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Whole egg consumption increases plasma choline and betaine without affecting TMAO levels or gut microbiome in overweight postmenopausal women

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ABSTRACT

As a crucial part of the symbiotic system, the gut microbiome is metabolically connected to many diseases and conditions, including cardiovascular diseases (CVD). Trimethylamine (TMA) is produced by gut bacteria from dietary choline, betaine, or L-carnitine, and is then converted in the liver to Trimethylamine N-oxide (TMAO), which in turn affects hepatic and intestinal lipid metabolism. Circulating TMAO is positively associated with CVD risk. Because eggs are rich in choline, it has been speculated that their consumption may increase plasma TMAO. In this study, we hypothesized that 2 eggs per day increases plasma TMAO level by altering gut microbiome composition in mildly hypercholesterolemic postmenopausal women. In this randomized, cross-over study, 20 overweight, postmenopausal women were given 2 whole eggs and the equivalent amount of yolk-free substitute as breakfast for 4 weeks, in randomized order, with a 4-week washout in between. Fasting blood draws and stool were collected at the beginning and end of each treatment period. Plasma TMAO, choline, betaine and other metabolites were analyzed using LC/MS, while gut microbiome composition was analyzed using 16S amplicon sequencing. Plasma choline and betaine were significantly increased after whole egg but not yolk-free substitute, however TMAO level was not significantly affected by treatments. Gut microbiome composition showed large inter-individual variability at baseline and in response to the treatments. The consumption of 2 eggs per day in overweight, postmenopausal mildly hypercholesterolemic women significantly increased plasma choline and betaine, but did not increase plasma TMAO or alter gut microbiome composition.

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1. Introduction

Eggs are enriched in a number of complex lipids including phospholipids. While these phospholipids are generally

health promoting, recent evidence is showing that these same phospholipids may be involved in the gut microbiota-mediated production of a metabolite that is strongly associated with heart disease risk. The primary phospholipid in

Abbreviations: TMAO, trimethylamine-N-oxide; TMA, trimethylamine; CVD, cardiovascular disease; ASV, amplicon sequence variant.

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eggs, phosphatidylcholine (PC), contains choline, which can be converted to trimethylamine (TMA) by gut bacteria. The TMA is then absorbed into the circulation and modified in the liver by flavin-containing monooxygenase to trimethylamine-N-oxide (TMAO). TMAO is a risk factor for cardiovascular disease (CVD) even after adjustment for other CVD risk factors [1]. Egg lipids as a source of choline in the form of PC have been shown to result in increased concentrations of TMAO in the postprandial hours after consuming 2 or more eggs [2]. However, there is high inter-individual variability in TMAO production following egg consumption depending on long-term dietary habits [2]. Vegans do not produce measurable concentrations of TMAO after dietary choline consumption whereas omnivores do [2]. Omnivores, on the other hand, although they do produce TMAO in measurable quantities in the hours following intake, are highly variable in their TMAO production ranging from near 0, similar to vegans, to as high as 30 $\mu\text{mol/L}$ [2]. Interestingly, although egg intake appears to increase plasma TMAO concentrations in the postprandial state, egg intake does not influence plasma TMAO in the long-term. In several studies in young, healthy individuals consuming up to 3 eggs per day for up to 4 weeks, plasma TMAO concentrations were not altered despite increases in plasma choline levels [3–5].

Due to concerns about the high cholesterol content of eggs and their potential negative impact on lipid profiles, eggs have not been recommended for individuals at increased risk for heart disease, however recent studies have shown that egg intake does not in fact increase circulating total cholesterol or LDL cholesterol concentrations and may even improve the cholesterol efflux capacity of HDL particles [3,6–9]. The question of whether eggs are a healthy choice in populations that are at risk for heart disease is particularly salient for postmenopausal women, in whom heart disease risk is higher than in premenopausal women. We have previously found that in overweight, mildly hypercholesterolemic, postmenopausal women consuming 2 eggs per day increases the cholesterol efflux capacity of HDL particles without altering lipid profiles or other cardiometabolic risk factors [8]. In this study we hypothesized that 2 eggs per day increases plasma TMAO concentration by altering gut microbiome composition in postmenopausal women. Twenty overweight to obese, mildly hypercholesterolemic but otherwise healthy women were recruited into this randomized cross-over study. TMAO and its precursors choline, betaine, and L-carnitine were measured from plasma, and gut microbiome composition was measured from feces.

2. Methods and materials

2.1. Subjects and study design

Twenty overweight/obese postmenopausal women (48–70 years old, non-smokers, HDL-C >50 mg/dL, BMI 25–35 kg/m²) were phone screened, consented, and enrolled in this randomized, cross-over study as previously reported [8]. Use of hormone replacement therapy, or any medications or supplements with known effects on lipid metabolism were included in the exclusion criteria. Subjects were included if

they had 2 or fewer traits of metabolic syndrome (MetS), and were free from any documented chronic diseases including diabetes, cancer, hypertension, or CVD. After a 2-week lead-in period, subjects were assigned to begin with either the whole egg or yolk-free egg treatment group for 4 weeks, followed by the other treatment for 4 weeks in randomized order, with a 4-week washout period between the 2 treatments. Subjects were given frozen breakfast made with 100 grams (equivalent to 2 eggs) of liquid whole egg or liquid yolk-free egg during the study. Subjects were asked not to consume any egg or egg-containing products during the lead-in and washout periods. During the study treatments, subjects were also instructed not to consume any additional egg or egg containing products and to otherwise maintain their regular diet. A 3-day diet record was administered by a dietitian at the beginning and end of each 4-week intervention treatment.

Overnight fasted blood draws were taken at the beginning and end of each intervention treatment using EDTA-containing tubes (Becton Dickinson). Plasma was isolated immediately after blood draw by centrifugation at 1000g (Sorvall-Legend RT) at 4°C for 15 min. Stool samples were collected within the 24-hour period preceding each blood draw, using a swab-based stool sample collection kit (uBiome) and stored with cooling gel packs before the study visit. Plasma and stool samples were stored in a –80°C freezer until analysis. All subjects were consented prior to enrollment in the study. The study was approved by the University of California Davis Institutional Review Board and the study followed all of the ethical standards of the Helsinki declaration. The study is registered at clinicaltrials.gov under identifier NCT02445638.

2.2. Biogenic amines analysis

The biogenic amines, including choline, betaine, and TMAO, were measured using a LCMS method at the UC Davis West Coast Metabolomics Center [10]. A 20 μL plasma sample was extracted through sequential addition of 225 μL methanol, 750 μL of MTBE (methyl tertiary butyl ether), and 188 μL LCMS water. Half of the polar layer at the bottom was then dried down and resuspended in 100 μL ACN (acetonitrile) water (8:2 volume ratio) containing internal standards. The internal standards spiked were D9-Choline, D9-Betaine, D9-TMAO, and D3-L-Carnitine. Resuspended samples were injected into a Waters Acquity UPLC BEH Amide column (1.7 μm , 2.1 mm \times 150 mm) by a 120-A Infinity UPLC system (Waters Technology Corporation, Milford, MA, USA), and analyzed on an Agilent 6530 QTOF mass spectrometer (Agilent Technology, Santa Clara, CA, USA). Raw data were first processed by mzMine 2.0 to select peaks, followed by Agilent's MassHunter for quantification. Nine plasma pool samples (BioIVT, Westbury, NY, USA) were analyzed along with the samples for the purpose of quality control.

2.3. Gut microbiome composition

Gut microbiome composition was analyzed using the 16S rRNA gene amplicon sequencing method as previously described [11]. DNA was extracted from swabs using the Zymo Research Fecal DNA kit (ZYMO Research Irvine, CA, USA) according to the manufacturer's instructions with a few modifications. Briefly, the Zymo lysis solution was not added

to the fecal swab-containing uBiome tubes as the tubes already contained the Zymo lysis solution. Samples were lysed by bead-beating using a SPEX Sample Prep 1600 MiniG automated mini tissue homogenizer (SPEX SamplePrep, Metuchen, NJ, USA). Samples were then processed in the MiniG at 1500 RPM for 1 minute, resting 3 minutes between sets for a total of 2 minutes of bead-beating. The 16S rRNA gene was PCR amplified from extracted fecal DNA using a previously reported method [12]. Barcoded primers F515 and R806 were used to amplify the V4 region with initial denaturation of 2 min at 94°C, followed by 30 cycles of 95°C for 45 s, 50°C for 60 s and 72°C for 90 s, and a final extension step at 72°C for 10 min. After evaluating by gel electrophoresis, PCR amplicons were combined, and purified with the Qiagen QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA). The purified DNA library was sequenced on an Illumina MiSeq instrument (Illumina, San Diego, CA, USA) at the UC Davis Genome Center DNA Technologies Core.

2.4. Statistical analyses

The 16S sequencing data was processed using a workflow developed in house, with various open source softwares. The fastq files were first processed by fastq-multx for demultiplexing [13], followed by fastx-toolkit to trim off primers [14]. A customized python script was used to remove PhiX sequences. The demultiplexed reads were then processed using the R package DADA2 (version 1.12.1) to detect amplicon sequencing variants (ASVs) [15,16]. DADA2 was also used to assign taxonomy labels to ASVs using a naive bayes algorithm and the SILVA ribosomal RNA gene database (version 132) [17]. Qiime2 (version 2019.7) was used to generate the phylogenetic tree of representative sequences of the ASVs [18], and the R package phyloseq was used to calculate the microbiome diversities [19]. Subjects for whom all 4 samples had more than 3,000 reads were included into the downstream analysis. The ASV table of read counts was converted into relative abundances for further statistical analysis and hypothesis testing in R (version 3.6.1).

A sample size of 20 was calculated for the primary endpoint of the study and the results from the primary analysis were recently published [8]. The current study is a secondary analysis thus a sample size calculation was not performed. The Shapiro-Wilk's test was first applied in R to test the normality of choline, betaine, TMAO and other biogenic amines, as well as the microbiome relative abundances. A log transformation was applied to the data if they failed to pass the Shapiro-Wilk's test. A linear mixed model was applied to test the differences in change after intervention of whole egg versus yolk-free egg, with the R package lme4 (version 3.4.0.6) [20]. The Benjamini-Hochberg test was applied for multiple test adjustment whenever more than 10 variables were tested.

3. Results

3.1. Clinical values and dietary records

Subjects who participated in this study were postmenopausal, overweight and mildly hypercholesterolemic but otherwise healthy women, with an average age of 57.7 (± 5.64) years and

average BMI of 28.34 (± 2.96). Subject baseline values of blood pressure, fasting glucose and circulating lipids were reported in detail previously, and they did not change across the study by interventions [8]. The average baseline plasma LDL cholesterol was 137.0 (± 23.3) mg/dL, HDL cholesterol 63.8 (± 9.1) mg/dL, and triglycerides 217.5 (± 28.5) mg/dL. Subjects were able to maintain their regular diet pattern throughout the study, with an average fat intake of 33.4 (± 6.6)% and carbohydrate intake of 49.6 (± 6.6)%. The cholesterol intake was significantly higher on the whole egg treatment (498.9 \pm 59.7 mg/d) compared to the yolk free egg treatment (126.6 \pm 53.8 mg/d) because of the study intervention [8].

3.2. Choline, betaine, and TMAO

A total of 101 biogenic amine molecules were monitored using the LCMS method in this population. Amine molecules that were monitored include fatty acid amides, indole derivatives, amino acids and derivatives, carnitines, niacin metabolites, xanthine metabolites, as well as choline metabolites. QC samples showed the coefficients of variance (CV) of all biogenic amines were from 0.4 to 21.2%, while only 2 were larger than 20% (Supplemental Figure S1). The baseline plasma choline and betaine measured in this particular population was 3.16 (± 0.77) μ mol/L and 44.11 (± 11.50) μ mol/L respectively. There was a significant increase in plasma choline and betaine concentration after 4 weeks of whole egg compared to yolk free egg treatment (Figure 1, $P = .01$ and $.011$, correspondingly). The plasma TMAO baseline level was 2.29 (± 0.129) μ mol/L. TMAO was not significantly affected by whole egg compared to yolk free egg treatment (Figure 1, $P = .778$). There was a significant positive correlation between plasma choline and betaine (Pearson's $R = 0.503$, $P < .001$, Figure 1), and a significant correlation between L-carnitine and TMAO (Pearson's $R = 0.294$, $P = .008$, Figure 1), however, there was no correlation between TMAO and choline (Pearson's $R = 0.174$, $P = .123$). The plasma concentration (mg/mL) of betaine, carnitine, choline, and TMAO are reported in Supplemental Table S1. The raw MS response of all measured biogenic amines is shown in Supplemental Table S2.

3.3. Fecal microbial composition

Based on the sequencing quality control, one subject was removed from downstream analysis because one of this subject's sample did not have enough sequence reads. The DADA2 algorithm was able to find 1465 ASVs from the sequence library. Most of the ASVs were able to be assigned to a genus (Supplemental Figure S2). There was a high degree of inter-individual variability in the microbiome composition. A total of 934 ASVs were found in only a single subject, and only 4 ASVs were present in all 19 subjects (Figure 2). Fourteen phyla were assigned to 99.4% of all ASVs, and 52 families were assigned to 92% ASVs (Figure 2). A total of 201 different genera were assigned to 73.9% of ASVs, however 40 genera were only observed in one subject, and only 15 genera were observed in all (Figure 2). There were no significant differences or changes in gut microbial diversity as shown in Figure 2. The weighted unifracs distance also showed that samples were clustered by subject and not by treatment

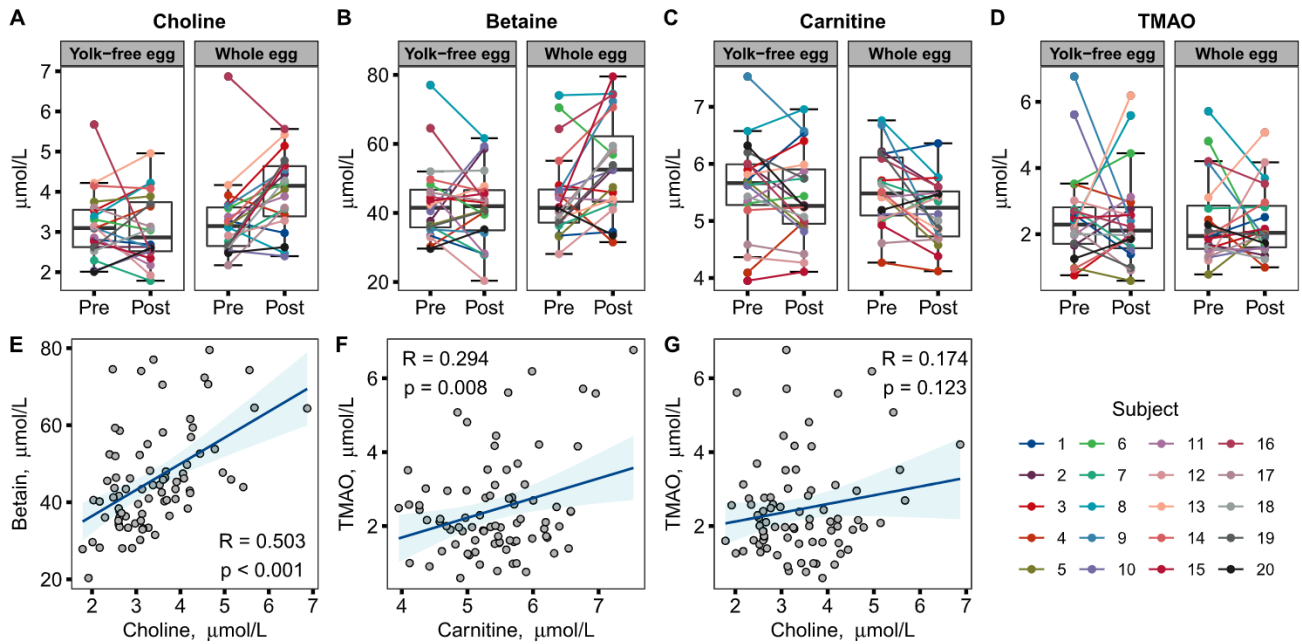


Figure 1 – A-D, Boxplot of plasma choline (A, $P = .01$), betaine (B, $P = .01$), carnitine (C, $P = .22$), and TMAO (D, $P = .99$) concentration in mg/L before and after yolk-free and whole egg treatment. Lines and points are color coded for individual subjects. E-G, scatter plot between plasma choline and betaine (E, Pearson's $R = 0.503$, $P < .001$), carnitine and TMAO (F, $R = 0.294$, $P = .008$), and choline and TMAO (G, $R = 0.174$, $P = .123$) concentration in mg/L with Pearson's correlation and P value.

group Figure 2. The bacterial genera *Prevotella*, *Anaeroplasm*, *Clostridium*, and family *Peptostreptococcaceae*, which were previously reported to be associated with plasma TMAO concentration [21], were not significantly affected differently

by whole egg compared to yolk-free substitute Figure 2. The relative abundance of all bacterial phyla and genera before and after yolk-free egg and whole egg as well as the unadjusted and adjusted P-values calculated using mixed-

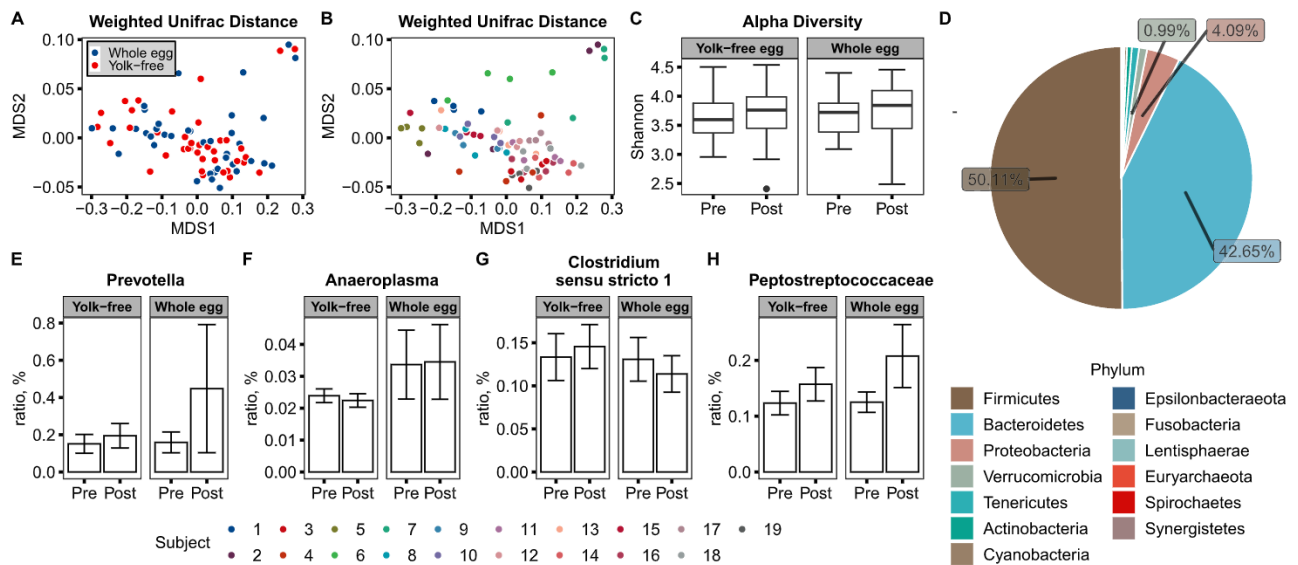


Figure 2 – A and B, The weighted-unifrac distance of gut microbiome community, color coded by treatment (A) and individual subject (B). C, Box plot of the Shannon alpha-diversity of the gut microbiome community before and after yolk-free and whole egg treatment. Lines and points are color-coded based on individual subject. D, Pie plot of gut microbial phyla composition. E-H, Bar plots of the relative abundances of bacterial genera *Prevotella* (E), *Anaeroplasm* (F), *Clostridium sensu stricto 1* (G), and *Lachnospira* (H) before and after yolk-free and whole egg treatment. None of the bacterial genus was significantly different before and after yolk-free or whole egg treatment. Error bars were drawn with standard errors.

linear model are shown in Supplemental Table S3 and S4. We did not find any bacterial taxa that were altered significantly differently by whole and yolk-free egg.

4. Discussion

The effects of egg intake on cardiovascular health have been highly controversial because of the potential of their high cholesterol content to raise plasma cholesterol concentrations [22]. Some observational studies found higher egg intakes were associated with increased CVD mortality and T2D [23,24], while others found it associated with lower hemorrhagic stroke and ischemic stroke [25]. Recent intervention studies found that egg with or without calorie restriction improves blood lipid profile, increases HDL cholesterol efflux capacity, and improves HDL lipid composition [6–8]. The US Department of Agriculture no longer includes the dietary cholesterol restriction in the dietary guidelines based on evidence that dietary cholesterol does not increase plasma cholesterol in most individuals [26]. However, the discovery of a positive correlation between plasma TMAO concentrations and CVD risk represents a new concern about the appropriateness of including eggs in the diet in populations at increased risk for heart disease.

High choline diets ranging in choline concentration from 0.1% to 1% (wt/wt) increased circulating TMAO concentration in a dose-dependent manner in ApoE^{-/-} mice [27]. A choline-enriched phospholipid-protein complex diet with an estimate of 0.5% (wt/wt) choline also increased circulating TMAO concentration in rats [28]. However, in human studies consuming up to 3 eggs per day (approximately 400 mg choline per day) for up to 4 weeks did not affect plasma TMAO concentrations despite increases in plasma choline levels [3–5]. In the current study, 2 eggs per day increased plasma choline and betaine concentrations in overweight, mildly hypercholesterolemic, postmenopausal women in 4 weeks, however, just as in the previously conducted studies, plasma TMAO was not increased. In a similar randomized crossover study, 10 healthy men and women aged between 18 and 30 with normal BMI were given either 2 eggs per day or 1 packet of oatmeal, and there was a significantly higher plasma choline level after egg while plasma TMAO was not different between treatments [5]. Our results and those of other human intervention studies conducted so far suggest several possibilities: that the choline in eggs may not be directly related to plasma TMAO, that the TMAO amount produced in response to 2 to 3 eggs per day is not sufficient to cause significant changes to the TMAO pathway in relatively healthy individuals consuming typical American diets, or that concentrations of TMAO are regulated by as yet poorly understood mechanisms not measured in these studies.

As a key component in TMAO metabolism, the gut microbiome also has a strong connection with CVD. A metagenome-wide association study found increased *Enterobacteriaceae* and *Streptococcus* spp in atherosclerotic and cardiovascular patients [29]. It was also found that *Collinsella* was elevated and *Eubacterium*, *Roseburia*, and *Bacteroides* were decreased in patients with systemic atherosclerotic plaques [30]. Increased *Bacteroides* and *Escherichia coli*, and decreased *Eubacterium*, *Roseburia*, and *Bacteroides* were observed in patients with T2D [31]. Diet is also an important factor of

gut microbiome composition. An animal-based diet (i.e., meat, egg, and cheese) increased bile-tolerant bacteria such as *Alistipes*, *Bilophila*, and *Bacteroides*, while a plant-based diet (i.e., grains, legumes, fruits and vegetables) increased fiber-consuming bacteria such as *Roseburia*, *Eubacterium*, and *Ruminococcus* [32]. In another human study, plasma TMAO was associated with *Clostridium*, *Anaeroplasm*, *Prevotella*, and *Peptostreptococcaceae* [21]. However, according to our results, none of the microbial taxa related to CVD, or associated with plasma TMAO concentrations, were significantly affected by egg consumption.

This study has several limitations. The participants were free-living, and foods except breakfast were not supplied by the study. Instead each participant was asked to follow their habitual diet, instructed to replace their usual breakfast with the egg breakfasts provided, and instructed by a dietitian on how to avoid egg-containing foods. The diet records collected for the study showed participants' diets were not significantly changed throughout the study, confirming the participants adhered with the study protocols. An advantage of the study is that with the randomized order cross-over study design, each participant acted as their own control, therefore the inter-individual differences between participants in their background diet should not affect the overall interpretation of the results.

In conclusion, consuming 2 eggs per day increased plasma choline and betaine but not TMAO level, and did not have a significant impact on gut microbiome composition in overweight to obese but generally healthy postmenopausal women over the course of 4 weeks. These data suggest that adding eggs to the diet is not likely to increase CVD risk associated with TMAO concentrations in postmenopausal women consuming background diets that conform to USDA dietary guidelines.

Supplemental materials

Supplemental material to this article can be found online at <https://doi.org/10.1016/j.nutres.2020.04.002>.

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