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Intravenous trimethylamine *N*-oxide infusion does not modify circulating markers of liver health, glucose tolerance, and milk production in early-lactation cows

William A. Myers,[1] Feiran Wang,[1,2] Crystal Chang,[1] Amanda N. Davis,[1] J. Eduardo Rico,[1] Brianna N. Tate,[1] Tanya L. France,[1] Linfeng F. Wang,[1,3] and Joseph W. McFadden[1]*

[1]Department of Animal Science, Cornell University, Ithaca, NY 14853
[2]China Agricultural University, Beijing, China 830052
[3]Henan Agricultural University, Zhengzhou, China 450046

ABSTRACT

In rodents and humans, the gut bacteria-derived metabolite trimethylamine *N*-oxide (TMAO) has been implicated in the progression of cardiovascular disease, chronic kidney disease, fatty liver, and insulin resistance; however, the effects of TMAO on dairy cattle health and milk production have not been defined. We aimed to determine whether intravenous TMAO infusion modifies measures of liver health, glucose tolerance, and milk production in early-lactation cows. Eight early-lactation Holstein cows (30.4 ± 6.41 d in milk; 2.88 ± 0.83 lactations) were enrolled in a study with a replicated 4 × 4 Latin square design. Cows were intravenously infused TMAO at 0 (control), 20, 40, or 60 g/d for 6 d. Washout periods lasted 9 d. Intravenous glucose tolerance tests (GTT) occurred on d 5. Blood was collected daily. Milk was collected on d −1, 0, 5, and 6. Urine was collected on d −1 and 6. Circulating metabolites, milk components, and TMAO concentrations in milk, urine, and plasma were quantified. Data were analyzed using a mixed model that included the fixed effects of treatment. Concentrations of TMAO in plasma, milk, and urine increased linearly with increasing dose. Dry matter intake and milk production were not modified by treatment. Daily plasma triacylglycerol, fatty acid (FA), and glucose concentrations were not modified. Serum albumin, total protein, globulin, total bilirubin, direct bilirubin, aspartate aminotransferase, γ-glutamyl transferase, and glutamate dehydrogenase concentrations were also not modified by treatment. Serum GTT glucose, FA, and insulin concentrations were not modified by treatment. Plasma total, reduced, and oxidized glutathione concentrations were also not modified by treatment. We conclude that a 6-d intravenous infusion of TMAO does not influence measures of liver health, glucose tolerance, or milk production in early-lactation dairy cows.

Key words: dairy cow, metabolic health, trimethylamine *N*-oxide

INTRODUCTION

In the small intestine of mammals, dietary choline, betaine, and L-carnitine are degraded to form trimethylamine (**TMA**) by bacterial choline trimethylamine lyase, betaine reductase, and carnitine oxidoreductase, respectively (Craciun and Balskus, 2012; Janeiro et al., 2018). The composition of the gut microbiota that express these enzymes is a major factor that influences TMA formation (Cho et al., 2017). Once TMA is absorbed and enters portal circulation, the microbial metabolite is converted to trimethylamine *N*-oxide (**TMAO**) in the liver by flavin-containing monooxygenase 3 (FMO3). In humans, researchers estimate that 95% of TMA is oxidized to TMAO in liver and excreted as waste in urine in a 3:95 TMA:TMAO ratio within 24 h of entry (Janeiro et al., 2018). However, TMAO has received interest in the scientific community because it has been implicated in the progression of cardiovascular disease, nonalcoholic fatty liver disease, type 2 diabetes, and chronic kidney disease (Chen et al., 2016; Missailidis et al., 2016; Heianza et al., 2017; Tan et al., 2019; León-Mimila et al., 2021). The mechanisms of TMAO action are believed to involve enhanced platelet reactivity, compromised reverse cholesterol transport, oxidative stress, inflammation, and reduced glucose tolerance (Tang et al., 2015; Xu et al., 2015; Barrea et al., 2018; Janeiro et al., 2018; DiNicolantonio et al., 2019).

In dairy cattle, unprotected dietary choline is broken down by rumen microbes to TMA, which is then used for methane production (Neill et al., 1978). Rumen-protected choline technologies were developed to reduce

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*Corresponding author: McFadden@cornell.edu

ruminal choline degradation and enhance the intestinal absorption of choline as a means to support hepatic health and milk production (Bobe et al., 2004; Arshad et al., 2020). However, we recently demonstrated that the abomasal infusion of choline chloride increases plasma TMAO concentrations in lactating dairy cows (Myers et al., 2019). Considering the potential aforementioned effects of TMAO on health in humans, our study objectives were to determine whether the intravenous infusion of TMAO modifies circulating markers of metabolic and liver health, glucose tolerance, or milk production in early-lactation dairy cows. This objective was further justified because Xu et al. (2016) observed an increase in circulating TMAO concentrations in postpartum dairy cows that developed fatty liver, relative to healthy controls. A secondary objective was to estimate the amount of TMAO that is excreted in urine versus milk. Such information has potential implications for better understanding the safety of bovine milk for human consumption.

MATERIALS AND METHODS

All experimental procedures were conducted in accordance with the Cornell University Institutional Animal Care and Use Committee (protocol no. 2017-0110; Ithaca, NY). At the Cornell University Dairy Research Center (Harford, NY), 8 early-lactation and multiparous Holstein dairy cows (30.4 ± 6.4 DIM; 2.88 ± 0.83 lactations) were managed in tiestalls and enrolled in a replicated 4 × 4 Latin square design. The inclusion criteria were early-lactation and multiparous cows without signs of clinical disease. Cows were intravenously infused TMAO solubilized in saline (1 L/d) at 4 concentrations: 0 (control), 20, 40, or 60 g/d for experimental periods lasting 6 d. Although data are limited, we chose the 20-g daily dose of TMAO to promote a plasma TMAO response that reflects the upper limit of what may be observed in lactating Holstein cows provided up to 37 g/d of choline chloride postruminally. Washout periods lasting 9 d were used to avoid carryover effects between experimental periods. Treatments were infused at a rate of 47.6 mL/h with the use of a controlled infusion pump (Abbot Plum XL Infusion Pump; Abbott Laboratories). Cows were fed a total mixed ration that was primarily corn silage formulated to meet or exceed nutrient requirements, provided at 0630 h daily (Table 1). Ad libitum access to water was provided. Intravenous jugular catheters were inserted 24 h before the start of infusions, and patency was maintained by flushing with heparinized saline every 12 h when not in use. Cows were milked 3 times daily at 0600, 1400, and 2200 h. The infusion of TMAO was continuous except when cows were milking (~2.5 h/d). No enrolled cows were excluded from the study.

Intravenous glucose tolerance tests (**GTT**) were performed on d 5 relative to the start of infusion, using previously described methods (Davis et al., 2021). Following the removal of feed at 0750 h, cows were intravenously infused with 300 mg of glucose (50% dextrose, wt/vol) per kilogram of body weight at 0950 h. Blood serum samples (10 mL) were collected at −10, 0, 10, 20, 30, 40, 60, 90, 120, 150, and 180 min, relative to initiation of glucose infusion. Feed was returned to the cow immediately following completion of the GTT.

Samples of diet were collected during each experimental period and stored at −20°C. Milk samples were collected on d −1, 0, 5, and 6 relative to the start of infusion. Milk samples were stored in tubes containing the preservative 2-bromo-2-nitropropane-1,3-diol and stored at 4°C for milk composition analysis within 3 d of collection. Preprandial blood samples were collected daily at 0500 h via coccygeal venipuncture on d −1 through d 6, relative to start of infusions. Blood samples were immediately placed on ice for ~30 min, followed by centrifugation at 2,171 × *g* for 15 min at 4°C. Separated plasma and serum samples were stored

Table 1. Ingredients and nutrient composition (% of DM unless otherwise noted) of the experimental diet for lactating Holstein dairy cows

Item	Value
Ingredient	
Corn silage	45.8
Mixed legume haylage	17.2
Corn meal	16.5
Concentrate mix[1]	20.3
Trace minerals[2]	0.08
Yeast culture[3]	0.03
Nutrient composition	
DM, %	36.9
CP	13.7
NDF	33.2
ADF	21.3
Lignin	2.89
Starch	27.1
Crude fat	3.6
Ash	7.35
NE_L, Mcal/kg of DM	1.70

[1]Mix contained 18% soybean hulls, 22.3% soybean meal, 19% bypass soybean product (AminoPlus, AGP), 11.2% blood meal, 7.4% dextrose, 4.22% ground corn fine, 4.7% calcium carbonate, 2% molasses, 3.7% sodium sesquicarbonate, stearic fat bypass (Energy Booster 100, Milk Specialties Global), 2.1% salt, 4.4% palm fat (Palmit 80, GlobalAgri Trade Corporation), 1% magnesium oxide, 0.43% 2-hydroxy-4-methylthio-butanoic acid (ALIMET, Novus International Inc.), 0.54% calcium sulfate, 0.09% vitamin A, D, and E (Cargill Animal Nutrition), and 0.008% vitamin E (Cargill Animal Nutrition).

[2]Trace mineral mix contained 88.2% sucrose, 6.3% calcium sulfate, and 5.4% calcium phosphate (Mercer).

[3]Celmanax (Arm and Hammer Animal Nutrition Co.).

at −80°C until analysis. Urine was collected repeatedly before each milking on d −1 and d 6, pooled within cow and day, and stored at −20°C.

Total mixed ration samples were analyzed for nutrient composition by near-infrared spectroscopy (Cumberland Valley Analytical Services, Cumberland, MD; AOAC International, 1995, method 989.03; Thompson et al., 1995). Milk samples were analyzed for fat, protein, and lactose content using Fourier-transform infrared spectroscopy (Dairy One, Ithaca, NY). Serum albumin, total protein, globulin, aspartate aminotransferase, γ-glutamyl transferase, and glutamate dehydrogenase were quantified using a Beckman AU480 clinical chemistry analyzer (Beckman Coulter; Veterinary Medical Diagnostic Laboratory, University of Missouri, Columbia, MO). Circulating total fatty acids (**FA**), glucose, and triacylglycerol (**TAG**) were quantified in duplicate using commercially available enzymatic colorimetry assay kits (Autokit Glucose no. 997-03001, HR series NEFA-HR no. 999-34691, 995-34791, 991-34891, 993-35191, Wako Chemicals USA Inc.; Triglyceride no. T7532, Pointe Scientific Inc.). Serum insulin concentrations were measured by bovine ELISA (Bovine Insulin ELISA no. 10-1201-01, Mercodia). Total glutathione and oxidized glutathione were quantified using a commercially available enzymatic colorimetry assay (Glutathione Colorimetric Detection Kit no. EIAGSHC no. 20G013C, Thermo Fisher Scientific). Intra- and interassay coefficients of variation (CV) were 4.1 and 9.0%, 6.2 and 10.2%, and 2.6 and 6.7% for plasma total FA, glucose, and TAG, respectively. Day-5 GTT serum metabolite sample intra- and interassay CV were respectively 4.0 and 7.0% for total FA and 6.3 and 5.6% for glucose. Intra- and interassay CV were 2.5 and 9.7% for total glutathione and oxidized glutathione concentrations.

Yields of ECM, 3.5% FCM, and milk components were calculated using milk yield and components for each milking, summed for daily total, and averaged for each collection period, as previously described (Myers et al., 2019). Feed efficiency was calculated as kilograms of ECM per kilogram of DMI. To determine plasma reduced glutathione concentrations, we subtracted the concentration of oxidized glutathione from total glutathione. Daily estimates of urinary output were calculated using measured creatinine concentrations and the following equation: estimated daily urinary output volume = 29 × BW (kg)/creatinine (mg/L; Tebbe and Weiss, 2018).

Milk production, plasma metabolites, and serum markers of liver health were analyzed in SAS (Version 9.4, SAS Institute Inc.) using the MIXED model procedure under the following model:

$$Y_{ijkl} = \mu + C_i + P_j + T_k + pVar_l + e_{ijkl},$$

where Y_{ijkl} = dependent variable, μ = overall mean, C_i = random effect of cow (i = 1–4), P_j = fixed effect of sampling period (j = 1–4), T_k = fixed effect of treatment (k = 1–4), $pVar_l$ = preliminary value for each variable used as a covariate, and e_{ijkl} = residual error. For production data and plasma TAG, glucose, and FA data, baseline d −1 and 0 measurements were averaged and used as covariates. Normality of the residuals was checked with normal probability and box plots, and homogeneity of variances were evaluated with plots of residual versus predicted values. Studentized residual values >3.5 or <−3.5 were considered outliers and removed from the analysis (typically ≤1 per variable). Significance was declared at $P < 0.05$ and trends at $P < 0.10$. All results are expressed as least squares means and their standard errors, unless stated otherwise.

RESULTS

We did not detect a significant difference in DM or energy intake, milk yield, yield or content of milk components (i.e., fat, protein, or lactose), ECM, 3.5% FCM, or feed efficiency with treatment (Table 2). The intravenous infusion of TMAO did increase plasma, urinary, and milk TMAO concentrations in a linear manner ($P < 0.01$; Figure 1A–C). The urinary and milk yields of TMAO also increased linearly with dose ($P < 0.01$; Figure 2A–C). Plasma TAG, FA, and glucose concentrations were not modified by treatment (Table 3). Serum markers of liver health, including albumin, total protein, globulin, aspartate aminotransferase, and γ-glutamyl transferase, were not modified by treatment (Table 4). However, serum glutamate dehydrogenase concentrations tended to be lowered by TMAO treatment ($P = 0.08$; Table 4). The plasma concentrations of total glutathione, reduced glutathione, oxidized glutathione, and the ratio of reduced to oxidized glutathione were not modified by treatment (Figure 3A–D). Serum GTT glucose, FA, and insulin concentrations were also not modified by TMAO infusion (Figure 4A and B).

DISCUSSION

Our experimental approach was able to increase plasma TMAO concentrations and TMAO excretion in urine and in milk. We demonstrate that >99% of TMAO is excreted in urine and <1% in milk. In humans, TMAO is generated in liver from TMA created by the gastrointestinal degradation of L-carnitine, choline, and betaine. Like cows, humans dispose of the near entirety of TMAO that enters circulation in urine (Al-Waiz et

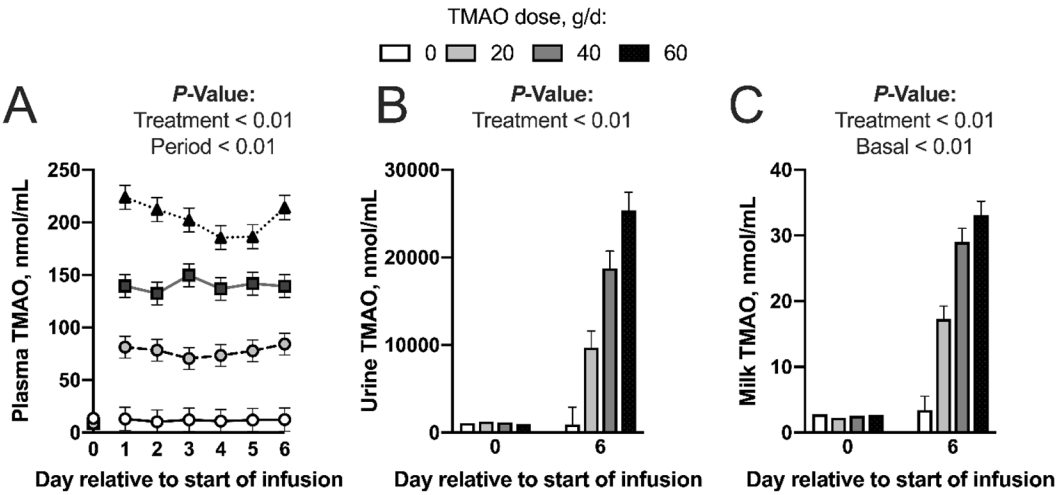

Figure 1. Changes in trimethylamine *N*-oxide (TMAO) concentrations of (A) plasma, (B) urine, and (C) milk in 8 early-lactation Holstein dairy cows intravenously infused with 0, 20, 40, or 60 g of TMAO/d for 6 d. Data are presented as LSM ± SEM. Main effect of treatment; $P < 0.05$ denotes significance.

al., 1987). A reduction in kidney glomerular filtration rate is considered the key cause for elevation in circulating TMAO concentrations in humans (Missailidis et al., 2016). Renal therapies such as dialysis and renal transplantation have been shown to lower circulating TMAO concentrations (Missailidis et al., 2016). The presence of TMAO in milk is also observed in women. Lactating women who received dietary choline chloride supplementation at 480 or 930 mg/d for 10 to 12 wk had breast milk TMAO concentrations of 2.5 and 2.2 μmol/L for each treatment, respectively (Davenport et al., 2015).

In nonruminants, TMAO has been implicated in the progression of adverse health outcomes and disease, including cardiovascular disease, fatty liver, and type 2 diabetes. Because the early-lactation dairy cow develops insulin resistance to support lactation but is prone to severe lipolysis and fatty liver, we sought to investigate the effects of TMAO on milk production as well as on measures of metabolic and liver health. In our study, we did not detect an effect of TMAO on milk production or circulating glucose, FA, or TAG concentrations. Moreover, circulating liver enzyme concentrations were not modified. These data do not suggest that TMAO

Table 2. Milk production responses in early-lactation Holstein dairy cows intravenously infused with trimethylamine *N*-oxide (TMAO)

	Dose,[1] g/d					
Item	0	20	40	60	SEM	*P*-value
DMI	23.7	24.0	23.0	24.1	1.16	0.89
Energy intake, Mcal/kg	40.2	40.8	39.0	40.9	1.98	0.88
Milk yield, kg/d	48.0	47.9	47.0	47.7	1.89	0.98
Milk solids, kg/d						
Fat	1.80	1.89	1.79	1.77	0.07	0.55
Protein	2.33	2.30	2.28	2.36	0.09	0.93
Lactose	1.28	1.27	1.25	1.27	0.05	0.97
Milk composition, %						
Fat	3.68	3.74	3.83	3.78	0.12	0.25
Protein	2.70	2.67	2.69	2.68	0.04	0.94
Lactose	4.89	4.83	4.88	4.95	0.04	0.11
3.5% FCM, kg/d	49.8	51.2	49.3	49.1	0.65	0.72
ECM, kg/d	47.7	48.7	47.2	47.1	1.55	0.80
Feed efficiency[2]	2.01	2.17	2.05	1.96	0.13	0.54
MUN	7.55	8.02	7.72	7.71	0.32	0.60

[1]Eight early-lactation Holstein dairy cows (30.4 ± 6.41 DIM) enrolled in a replicated 4 × 4 Latin square design were intravenously infused with 0, 20, 40, or 60 g/d of TMAO continuously for 6 d.

[2]Feed efficiency = ECM (kg)/DMI (kg).

Table 3. Plasma triacylglycerol (TAG), glucose, and total fatty acid (FA) responses in early-lactation Holstein dairy cows intravenously infused with trimethylamine *N*-oxide (TMAO)

	Dose,[1] g/d					
Item	0	20	40	60	SEM	*P*-value
TAG, mg/dL	19.5	19.8	15.9	18.6	3.45	0.82
Glucose, mg/dL	63.1	60.9	63.0	67.4	3.81	0.67
Total FA, μmol/L	166	177	145	143	22.4	0.21

[1]Eight early-lactation Holstein dairy cows (30.4 ± 6.41 DIM) enrolled in a replicated 4 × 4 Latin square design were intravenously infused with 0, 20, 40, or 60 g/d of TMAO continuously for 6 d.

impairs liver health, although treatment with TMAO has been able to cause liver injury in nonruminants fed TMAO for 8 wk (Hu et al., 2015). We also did not detect a difference in glucose tolerance in response to TMAO infusion in cows. This focus had scientific merit because TMAO has been shown to reduce glucose clearance in mice fed a high-fat diet for 4 wk (Gao et al., 2014), and people with diabetes have higher circulating TMAO concentrations (Dambrova et al., 2016).

In rodents, TMAO disrupts systemic redox homeostasis to promote oxidative stress (Li et al., 2017). In mice fed beef containing L-carnitine, urinary TMA and TMAO concentrations were elevated, as were blood glutathione and plasma superoxide dismutase activity (Van Hecke et al., 2016). In our present study, we did not detect any differences in glutathione status with TMAO treatment, which suggests that TMAO does not promote oxidative stress in early-lactation cows. Such findings are important, considering that choline is fed to early-lactation dairy cows, which likely increases circulating TMAO concentrations (Myers et al., 2019), and redox imbalance develops during this stage of lactation (Castillo et al., 2005). The lack of an effect of TMAO on liver health and redox status align with the lack of an effect of TMAO on milk yield or composition.

We realize that the short duration of our intravenous TMAO infusion and the use of early-lactation cows starting at ~30 DIM are potential limitations of our study, considering that rodent studies often involve extended TMAO exposure (i.e., weeks) and hepatic TAG deposition peaks by wk 2 to 3 postpartum. Indeed, dietary TMAO administration for 18 wk was able to accelerate liver steatosis in mice fed a high-fat diet (Tan et al., 2019). The effect was attributed to an increase in de novo lipogenesis, due in part to increased expression of lipogenic genes, including FA synthase. It remains uncertain whether this would also be observed in transition cows that develop fatty liver because of increased hepatic FA uptake and esterification, and reduced TAG export. Future research should investigate whether increases in circulating TMAO concentrations throughout the transition period predispose cattle to fatty liver and other diseases. However, we consider this unlikely because our results suggest otherwise, and rumen-protected choline supplementation is a common approach to enhance health and milk production in early-lactation cows.

Recent scientific work has focused on the ability of milk consumption to increase TMAO formation in humans, because of the presence of choline and lipids

Table 4. Serum liver panel metabolite responses in early-lactation Holstein dairy cows intravenously infused trimethylamine *N*-oxide (TMAO)

	Dose,[1] g/d					
Item	0	20	40	60	SEM	*P*-value
Albumin, g/dL	3.54	3.44	3.55	3.44	0.09	0.56
Total protein, g/dL	6.88	6.82	7.08	6.92	0.34	0.78
Globulin, g/dL	3.38	3.36	3.50	3.53	0.23	0.53
AST,[2] U/L	65.2	65.3	69.4	64.2	0.04	0.57
GGT,[3] U/L	23.6	23.1	24.7	23.3	0.76	0.28
GLDH,[4] U/L	21.8	23.3	20.4	17.0	1.79	0.08
Globulin, g/dL	3.38	3.36	3.50	3.53	0.23	0.53

[1]Eight early-lactation Holstein dairy cows (30.4 ± 6.41 DIM) enrolled in a replicated 4 × 4 Latin square design were intravenously infused with 0, 20, 40, or 60 g/d of TMAO continuously for 6 d.

[2]Aspartate aminotransferase.

[3]γ-Glutamyl transferase.

[4]Glutamate dehydrogenase.

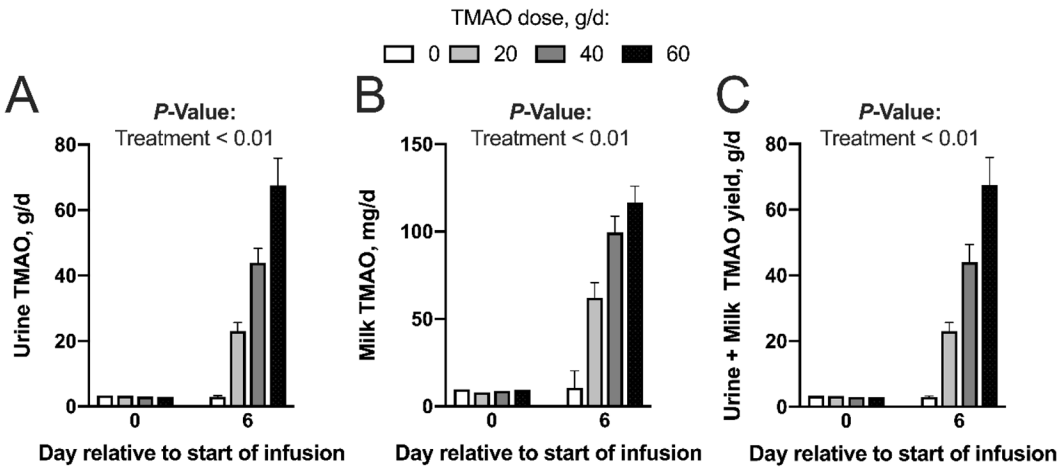

Figure 2. Changes in (A) urine, (B) milk, and (C) total urine + milk yields of trimethylamine *N*-oxide (TMAO) in 8 early-lactation Holstein dairy cows intravenously infused with TMAO at 0, 20, 40, or 60 g/d for 6 d. Data are presented as LSM ± SEM. Main effect of treatment; $P < 0.05$ denotes significance.

with the choline moiety in milk. Among a German adult population, Rohrmann et al. (2016) found that increased milk and dairy food consumption was related to increased plasma TMAO concentrations. In another study, the consumption of fermented dairy products lowered plasma and urinary TMAO concentrations, compared with the consumption of nonfermented milk (Burton et al., 2020). In regard to the observed TMAO concentrations in milk, milk TMAO yield peaked at around 100 mg/d or 2.1 mg/kg of milk. Per 8-oz serving of milk, we estimate that 0.5 mg of TMAO is provided, which is far less than cod (i.e., 529 mg per 6-oz serving; Cho et al., 2017). Therefore, consumers should not be concerned by the relatively low levels of TMAO in milk. Because dairy, meat, eggs, and fish are enriched in choline, betaine, L-carnitine, and TMAO, research has demonstrated that the consumption of these animal foods enhances circulating TMAO concentrations in humans. However, consumers should be cautious about downplaying the importance of choline, because this nutrient has been recognized as essential for human health (Zeisel and Da Costa, 2009). The observed increases in circulating TMAO concentrations during disease in humans may be due to gut microbial dysbiosis, increased gut permeability, and impaired renal function (Farhadi et al., 2008; Boursier et al., 2016; Cho and Caudill, 2017; Soderborg and Friedman, 2019), which may also explain the true underlying cause of disease.

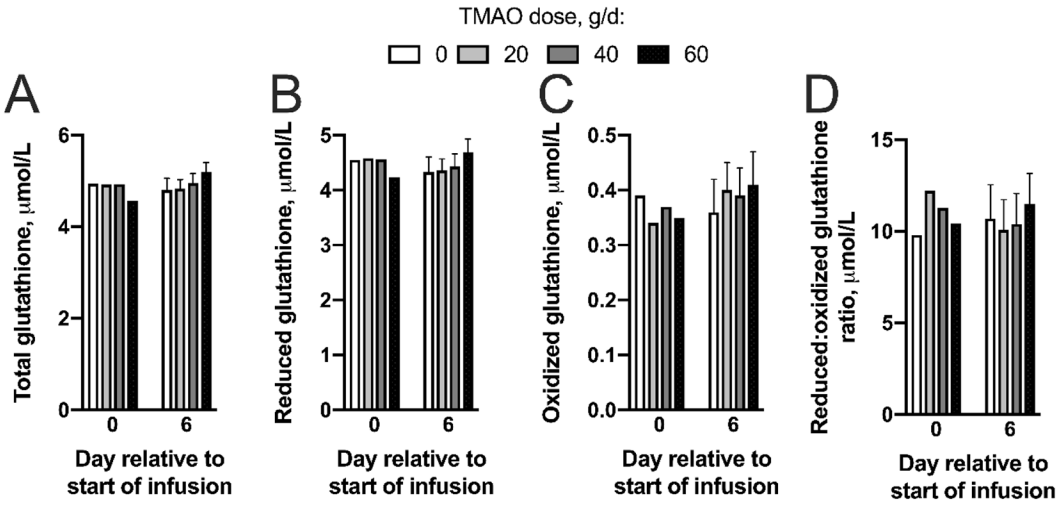

Figure 3. Changes in (A) total, (B) reduced, and (C) oxidized glutathione concentrations and (D) ratio of reduced to oxidized glutathione concentration in 8 early-lactation Holstein dairy cows intravenously infused with trimethylamine *N*-oxide (TMAO) at 0, 20, 40, or 60 g/d for 6 d. Data are presented as LSM ± SEM. Main effect of treatment; $P < 0.05$ denotes significance.

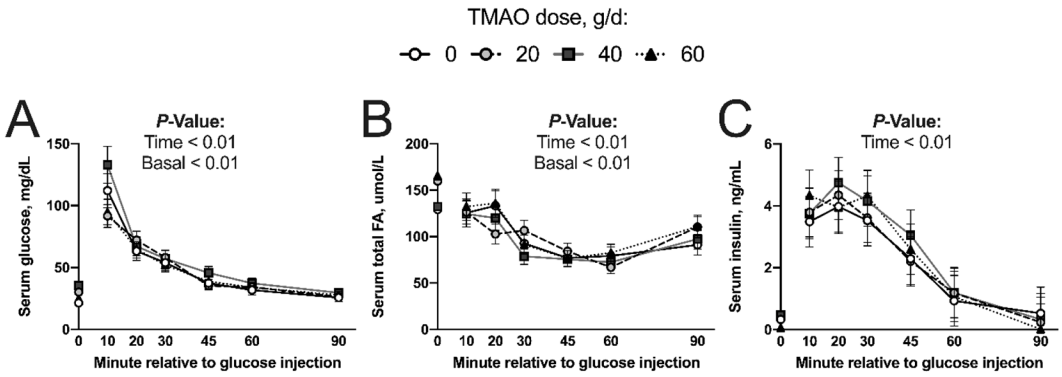

Figure 4. Changes in serum (A) glucose, (B) total fatty acids (FA), and (C) insulin following an intravenous glucose tolerance test administered in 8 early-lactation Holstein dairy cows intravenously infused trimethylamine *N*-oxide (TMAO) at 0, 20, 40, or 60 g/d for 6 d. The test occurred on d 5 of TMAO treatment. Data are presented as LSM ± SEM. Main effect of treatment; $P < 0.05$ denotes significance.

CONCLUSIONS

We conclude that short-term intravenous infusion of TMAO does not change circulating markers of liver function and health, glucose tolerance, glutathione status, or milk production. We confirm that almost all TMAO is excreted as urine in cows. The limited presence of TMAO in milk should be of little concern for human consumption. Future work should evaluate the influence of choline, L-carnitine, or betaine degradation to TMA on the bioavailability of these nutrients to the cow.

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ORCIDS

Amanda N. Davis https://orcid.org/0000-0002-6370-3075
J. Eduardo Rico https://orcid.org/0000-0003-0286-1747
Linfeng F. Wang https://orcid.org/0000-0001-6823-8304
Joseph W. McFadden https://orcid.org/0000-0001-5147-9013