Probiotic Supplementation and Trimethylamine-N-Oxide Production Following a High-Fat Diet

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Objective: The objective of this study was to test the hypothesis that the multi-strain probiotic VSL#3 would attenuate the increase in fasting plasma concentrations of trimethylamine-N-oxide (TMAO) following a high-fat diet.

Methods: Nineteen healthy, non-obese males (18-30 years) participated in the present study. Following a 2-week eucaloric control diet, subjects were randomized to either VSL#3 (900 billion live bacteria) or placebo (cornstarch) during the consumption of a hypercaloric (+1,000 kcal day⁻¹), high-fat diet (55% fat) for 4 weeks. Plasma TMAO, L-carnitine, choline, and betaine (UPLC-MS/MS) were measured at baseline and following a high-fat diet.

Results: Plasma TMAO significantly increased 89% ± 66% vs. 115% ± 61% in both the VSL#3 and placebo groups, respectively; however, the magnitude of change in plasma TMAO was not different (P > 0.05) between them. Plasma L-carnitine, choline, and betaine concentrations did not increase following the high-fat diet in either group.

Conclusions: A high-fat diet increases plasma TMAO in healthy, normal-weight, young males. However, VSL#3 treatment does not appear to influence plasma TMAO concentrations following a high-fat diet. Future studies are needed to determine whether other therapeutic strategies can attenuate the production of TMAO.

Introduction

Cardiovascular disease (CVD) is the leading cause of death in the United States (1). The high saturated fat and cholesterol content in high-fat foods has been implicated in the increased CVD risk associated with Westernized diets (2). However, the results of recent studies suggest that other components present in high-fat foods are metabolized by gut bacteria to produce metabolites that appear to play a role in the pathophysiology of atherosclerosis and CVD (3-6). The gut microbiota play an obligatory role in the metabolism of nutrients containing trimethylamine (TMA) structures such as L-carnitine, choline, phosphatidylcholine, and betaine (4-6). The metabolism of these compounds leads to the production of TMA (7), which is then readily absorbed into systemic circulation and oxidized by hepatic flavin monooxygenases (FMO) 3 to trimethylamine-N-oxide (TMAO) (8). Importantly, TMAO is independently associated with incident major adverse cardiovascular events (MACE), even after accounting for traditional risk factors (5). Furthermore, prospective studies have shown that fasting plasma L-carnitine, choline, and betaine levels independently predict an increased incident MACE, but only in individuals with concurrently high TMAO levels (4,6).

Atherosclerosis is accelerated in ApoE⁻/⁻ mice fed a normal chow diet supplemented with L-carnitine or choline compared with animals on a standard chow diet (4,5). Mice on these diets are characterized by elevated plasma TMAO, reduced cholesterol transport, and increased “forward” cholesterol transport. Conversely, mice administered broad-spectrum antibiotics while on these diets produce significantly less TMAO and are protected from atherosclerosis (4,5). Furthermore, fasting plasma TMAO concentrations increase significantly following an acute oral L-carnitine or phosphatidylcholine challenge in humans. In addition, gut flora suppression with broad-spectrum antibiotics abolishes the TMAO response following a TMA-laden meal challenge (3,4). Probiotic supplementation has also been shown to alter liver concentrations of TMAO in a humanized microbiome mouse model (9). However, whether modification of gut microbial communities attenuates the increase in fasting plasma TMAO in response to a high-fat diet in humans is unknown. Accordingly, we hypothesized that probiotic supplementation (VSL#3) would attenuate the increase in plasma TMAO concentrations induced by a high-fat diet.

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Methods

Participants

Nineteen non-obese (body mass index [BMI], 18-30 kg m⁻²), college aged males (18-30 years old) who were included in a larger study examining the effects of probiotics on body weight and body composition comprised the study sample. We excluded females in this initial investigation to eliminate the potential confounding due the transient trimethylaminuria (elevated urinary TMA) that has been reported to occur during menstruation (10). Participants were weight stable (±2.5kg), sedentary to recreationally active (<2 days, 20 min day⁻¹ of low-intensity physical activity), and abstained from antibiotic use for at least 6 months prior to study commencement. All participants were normotensive (BP <140/90 mm Hg), normoglycemic (fasting glucose <100 mg dl⁻¹), normolipemic (total cholesterol <200 mg dl⁻¹, triglycerides <150 mg dl⁻¹), and were not taking any medications or supplements (e.g., prebiotics/probiotics) for at least 4 weeks. Participants were excluded if their total daily fat consumption was >40% and/or their total daily saturated fat consumption was >15%. The Virginia Polytechnic and State University Institutional Review Board approved the study protocol. The nature, purpose, risks, and benefits of the study were explained before obtaining informed consent.

Experimental design

We utilized a double-blind, placebo-controlled, randomized design for the present study. All participants completed baseline testing following completion of a 2-week eucaloric control diet (55% carbohydrate, 30% fat, 15% protein). Subsequently, participants were randomized to either VSL#3 (n = 9, 900 billion live bacteria) or placebo (n = 10, cornstarch) during the consumption of a hypercaloric (+1,000 kcal day⁻¹), high-fat diet (55% fat, 30% carbohydrate, 15% protein) for 4 weeks. Participants repeated baseline testing immediately following the high-fat diet. We selected the 4-week duration to allow adequate time for changes in the gut microbiota to exert its hypothesized effects, since previous studies have shown that this time is adequate to alter the gut microbiota in human subjects (11-14).

Identical and unmarked sachets of VSL#3 and cornstarch (placebo) were supplied to us from VSL Pharmaceuticals (Gaithersburg, MD), and coded by an individual not involved in the collection or analysis of study data. The sachets were stored at 4°C prior to ingestion. Each sachet of VSL#3 contained 450 billion live bacteria and included the strains: streptococcus thermophiles DSM24731, lactobacillus acidophilus DSM24735, lactobacillus delbrueckii ssp. bulgaricus DSM24734, lactobacillus paracasei DSM24733, lactobacillus plantarum DSM24730, bifidobacterium longum DSM24736, bifidobacterium infantis DSM24737, and bifidobacterium breve DSM24732. We selected 900 billion live bacteria/day of VSL#3 as the dose and type of probiotic for the present study because this dose has been shown to be safe and effective in previous reports (15) and VSL#3 has documented efficacy in modulating the gut microbiota (16-19).

Controlled diet

All diets were controlled to minimize the potential impact of interindividual variability in habitual dietary intake. Energy requirements were estimated based on height, weight, age, and activity level using the Institutes of Medicine equation (20). Subsequently, a 7-day cycle menu was constructed for each participant with the appropriate macronutrient and caloric content for each diet (lead-in and high-fat) using Nutritionist Pro™ software (Axxya Systems, Stafford, TX). Food modules (250 kcal) with the same macronutrient composition as the lead-in diet were added or subtracted if weight changed >1 kg. In addition, participants were weighed each morning during the lead-in and high-fat diet periods.

Participants consumed breakfast in the metabolic kitchen of the Department of Human Nutrition, Foods, and Exercise each day and were provided a cooler with food for the remainder of the day. Participants were instructed to only consume provided food for the duration of the study and were instructed to report all non-study foods, if consumed, to research staff. Participants were instructed to return any uneaten food and all unwashed food containers to monitor compliance. During the high-fat diet period the surplus 1,000 kcal were provided in the form of a high-fat (81 g fat), ice cream-based (chocolate ice cream and coconut milk) shake that contained either two packets of VSL#3 or two identical packets of placebo (cornstarch). Each morning research staff delivered the shake to participants and supervised its complete consumption.

Experimental testing

All testing took place at the Human Integrative Physiology Lab between the hours of 5:00 and 11:00 am. Participants were fasted for the prior 12 h (included caffeinated and alcoholic beverages), performed no vigorous physical activity for the prior 48 h, and were free from acute illness for the prior 2 weeks.

Measurements

Body mass was measured on a digital scale (Model 5002, Scale-Tronix, Inc.) and height was measured using a stadiometer. Brachial arterial pressure was measured in a seated position using automated sphygmomanometer (Pilot model 9200, Colin Instruments Corp.) (21). Habitual dietary intake was assessed using detailed 4-day diet records. Participants were instructed on the proper way to weigh and record food intake for 3 weekdays and 1 weekend day. Habitual dietary intakes as well as the controlled diets were analyzed with Nutrition Data System for Research (NDS-R) software (University of Minnesota) by a trained diet technician.

The daily intake of L-carnitine was estimated from the average servings of foods known to contain L-carnitine by first converting the average of these food servings to gram amounts, and then converting these gram quantities to milligrams of L-carnitine with the use of previously published conversion tables (22,23). The daily intake of choline and betaine for each participant was determined from the averaged nutrient totals report output in NDS-R for each diet.

Stool samples were collected from all subjects in sterile containers (Commode Collection Systems, Thermo Fisher Scientific, Waltham, MA), delivered to the laboratory within 24 h of collection, and immediately stored at 80°C. At the conclusion of the study, DNA was extracted from samples using the QIAamp Fast Stool DNA Mini Kit (QIAGEN, Venlo, Limburg). Two of the nine VSL#3 bacterial species (Streptococcus thermophilus and Lactobacillus acidophilus) were selected to be amplified and quantified with the use of previously described 16 S rDNA primers (Integrated DNA
Central and peripheral arterial pressures were obtained using a high-fidelity, non-invasive applanation tonometer and a semi-automated computer controlled device (NIHem, Cardiovascular Engineering) as previously described (21,25). β-stiffness index, a relatively blood pressure independent index of carotid artery stiffness, was measured using an ultrasound unit (Sonos 7500, Phillips Medical Systems) equipped with a high-resolution linear array transducer (3-11 MHz) and applanation tonometry (NIHem, Cardiovascular Engineering) as previously described (21).

Fasting plasma concentrations of TMAO, L-carnitine, choline, and betaine were quantified by isocratic ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) using the stable isotope dilution method against internal standards as described previously by Kirsch et al. (26) with modifications.

For the analysis of TMAO, L-carnitine, choline, and betaine, a stock solution of the IS (29.4 ± 1 pmol) was prepared in water and stored at -20°C. Immediately prior to sample preparation, the IS stock solution was diluted 25-fold in acetonitrile (ACN). For the analysis of L-carnitine, a stock solution of the IS (29.4 µM L-carnitine-d₃) was prepared in water and stored at -20°C. Immediately prior to sample preparation, the IS stock solution was diluted 25-fold in ACN.

Following dilution of the IS stock solution, 300 µl of the ACN/IS was combined with 25 µl of plasma and was vigorously vortexed (30 s) to remove the analytes. Samples were then centrifuged at 17,000 g for 3 min at 21°C and the resultant supernatant was then vacuum filtered into HPLC vials and analyzed immediately by UPLC-MS/MS. UPLC-MS/MS analyses were carried out using a Waters Acquity UPLC system coupled to a Waters TQD triple quadrupole mass spectrometer equipped with MassLynx software (Waters, Milford, MA). The samples were separated on a Waters BEH HILIC analytical column (2.1 × 100 mm², 1.7 µm particle size) with a Waters BEH HILIC VanGuard pre-column (5 × 2.1 mm²; 1.7 µm). The column temperature at 30°C and the sample compartment was 10°C. The mobile phases were 15 mM ammonium formate, pH 3.5 (phase A) and acetonitrile (phase B). The system flow rate was 0.65 ml min⁻¹, and isotropic elution was achieved using 20% A/80% B over 3 min. Following UPLC separation, the target analytes and their respective internal standards were identified and quantified using positive electrospray ionization (ESI) in (+)-mode. The source and capillary temperatures were temperature 150 and 400°C, respectively. The capillary voltage was 0.60 kV, and the desolvation and cone gas (N₂) flow rates were 800 and 20 L h⁻¹, respectively. The compounds were quantified using multi-reaction monitoring (MRM) functions optimized by Intellistart as shown in Supporting Information Table 1. MRM functions used the Autodwell function to optimize the number of points per peak (12 points for a 10 s peak). The detection span was ±0.2 amu for each mass.

Quantification was performed using QuanLynx (Waters, Milford, MA) by taking the ratio of the target analyte and respective IS peak areas, based on external standard curves prepared using a wide range of target analyte concentrations (bracketing the peak areas observed in the plasma samples) and the same IS concentrations used to prepare the plasma samples.

Fasting plasma triglyceride, very low-density lipoprotein (VLDL), and high-density lipoprotein (HDL) concentration, and lipoprotein particle number and size were determined by nuclear magnetic resonance (The Vantera® Clinical Analyzer) by a commercial laboratory (Liposcience, Raleigh, NC). Total cholesterol and low-density lipoprotein (LDL) concentrations were determined by conventional enzymatic techniques by a commercial laboratory (Liposcience, Raleigh, NC). Oxidized (ox) LDL was measured in fasting plasma samples with the use of an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s instructions (MERCodia, Winston-Salem, NC).

Statistical analysis
Repeated measures analysis of variance was used to compare subject characteristics and dependent variables over time between the two groups. A Tukey’s post hoc analysis was used for multiple comparisons. TMAO concentrations were not normally distributed and as such were log transformed. Independent T tests were used to compare the magnitude of change in dependent variables between groups. Pearson’s Product Moment correlations were used to determine relationships among variables of interest. All of the data are expressed as mean ± standard error (SE). The significance level was set a priori at α = 0.05.

Results
Participant characteristics at baseline and following the high-fat diet are shown in Table 1. There were no differences in age, weight, or BMI (all P > 0.05) at baseline. There was a significant increase in body mass and BMI following the high-fat diet in both groups; the magnitude of increase in body mass and BMI was significantly smaller in the VSL#3 compared with placebo group. Resting heart rate, supine resting brachial systolic blood pressure (SBP), brachial diastolic blood pressure (DBP), brachial pulse pressure (PP), carotid SBP, carotid DBP, carotid PP, and β-stiffness index were not different (all P > 0.05) between groups at baseline. Resting heart rate increased significantly following the high-fat diet; however, the magnitude of increase in resting heart rate was not different in both groups (P > 0.05). There were no changes in supine resting brachial SBP, brachial DBP, brachial PP, carotid SBP, carotid DBP, carotid PP, and β-stiffness index following the high-fat diet in either group (all P > 0.05).
The dietary composition of the lead-in and high-fat diet periods is shown in Table 2. Total energy, total fat, saturated fatty acid (SFA), protein, L-carnitine and choline intake was higher (all \( P < 0.05 \)) on the high-fat diet compared with the lead-in (all \( P < 0.05 \)). Carbohydrate, total fiber, relative fiber per 1,000 kcal day\(^{-1} \), and betaine intake were significantly lower on high-fat diet compared with the lead-in.

There were no differences in the fecal enrichment of the probiotic species, *Streptococcus thermophilus* (0.85 \pm 0.2 vs. 1.9 \pm 0.7 U) and *Lactobacillus acidophilus* (0.8 \pm 0.4 vs. 0.8 \pm 0.3 U) between the placebo and VSL\#3 groups, respectively at baseline (All \( P > 0.05 \)). *Streptococcus thermophilus* enrichment increased significantly in the placebo and VSL\#3 groups; the magnitude of increase in fecal bacterial enrichment was greater in VSL\#3 compared with placebo (16.6 \pm 6.0 vs. +2.2 \pm 1.0 U, \( P < 0.05 \)). In contrast, *Lactobacillus acidophilus* enrichment increased in the VSL\#3 compared with the placebo group (12.4 \pm 5.0 vs. +0.6 \pm 0.2 U, \( P < 0.05 \)) following the high-fat diet.

Fasting plasma lipid and lipoproteins concentrations at baseline and following the high-fat diet are shown in Table 3. There were no differences in plasma lipids and lipoproteins at baseline (all \( P > 0.05 \)). There was an increase in total and HDL cholesterol in the placebo and VSL groups following the high-fat diet; however, the magnitude of change was not significantly different between groups (Table 3). There were no changes in triglyceride, LDL, VLDL, and oxLDL concentrations following the high-fat diet (all \( P > 0.05 \)). Total HDL particles (+3.2 \pm 1.4 and 2.0 \pm 1.4 \( \mu \)mol l\(^{-1} \), \( P < 0.05 \)), large HDL particles (+1.5 \pm 1 and 1.3 \pm 0.7 \( \mu \)mol l\(^{-1} \), \( P < 0.05 \)) and HDL particle size (+0.07 \pm 0.14 and 0.14 \pm 0.07 nm, \( P < 0.05 \)) increased following the high-fat diet; however, the magnitude of increase in all of these were not different between groups (\( P > 0.05 \)). All other number and sizes of other lipid species particles were not influenced by the high-fat diet nor VSL\#3 treatment (All \( P > 0.05 \), data not shown).

Fasting plasma concentrations of TMAO, L-carnitine, choline, and betaine at baseline and following the high-fat diet are shown in Figure 1. There were no differences in TMAO, L-carnitine, choline, and betaine between the groups at baseline (\( P > 0.05 \)). Plasma TMAO increased significantly (89\% \pm 66\% vs. 115\% \pm 61\%) in VSL\#3 compared with placebo, respectively. However, the magnitude of increase in TMAO was not different (\( P > 0.05 \)) between groups (Figure 1A). Plasma L-carnitine, choline, and betaine concentrations did not increase following the high-fat diet (\( P > 0.05 \)) (Figure 1B-D). The plasma concentrations of TMAO, L-carnitine, choline, and betaine at baseline and following the high-fat diet for each individual participant are shown for each study participant in Supporting Information Table 2.
The magnitude of change in plasma TMAO was correlated to the magnitude of change in carotid SBP \((r = 0.595, P < 0.01)\) and carotid PP \((r = 0.471, P < 0.05)\), but not to brachial SBP \((r = 0.308, P = 0.107)\) nor brachial PP \((r = 0.084, P = 0.371)\) (Figure 2). The magnitude of change in plasma TMAO was not correlated to the magnitude of change in any other hemodynamic variables following the high-fat diet.

**Discussion**

The major finding from the present study is that, in contrast to our hypothesis, probiotic supplementation did not attenuate the rise in plasma TMAO following a high-fat diet. In addition, neither the high-fat diet nor VSL#3 treatment influenced plasma concentrations of L-carnitine, choline, or betaine. Interestingly, the magnitude of change in plasma TMAO was correlated with the magnitude of change in indices of arterial stiffness following the high-fat diet.

Stella et al. (27) reported that consumption of a diet high in red meat (420 g day\(^{-1}\), [30% of total energy as fat]) for 15 days increased urinary TMAO in non-obese, healthy males. Our findings are consistent with these prior observations. L-carnitine and choline are substrates for TMAO production and found in foods such as red meat, milk, liver, and eggs (28). Adults eating a mixed diet consume between 60 and 180 mg day\(^{-1}\) of L-carnitine (29) and \(\sim 300\) mg day\(^{-1}\) of choline (30). The high-fat diet we provided in the present study resulted in an increase of 45 \(\pm 6\) mg day\(^{-1}\) in L-carnitine and 176 \(\pm 6\) mg day\(^{-1}\) in choline. Importantly, this significantly higher intake of L-carnitine and choline intake was sufficient to

### Table 3: Fasting plasma lipid and lipoprotein concentrations at baseline (PRE) and following the high-fat diet (POST)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Placebo (N = 10)</th>
<th>VSL#3 (N = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PRE</td>
<td>POST</td>
</tr>
<tr>
<td>Total cholesterol (mg dL(^{-1}))</td>
<td>153.9 (\pm 7.0)</td>
<td>180.0 (\pm 6.8)</td>
</tr>
<tr>
<td>LDL (mg dL(^{-1}))</td>
<td>92.3 (\pm 6.2)</td>
<td>104.2 (\pm 6.8)</td>
</tr>
<tr>
<td>VLDL (mg dL(^{-1}))</td>
<td>75.6 (\pm 9.8)</td>
<td>66.9 (\pm 6.2)</td>
</tr>
<tr>
<td>HDL (mg dL(^{-1}))</td>
<td>53.1 (\pm 3.0)</td>
<td>65.7 (\pm 5.1)</td>
</tr>
<tr>
<td>Triglycerides (mg dL(^{-1}))</td>
<td>105.7 (\pm 8.9)</td>
<td>103.5 (\pm 6.6)</td>
</tr>
<tr>
<td>oxLDL (IU mL(^{-1}))</td>
<td>57.6 (\pm 5.8)</td>
<td>66.9 (\pm 7.0)</td>
</tr>
</tbody>
</table>

Values expressed as mean \(\pm\) SE.

LDL = low-density lipoprotein; VLDL = very low-density lipoprotein; HDL = high-density lipoprotein; oxLDL = oxidized low-density lipoprotein.

\(^* P < 0.05, \text{time effect}; ^{**} P < 0.01, \text{time effect.}\)
increase plasma TMAO concentrations in the present study. However, whether other substrates associated with high-fat diets contributed to the increase in TMAO in the present study is unknown.

VSL#3 has been reported to have cardiovascular (31), hepatic (32), large intestinal (33), and anti-inflammatory (34) pleiotropic actions. However, in this study, probiotic supplementation did not attenuate the increase in plasma TMAO following a high-fat diet. Several bacterial genera, such as, *Peptostreptococcaceae Incertae Sedis* and *Clostridium*, have been associated with plasma TMAO concentrations (4). In addition, the proportions of the genera *Peptostreptococcaceae Incertae Sedis* and *Clostridium* are increased in C57BL/J6 mice following a high-fat diet relative to littermates receiving a high-fat diet supplemented with vancomycin (35). Our findings suggest that VSL#3 did not exert a significant influence on the number or function of these TMA-producing species. Future studies are needed to determine whether other therapies can be designed to specifically target TMA-producing bacteria.

Plasma TMAO is associated with an increased risk of incident MACE (5). In addition, elevated TMAO production from L-carnitine and choline supplemented diets accelerates atherosclerosis in rodent models (4,5). In the present study, we report for the first time that the magnitude of change in plasma TMAO concentration following a high-fat diet is correlated to the magnitude of change in both carotid SBP and carotid PP. Importantly, both central SBP and PP are associated with an increased risk for cardiovascular events, cardiovascular mortality and all-cause mortality (36-38). Thus, it is possible that elevated TMAO may increase CVD risk, at least in part, by influencing central hemodynamics. Future studies are necessary to better understand the relation between plasma TMAO concentration and central artery hemodynamics.

There are some limitations of the present study that should be discussed. First, our findings in our sample of healthy, non-obese males may not be generalizable to the general population. Therefore, it is possible that VSL#3, or other therapies that modify gut bacterial communities, may influence TMAO concentration in clinical populations in which TMAO concentrations are elevated. Second, we did not assess endothelial function, which is a strong predictor of atherosclerosis (39). Mechanistic studies in rodents implicate TMAO in the pathophysiology of atherosclerosis. Therefore, assessing endothelial function may provide more robust information on the influence of elevated TMAO on vascular function in humans. Finally, our intervention time and treatment dose may not have been sufficient enough to influence fasting plasma TMAO concentrations.

In conclusion, the major findings from the present study are that plasma TMAO concentrations increased following a high-fat diet but the increase was not attenuated by VSL#3 treatment. In addition, we observed that the magnitude of increase in plasma TMAO concentration was correlated to the magnitude of increase in carotid SBP and PP following a high-fat diet. Future studies are needed to determine effective therapeutic strategies for reducing the number and/or function of TMA-producing bacteria.

![Figure 2 Relation between the magnitude of change in plasma TMAO and the magnitude of change in (A) carotid SBP, (B) carotid PP, (C) brachial SBP, and (D) brachial PP following the high-fat diet in the pooled sample. SBP = systolic blood pressure; PP = pulse pressure; TMAO = trimethylamine-N-oxide.](image-url)
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References


