]. In addition, a recent in vitro study concluded that the addition of a prebiotic to fecal fermentation inhibited proteolysis [42]. In the present study, a 5-g supplement of prebiotic was provided, and although this dose increased total fiber and thus carbohydrate substrate provision to the microbiota, a higher dose may be needed to elicit an effect in vivo.

We assessed the TMAO response of older women to a controlled HPD high in L-carnitine, a potential substrate for the microbial bioconversion to TMA and subsequent postabsorption oxidation to TMAO. Animal studies demonstrating increased TMAO, and its effects have exclusively tested supplemental L-carnitine [43]. Similarly, in some human trials, supplemental L-carnitine, known to have very low bioavailability [44], has been evaluated vs food sources with much higher bioavailability. For example, in older women, 1500 mg L-carnitine–L-tartrate supplementation elevated TMAO to 33.6 ± 6.7 *μmol/L* compared to 2.9 ± 0.3 *μmol/L* in the placebo group [45]. In the present study, wherein only dietary sources of L-carnitine were provided, mean serum TMAO was 8.1 *μmol/L* at baseline and 8.5 *μmol/L* on the HPD, suggesting little potential to contribute to chronic disease risk for the group as a whole. The baseline serum TMAO of our participants was similar to mean of 8.3 *μmol/L* reported by Mitchell et al in older men [46] but higher than the reported reference value of 3.5 *μmol/L* for healthy individuals in the United States [47]. In general, subjects with lower levels at baseline tended to increase with the HPD, whereas those with higher levels at baseline decreased or remained relatively unchanged during HPD periods. Thus, if the HPD had been tested on participants with lower baseline values, a significant TMAO increase may have been seen. Ge et al reported a

Table 1 – Urinary and serum metabolites of healthy older women at baseline and in response to four high protein diet periods.

	Baseline	High protein diet control+placebo	High protein diet control+probiotic[1]	High protein diet prebiotic[2]+placebo	High protein diet probiotic[1]+ prebiotic[2]	Model 1 P-value	Model 2 P-value
Urinary metabolites				mmol(mean±SE)			
	0.63±0.10 [a]	1.05±0.36 [a]	0.93±0.18 [a]	0.89±0.16 [a]	0.70±0.11 [a]	S:0.05 T:0.12 S*T:0.71	I:0.90 T:0.91
p-Cresyl sulfate	0.33±0.04 [b]	0.57±0.16 [a]	0.50±0.07 [a]	0.43±0.06 [a]	0.38±0.04 [a]	S:0.46 T:0.005 S*T: 0.46	I:0.17 T:0.19
Indoxyl sulfate	1.08±0.14 [b]	1.76±0.40 [a]	1.70±0.31 [a]	1.49±0.21 [a]	1.46±0.20 [a]	S:0.14 T:<0.001 S*T:0.56	I:0.56 T:0.40
Phenylacetylglutamine	73.9±14.9 [b]	194.6±32.2 [a]	192.4±27.4 [a]	190.7±22.5 [a]	151.3±20.9 [a]	S:0.43 T:<0.0001 S*T:0.72	I:0.14 T:0.03
L-Carnitine	8.7±1.3 [b]	420.4±324.3 [a]	10.3±1.6 [a]	12.2±2.7 [a]	9.2±1.6 [a]	S:0.83 T:0.04 S*T:0.10	I:0.22 T:0.22
Trimethylamine	1165±298 [a]	1104±237 [a]	1274±156 [a]	1255±225 [a]	1077±84 [a]	S:0.26 T:0.14 S*T: 0.27	I:0.06 T:0.59
Trimethylamine-*N*-oxide							
Serum metabolites				μmol/L^{-1}(mean±SE)			
	0.44±0.06 [a]	0.51±0.06 [a]	0.53±0.08 [a]	0.55±0.07 [a]	0.47±0.07 [a]	S:0.02 T:0.13 S*T: 0.07	I:0.38 T:0.41
p-Cresyl sulfate	0.050±0.013 [b]	0.055±0.007 [a]	0.078±0.030 [a]	0.077±0.026 [a]	0.075±0.028 [a]	S:0.17 T:0.001 S*T: 0.88	I:0.86 T:0.04
Indoxyl sulfate	0.026±0.003 [b]	0.032±0.003 [a]	0.032±0.004 [a]	0.033±0.004 [a]	0.036±0.008 [a]	S:0.004 T:0.003 S*T: 0.45	I:0.65 T:0.01
Phenylacetylglutamine	50.7±2.3 [b]	52.8±2.3 [a]	52.6±2.5 [a]	53.9±2.6 [a]	51.7±2.5 [a]	S:0.14 T:0.04 S*T:0.33	I:0.23 T:0.14
L-Carnitine	8.9±1.6 [a]	9.4±1.0 [a]	12.7±1.9 [a]	11.8±1.4 [a]	10.0±1.0 [a]	S:0.46 T:0.09 S*T:0.80	I:0.20 T:0.92
Trimethylamine-*N*-oxide							

Values with superscript letters that differ are significantly different.

[a] Probiotic containing *Bifidobacterium bifidum* HA-132, *B. breve* HA-129, *B. longum* HA-135, *Lactobacillus acidophilus* HA-122, and *L. plantarum* HA-119;

[b] Prebiotic: inulin; S: Latin Square sequence; T: Timepoint; I: Intervention; Alpha: 0.05; Model 1: baseline + 4 high protein; Model 2: 4 high protein periods.

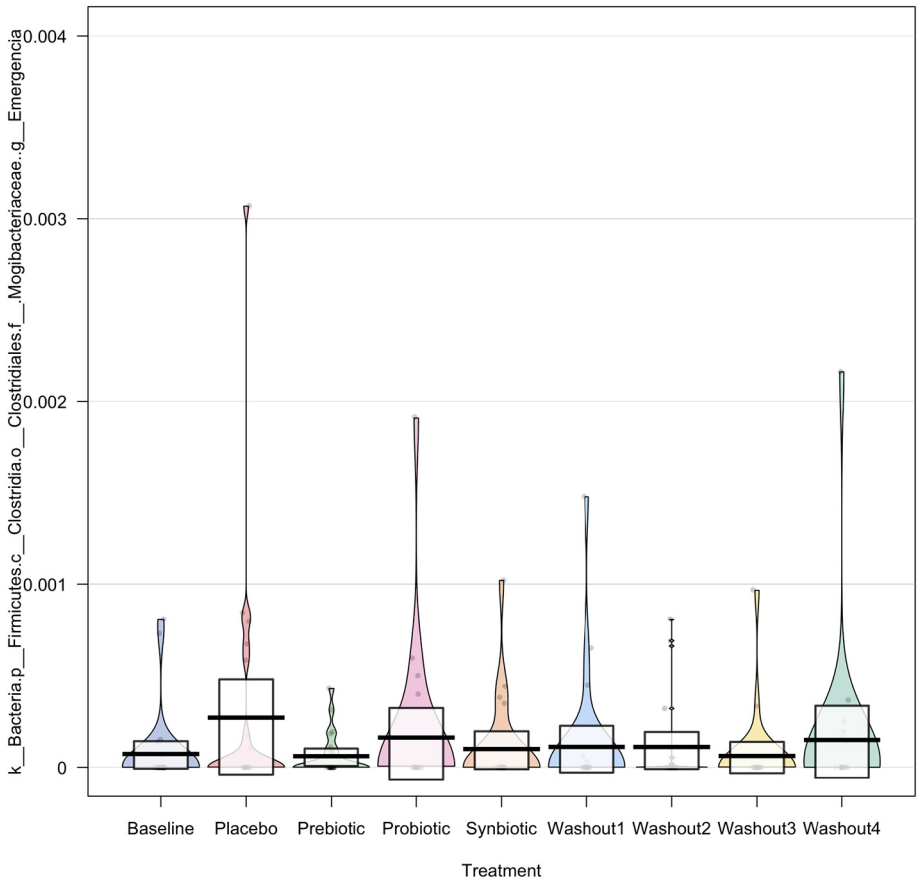

Fig. 2 – Pirate plot of the relative abundance of *E timonensis* for participants (N = 20) at baseline and during the HPD periods and washouts.

9% increase in risk for hypertension per 5-μmol/L increment [14], whereas Schiattarella et al reported a 7.6% increase in all-cause mortality for each 10-μmol/L increase in TMAO [48]; thus, some participants in the present study exhibited TMAO levels associated with higher chronic disease risk, and this risk may change with exposure to a high-protein diet.

Notably, the mean serum and urinary excretion of TMAO of the group did not change with the HPD. As TMAO is readily excreted in the urine [49], these findings suggest that the production of TMAO did not significantly increase in participants. Instead, the provision of the HPD resulted in a substantial increase in urinary L-carnitine, confirming that, in the older women studied, the bioavailability of L-carnitine from the food sources was high and thus provided little substrate for the bioconversion to TMA and subsequent oxidation to TMAO. In contrast, Mitchell et al showed reduced urinary TMAO levels and reduced fractional clearance of TMAO, suggesting renal clearance as factor affecting TMAO levels beyond diet in individuals exhibiting normal renal function [45], whereas Wang et al demonstrated significant increases in urinary TMAO excretion with 4-week red meat vs white meat or no-meat interventions in a crossover trial in men and women [50]. Conflicting urinary TMAO outcomes, reflective of overall absorption, may be due to differences in participant' characteristics (eg, hepatic expression of FMO3 [16]) and kidney function, but also the diet provided. Mitchell et al noted that seafood intake may have confounded their TMAO findings [45]. Wang et al included men and women with a median age of 45 and none over 65 years of age [49]. Their diet provided 25% protein with 12% of energy from red meat, similar to what was provided in the present study, but also provided 37% energy from fat and only 38% of energy from carbohydrate. The authors did not comment on the provision of fiber in their low-carbohydrate diet, but if the diet provided low levels of microbial-available carbohydrate, this may have affected the gut microbiota and its activity, and possibly the production of TMA. In contrast, Mitchell et al provided a higher-protein diet along with 50 g/d dietary fiber, a level almost twice the participants' usual intake [45]. In the present study, 15.5 g/d of fiber was provided from varied sources including grains, fruit, and vegetables, a level similar to the intake of women in the United States [51] and the participants' usual intake. It has recently been shown that individuals with strict adherence to a paleolithic diet (lacking grains) exhibited higher TMAO levels than those consuming a well-balanced diet in line with dietary recommendations [52]. An inverse relationship between serum TMAO and fiber has been recently reported [53]. It is possible that diets providing diverse microbial-available carbohydrate substrates, such as whole grains and legumes, may suppress TMA production.

The supplementation of prebiotic, probiotic, or synbiotic provided no evidence of suppression of TMAO in the context of the HPD. Recent studies examining efficacy of probiotics in

moderating TMAO have also not demonstrated any significant effects [54-56]; however, these studies were small and performed with various patient populations and without adequate control of dietary precursors of TMA. Similarly, studies administrating the prebiotics inulin and arabinoxylan have not resulted in suppression of TMAO [57,58]. This is not surprising given the selectivity of prebiotics and their lack of impact on microbial diversity [59]. Enhancement of diverse taxa may be needed to suppress microbial TMA production and proteolytic activity.

We previously reported the general microbiome response to the HPD was a higher abundance of *Lactococcus* and *Streptococcus*, and a lower abundance of butyrate-producing genera, *Roseburia* and *Anaerostipes* [24]. In the present analysis, we focused on the relative abundance of *E timonensis*, a species recently identified as capable of the transformation of γ-butyrobetaine to TMA, potentially the rate-limiting step of the bioconversion of L-carnitine to TMA [11]. However, only 50% of the older women had detectable levels of *E timonensis* during any of the HPD periods, suggesting that other low-abundance taxa were responsible for TMA generation as has previously been suggested [60].

Strengths of this study include the controlled weight-maintenance HPD, specifically the provision of both animal and plant sources of protein, but not marine sources that would confound the results by providing high levels of preformed TMA. Measurement of TMAO following 4 HPD periods strengthens the validity of the HPD response, as TMAO exhibits considerable day-to-day variation [49]. A potential limitation of the study was the 2-week HPD intervention periods. It is possible that with longer exposure and further modulation of the microbiota, proteolytic fermentation and TMA production may further change. In addition, comparisons were made between baseline samples taken following the consumption of a usual diet. As stated above, inclusion criteria included limits on fiber and protein intake, but there was no restriction of marine-sourced foods, and other differences in their usual diets may have contributed to intraindividual variation in TMAO and other values at baseline. Furthermore, choline was not analyzed, although other studies have done so [45]. However, choline has been shown to not significantly contribute to TMA generation [49], and intake is not associated with cardiovascular disease [61].

Few interventional trials have examined the impact of a high-protein diet and its microbial-generated metabolite response, although health associations with these changes are probable. In this study of older women consuming a controlled high-protein diet and without overt kidney and liver dysfunction, serum TMAO exhibited considerable intraindividual variation, but overall, the TMAO response to high protein was limited. As DiNicolantonia et al have recently suggested, diet may not be the major contributing factor to blood levels of TMAO in non-CKD populations [62]. However, the health impacts of increased proteolytic activity and metabolite production resulting from higher protein intakes require further investigation.

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