Changes in plasma and milk choline metabolite concentrations in response to the provision of various rumen-protected choline prototypes in lactating dairy cows

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ABSTRACT

Choline feeding in the form of rumen-protected choline (RPC) has been shown to increase milk production and improve measures of metabolic health (e.g., liver triglyceride) in dairy cows. The objective was to characterize changes in plasma and milk choline and choline metabolite concentrations, including microbial-derived trimethylamine N-oxide (TMAO), in response to increasing ruminal spot-doses, different types of RPC, and ruminal stability of RPC in lactating cows. For experiment 1, 12 mid-lactation (121 ± 16.3 d in milk) Holstein cows were balanced by total plasma choline concentrations and milk yields. Cows were assigned to 1 of 3 lipid-encapsulated RPC products (main plots): prototypes P1, P2, and P3 (containing 59, 56, and 30% choline chloride, respectively). Within each main plot, cows were assigned to a sequence of doses in a 4 × 4 Latin square design: 0, 18, 36, or 54 g of choline chloride. Treatments were preconditioned with ground corn and administered as a single ruminal bolus once per experimental period 1 h postfeeding of a total mixed ration. For experiment 2, we compared a control (0 g of choline chloride) versus P2, and P4 and P5 (60 and 62% choline chloride, respectively) in a repeated 4 × 4 Latin square design. Experiment 2 followed a similar design as experiment 1 with modifications: 12 late-lactation (228 ± 7.10 d in milk) Holstein cows were used; treatments were administered as part of a premeal; and cows received a daily allowance of a total mixed ration as equal provisions every 4 h within 24 h before and after treatment. For both experiments, plasma and milk samples were collected for choline and choline metabolite quantification. Data were analyzed using a mixed model including fixed effects of treatment, period, and time. Contrast statements were used to test for linearity of dose and differences between prototypes for experiment 1 and 2, respectively. Plasma and milk TMAO concentrations increased with RPC dose (peak by h). Milk choline and betaine yields increased with RPC dose in a quadratic manner; albeit, dependent upon RPC type. Milk phosphocholine (PCho) and glycerophosphorylcholine (GPC) yields changed by select RPC dose (experiment 1), however Met, PCho, GPC, phosphatidylcholine, and total choline concentrations in milk, and plasma Met and sphingomyelin concentrations were not responsive. We conclude that plasma or milk choline, betaine, and TMAO concentrations are responsive to RPC type, dose, and stage of lactation evaluated.

Key words: choline, dairy cow, methyl donor, trimethylamine N-oxide

INTRODUCTION

Dietary rumen-protected choline (RPC) supplementation has been shown to increase milk production in dairy cattle (Sales et al., 2010; Arshad et al., 2020). The mode of action by which choline elicits this response is likely attributed in part to how choline is metabolized. Mitochondrial oxidation of choline forms betaine via the actions of choline dehydrogenase and betaine aldehyde dehydrogenase (Eklund et al., 2005; Li and Vance, 2008). Further oxidation of betaine by betaine homocysteine S-methyltransferase catalyzes the transfer of a methyl group from betaine to homocysteine to form Met and support methylation (Garrow, 2001). Phosphatidylcholine (PC), lysophosphatidylcholine (LPC), and sphingomyelin (SM) are complex lipids that have a vital role in membrane synthesis, immunity, and cell signaling (Zeisel, 1990; Jiang et al., 2014). An increase in PC synthesis has potential to aid in the assembly and secretion of very low-density lipoproteins; thereby contributing to hepatic triglyceride export and transport to the mammary gland (Grummer et al., 1987; Erdman and Sharma, 1991; Fagone and Jackowski, 2013). Unprotected dietary choline is degraded by bacterial trimethylamine lyase in the gut to trimethylamine. Trimethylamine is then converted to trimethylamine N-oxide (TMAO) in the liver by flavin-containing
monooxygenase 3 (Zeisel et al., 1983). We have shown that the abomasal infusion of choline chloride or dietary supplementation of lecithin, which contains PC, increases plasma TMAO concentrations in dairy cattle (Myers et al., 2019; Wang et al., 2021). Although TMAO is a health concern in humans (Zhu et al., 2016, 2017; Gatarek and Kaluzna-Czapinska, 2021), the acute intravenous infusion of TMAO has not been shown to impair health in early lactation dairy cows (Myers et al., 2021). To the best of our knowledge, the effects of dietary RPC on plasma or milk TMAO status has not yet been characterized in the dairy cow. However, this question is important because changes in plasma or milk TMAO concentrations are likely to reflect changes in choline gastrointestinal degradation and endogenous bioavailability in cows fed RPC (Romano et al., 2015; Li et al., 2021).

Effective dietary RPC supplements aim to achieve (1) a high degree of protection against ruminal choline degradation and (2) provide a high degree of intestinal release for absorption and bioavailability. Although standardized methodologies to measure choline bioavailability that consider the influence of postruminal choline degradation to trimethylamine don’t currently exist, it has been suggested that lipid-encapsulated RPC products may have low bioavailability, relative to no RPC supplementation (Elek and Husveth, 2007; Sales et al., 2010; de Veth et al., 2016). This can be due to inadequate or excessive lipid-encapsulation that limits ruminal protection or intestinal digestibility, respectively. In addition, the conversion of choline to trimethylamine in the rumen or intestine could limit choline bioavailability. Therefore, our objectives were to investigate (1) the effects of increasing spot-doses and different choline chloride contents of RPC, and (2) differences in ruminal stability of RPC on the responses of plasma and milk choline and choline metabolites including TMAO. We hypothesized that plasma and milk choline concentrations would increase with increasing dose of RPC. We also hypothesized that plasma and milk TMAO concentrations would be responsive to RPC delivery.

MATERIALS AND METHODS

To test our objective, we conducted 2 independent experiments. For experiment 1, we examined the effects of 3 RPC supplements at increasing dose in mid-lactation cows. We identified a superior product based on plasma and milk choline and betaine response. Experiment 2 was performed to compare this product with 2 additional alternative RPC supplements, that differed in the degree of ruminal stability, on the plasma and milk choline and choline metabolite response in late-lactation cows. For both experiments, the choline chloride percentages for each RPC product were unique with the realization that small or large differences can likely affect ruminal degradation and intestinal release. All procedures were carried out in compliance with Cornell University’s Institutional Animal Care and Use Committee (protocol #2017–0110).

Experiment 1 Design

Twelve mid-lactation, multiparous, Holstein dairy cows (121 ± 16.3 DIM, ± SD; 3.1 ± 0.3 BCS; 2.8 ± 0.7 lactations; 661.9 ± 32.5 kg of BW) were housed in tiestalls at the Cornell University Dairy Research Center (Harford, NY). Cows were acclimated to the facility and diet for 10 d. Cows were provided ad libitum access to TMR and water. Diets primarily contained corn silage, ground corn, haylage, and soybean meal (Supplemental Table S1; https://doi.org/10.17632/5ntvj7j4f.2; France, 2022). Diets were offered once daily at 0700 h to achieve 10% orts. Cows were milked thrice daily at 0600, 1400, and 2200 h. Body weights were measured weekly.

Cows were balanced by plasma total choline concentrations and milk yields, measured during acclimation, and assigned to one of 3 RPC products (main plots): prototype 1, 2, and 3 (P1, P2, and P3, respectively). Within each main plot, cows were assigned to a sequence of doses in a study with a 4 × 4 Latin square design: unsupplemented (control), low, moderate, or high RPC (0, 18, 36, or 54 g of choline chloride, respectively). Each RPC supplement was provided by Balchem Corporation. Each RPC supplement was manufactured by a patented microencapsulation technique, which protects choline from ruminal degradation, yet releases it for absorption in the small intestine. The lipid coating used in these prototypes is similar to coatings used in other Balchem products (Ji et al., 2016). P1, P2, and P3 contained 59, 56, and 30% choline chloride, respectively. Ruminal stability of each prototype was determined by a third-party commercial laboratory (Cumberland Valley Analytical Services, Waynesboro, PA) using an in situ procedure. P1, P2, and P3 were 76, 75, and 73% rumen-protected at 8 h of incubation. Treatments were preconditioned in a tumbler mill (Dual Drum Rotary Rock Tumbler, 3400 Motor RPM) with 33% (wt/wt) ground corn for 10 min before loading into gelatin capsules (catalog #A-35110 CT; Torpac Inc.). Intravenous jugular catheters were inserted 24 h before bolus administration and were removed immediately following the last blood sample. Catheters were monitored 12 h postinsertion, flushed with saline, and then 1:10 or 1:100 diluted heparin was added to maintain patency depending on stage of use. Spot-doses were adminis-
tered once per experimental period as a ruminal bolus 1 h postfeeding via the esophagus using an oral balling gun. Plasma samples were collected from jugular catheters at 0, 2, 4, 6, 8, 10, 12, 14, 16, 20, and 36 h, relative to bolus administration. Milk samples were individually collected in accordance with normal milking times, where 0 h denotes the milking immediately before bolus administration, and 8, 16, and 24 h samplings were in alignment with the 3 following milking times (approximately 1400, 2200, and 0600 h). A 7-d washout was utilized between bolus administrations.

**Experiment 2 Design**

Twelve late-lactation multiparous Holstein dairy cows (228 ± 7.1 DIM; 2.92 ± 0.38 BCS; 2.5 ± 0.5 lactations; 757.2 ± 52.6 kg of BW) were housed and managed in similar conditions as experiment 1. The diet fed is described in Supplemental Table S1; however, cows received their daily feed allotment as equal provisions every 4 h starting 24 h prior and ending 24 h following treatment administration. This frequent feeding approach was an attempt to reduce diurnal fluctuations in metabolite concentrations in response to variable intake that may occur throughout a day when feed is provided once daily. The total amount of TMR provided reflected the average voluntary feed intake measured the 5-d preceding baseline measurements when fed once daily at 0800 h to achieve 10% ors. Cows were milked thrice daily at 0600, 1400, and 2200 h. Cows were provided ad libitum access to water. Body weights were measured weekly.

Cows were balanced by plasma total choline concentrations and milk yields, measured during the acclimation period, and assigned to a sequence of 4 treatments in a replicated 4 × 4 Latin square design: unsupplemented (control), or prototype 2, 4, or 5 (P2, P4, and P5, respectively), which contained 56, 60, and 62% choline chloride, respectively, and were 75, 60, and 79% rumen-protected at 8-h of incubation, respectively. Similar to experiment 1, all prototypes were provided by Balchem Corporation. All treatments provided an equivalent of 36 g of choline chloride and were preconditioned with 33% (wt/wt) ground corn following the same protocol as described in experiment 1. Treatments were fed as part of a 1 kg TMR pre-meal at 0800 h (control treatments only received 1 kg of TMR). The change in RPC administration method from a bolus for experiment 1 to a premeal for experiment 2 was an improvement in study design to account for the effect of mastication. Cows were provided 20 min to consume the pre-meal before the delivery of their 4 h dietary allowance. Intravenous jugular catheters were inserted 48 h before treatment delivery and were removed immediately following the last blood sample. Patency was maintained as described for experiment 1. Milk samples were collected at 0600, 1400, and 2200 h the day before and immediately following the pre-meal. Plasma samples were collected through jugular catheters at 0, 2, 4, 6, 8, 10, 12, 14, 16, 20, and 24 h, relative to the provision of the pre-meal. We provided a 7-d washout between the choline-supplemented pre-meals.

**Sample Collection and Analyses**

Milk samples (~40 mL), initially stored at 4°C with 2-bromo-2-nitropropane-1,3-diol for preservation, were analyzed for true protein, lactose, and fat concentrations by mid-infrared analysis within 2 d of collection for descriptive purposes (Dairy One, Ithaca, NY). All plasma and milk samples (~1.5 mL) for choline and choline metabolite quantification were stored at ~−20°C, without 2-bromo-2-nitropropane-1,3-diol, for no more than 48 h, and then moved to −80°C until analysis. Liquid chromatography coupled with tandem mass spectrometry was used to quantify plasma and milk choline, betaine, dimethylglycine, Met, and TMAO, as previously described by Holm et al. (2003). Glycero-phosphorylcholine (GPC), phosphocholine (PCho), PC, LPC, and SM were quantified by liquid chromatography–mass spectrometry according to Koc et al. (2002) and Yan et al. (2013). Both methods were used to measure plasma and milk choline and choline metabolites for all prototypes studied with the exception that the plasma PCho, PC, LPC, and SM concentrations were only measured in response to P2 in experiment 1.

**Calculations and Statistical Analysis**

We calculated total choline concentrations as the sum of choline, GPC, PCho, PC, LPC, and SM. The areas under the curve (AUC) for plasma TMAO concentrations were calculated using the trapezoidal method as described by Pires et al. (2007) and Cardoso et al. (2011).

For experiment 1, choline and choline metabolite quantification data were analyzed in SAS (version 9.4; SAS Institute Inc.) using the MIXED model procedure under the following model:

\[
Y_{ijklmn} = \mu + C_i(T_k) + D_j + T_k + P_l + H_m + (D_j \times T_k) + (D_j \times T_k \times H_m) + pV_{arn} + e_{ijklmn},
\]

where \(Y_{ijklmn}\) was the dependent variable; \(\mu\) was the overall mean; \(C_i(T_k)\) was the random effect of cow \((i = 1–12)\) nested within prototype \((k = 1–3)\); \(D_j\) was the fixed effect of dose \((j = 1–4)\); \(T_k\) was the fixed effect...
of RPC prototype \((k = 1–3)\); \(P_i\) was the fixed effect of period \((i = 1–4)\); \(H_m\) was the fixed effect time \((m = h 1–11)\); \(D_j \times T_k\) was the interaction between dose and RPC prototype; \(D_j \times T_k \times H_m\) was the interaction between dose, RPC prototype, and time; \(p\)Var was the value for each variable used as a covariate (i.e., baseline measurements); and \(e_{ijkmn}\) was the residual error. Time was also included as a repeated measure with cow as the subject nested within prototype. Contrast statements were included to determine whether the effects of dose were linear or quadratic. For analysis of only prototype 2 plasma metabolites, we followed the same mixed model parameters as stated above, however the fixed effect of prototype was removed, the random effect was cow \((i = 1–4)\), and the fixed effect of time did not include \(h\) 36.

For experiment 2, choline and choline metabolite quantification data were analyzed in SAS using the MIXED model procedure under the following model:

\[
Y_{ijklmn} = \mu + C_i(S_l) + P_j + T_k + H_m + S_l + (H_m \times T_k) + [P_j \times T_k \times C_i(S_l)] + p\text{Var} + e_{ijklmn},
\]

where \(Y_{ijklmn}\) was the dependent variable; \(\mu\) was the overall mean; \(C_i(S_l)\) was the random effect of cow \((i = 1–12)\) nested within square \((l = 1–3)\); \(P_j\) was the fixed effect of period \((j = 1–4)\); \(T_k\) was the fixed effect of prototype \((k = 1–4)\); \(H_m\) was the fixed effect of time \((m = 1–11)\); \(S_l\) was the fixed effect of square; \(H_i \times T_k\) was the interaction between time and prototype; \([P_j \times T_k \times C_i(S_l)]\) was the random effect of the interaction between period, prototype, and cow nested within square; \(p\)Var was the value for each variable used as a covariate (i.e., baseline measurements); and \(e_{ijklmn}\) was the residual error. Time was also included as a repeated measure with cow as the subject nested within square. Contrast statements were included to determine differences between control versus rest (all prototypes) and prototype 2 versus prototype 4 and 5.

Three common covariance structures (variance components, first-order autoregressive, and compound symmetry) for repeated measures analysis were evaluated and the structure with the smallest Akaike’s information criterion coefficient was selected for analysis. Plasma TMAO AUC concentrations in both experiments followed their respective mixed model statistical approach stated above, without the fixed effect of time and any time interaction effect. Main effects were declared significant at \(P \leq 0.05\) and trends at \(P < 0.10\). Interactions were declared significant at \(P \leq 0.05\), and tendencies were declared at \(0.10 \leq P \leq 0.15\). Mean separations were protected by the initial \(F\)-test of dose \(\times\) time (experiment 1) or time \(\times\) prototype (experiment 2) at trends \((P \leq 0.10)\) or significance \((P \leq 0.05)\). Mean separations at a given time point were performed using the PDIF statement, with significance at \(P \leq 0.05\). Studentized residual values > 3.0 or < −3.0 were considered outliers and removed from analysis.

**RESULTS**

**Experiment 1**

Daily DMI (mean ± SD) was 21.6 ± 0.71 kg/d. Mean daily milk yield, fat %, and protein % for the duration of the study were 37.3 ± 1.14 kg/d, 3.96 ± 0.15%, and 3.07 ± 0.15%, respectively. Dose of RPC quadratically increased plasma choline concentrations \((P = 0.01); Table 1; Supplemental Figure S1, https://doi.org/10.17632/5ntvjr7j4f2; France, 2022). Similarly, RPC dose quadratically increased milk choline yield \((P = 0.04); Table 2\). Dose of RPC linearly increased plasma betaine concentrations \((P < 0.01; Table 1; Supplemental Figure S1) and an effect of the interaction between prototype and dose was observed \((P < 0.01; Table 1\). Dose of RPC quadratically increased milk betaine yields \((P = 0.04); Table 2\). Prototype of RPC did not modify choline or betaine concentrations in plasma or milk. Dose of RPC did not modify milk choline and betaine concentrations. We determined P2 was the superior product because of the increased milk choline concentrations and yields over time \((Type \times Dose, P = 0.15 and 0.04, respectively; Table 2)\).

We observed a linear dose effect for plasma TMAO concentrations across dose \((P < 0.01; Table 1)\) and an effect of RPC prototype \((P = 0.02)\) and the interaction between prototype and dose \((P < 0.01)\). Plasma TMAO had the highest response for P2, with the peak concentration observed at h 4 (Figure 1). Dose linearly increased milk TMAO concentrations \((P < 0.01; Table 2\). Peak milk TMAO concentrations were observed at h 16 across all prototypes, with milk TMAO concentrations in cows provided P2 being the most responsive (Figure 1). Dose linearly increased milk TMAO yields \((P < 0.01; Table 2\). Prototype and the interaction between prototype and dose affected milk TMAO yields \((P < 0.05; Table 2; Figure 1)\). Rumen-protected choline dose and prototype modified AUC for plasma TMAO concentrations \((P < 0.05; Figure 2)\). In addition, the interaction between RPC prototype and dose tended to modify plasma TMAO AUC \((P = 0.06; Figure 2\). Prototype 2 had the greatest plasma TMAO AUC for all doses studied.

With increasing P2 dose, we observed a decrease in plasma PC and total choline concentrations in mid-lactation cows \((P < 0.01; Table 3)\). Prototype of RPC tended to modify milk PCho concentrations \((P = 0.08;
Table 2); however, no changes were observed across dose. Dose of RPC linearly increased milk PCho yields ($P = 0.05$; Table 2). Dose of RPC linearly decreased milk GPC yields ($P = 0.03$; Table 2), but milk GPC yields were not modified by RPC prototype. Plasma Met concentrations were not modified by dose for all prototypes. Plasma SM and LPC concentrations were not modified by dose of P2. Milk Met, GPC, PC, SM, and total choline concentrations were not modified by RPC prototype or dose. Moreover, RPC dose did not alter milk Met, PC, SM, or total choline yields.

**Experiment 2**

Over 90% of late-lactation cows studied consumed their entire premeal including treatment within 10 min. Daily DMI (mean ± SD) was 27.7 ± 3.01 kg/d. Mean daily milk yield, fat percentage, and protein percentage for the duration of the study were 41.2 ± 7.7 kg/d, 4.60 ± 0.83%, and 3.12 ± 0.25%, respectively. Plasma choline concentrations increased in cows provided RPC, relative to unsupplemented control (control vs. rest, $P < 0.05$; Figure 3; Table 4). Plasma LPC concentrations tended to be modified by prototype (control vs. rest, $P = 0.09$; Table 4), where the control cows had higher LPC plasma concentrations as compared with those provide RPC. We did not observe differences in plasma betaine, Met, PC, SM, and total choline concentrations with prototype treatment. Plasma PCho was not detected in cows that received any RPC prototype. Milk choline concentrations ($P = 0.11$) and milk total choline yields ($P = 0.14$) tended to increase with the provision of P2 compared with P4 and P5 (Table 5). Milk betaine concentrations tended to be greater in cows fed RPC versus control (control vs. rest, $P = 0.08$; Figure 4, Table 5). Milk betaine yields were different when comparing cows that received RPC versus those that received the control treatment (control vs. rest, $P = 0.05$; Table 5). We did not detect an effect of RPC for milk choline, Met, PCho, PC, GPC, and total choline concentrations and yields, relative to control.

Plasma TMAO concentrations were different between control and prototype treatments (control vs. rest, $P < 0.05$; Table 4). Moreover, cows provided RPC had increased milk TMAO concentrations and yields, relative to control (control vs. rest, $P < 0.01$; Table 5). Peak milk TMAO concentrations and yields were observed at h 8 and 16, respectively (Figure 4). Plasma TMAO concentrations peaked at h 4 in cows that received RPC, whereas plasma choline concentrations remained consistently higher in RPC-treated versus control.
Table 2. Experiment 1 concentrations and yields of choline and choline metabolites in milk when mid-lactation dairy cows were provided different doses of choline chloride and prototypes of rumen-protected choline (RPC) as a ruminal bolus.

| Metabolite          | P1  | P2  | P3  | SEM | Type  | Dose  | Type × Dose |
|---------------------|-----|-----|-----|-----|-------|-------|-------------|
|                     | 0   | 18  | 36  | 54  |       |       |             |
| Concentration, μM   |     |     |     |     |       |       |             |
| Choline             | 243 | 250 | 240 | 235 | 215<sup>a</sup> | 243<sup>b</sup> | 265<sup>b</sup> | 267<sup>b</sup> | 243<sup>b</sup> | 254<sup>b</sup> | 263<sup>b</sup> | 224<sup>b</sup> | 14.5 | 0.85 | 0.26 | 0.15 |
| Betaine             | 47.9 | 48.2 | 45.4 | 47.5 | 47.0 | 49.5 | 49.3 | 49.4 | 48.6 | 51.3 | 54.2 | 47.5 | 3.58 | 0.82 | 0.32 | 0.94 |
| TMAO                | 2.17 | 2.60 | 3.17 | 4.65 | 2.59 | 3.86 | 5.43 | 7.46 | 2.24 | 2.80 | 4.40 | 5.29 | 0.50 | 0.01 | <0.01<sup>1</sup> | 0.21 |
| Met                 | 0.83 | 0.60 | 0.64 | 0.54 | 0.59 | 0.66 | 0.71 | 0.80 | 0.63 | 0.60 | 0.50 | 0.64 | 0.64 | 0.51 | 0.84 | 0.38 |
| PCho                | 507 | 540 | 512 | 491 | 565 | 557 | 530 | 557 | 548 | 539 | 559 | 540 | 327 | 0.58 | 0.71 | 0.52 |
| GPC                 | 67.0 | 68.5 | 72.4 | 80.0 | 75.8 | 76.8 | 77.4 | 64.4 | 68.2 | 71.6 | 69.4 | 63.9 | 5.84 | 0.41 | 0.85 | 0.34 |
| PC                  | 123 | 128 | 132 | 139 | 135 | 143 | 142 | 117 | 130 | 131 | 126 | 117 | 9.82 | 0.57 | 0.77 | 0.66 |
| SM                  |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Total choline       | 1,144 | 1,091 | 1,090 | 1,069 | 1,114 | 1,139 | 1,134 | 1,151 | 1,104 | 1,095 | 1,161 | 1,070 | 42.9 | 0.57 | 0.77 | 0.66 |
| Yield, mg/milking   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Choline             | 301 | 296 | 297 | 282 | 269<sup>a</sup> | 316<sup>b</sup> | 323<sup>b</sup> | 340<sup>b</sup> | 303<sup>b</sup> | 338<sup>a</sup> | 326<sup>b</sup> | 282<sup>b</sup> | 22.0 | 0.62 | 0.04<sup>1</sup> | 0.04 |
| Betaine             | 53.9 | 56.1 | 69.9 | 52.7 | 54.8 | 62.4 | 57.8 | 61.0 | 57.4 | 68.2 | 66.0 | 61.9 | 6.27 | 0.62 | 0.04<sup>1</sup> | 0.38 |
| TMAO                | 1.97<sup>a</sup> | 2.33<sup>a</sup> | 3.02<sup>b</sup> | 3.49<sup>b</sup> | 2.42<sup>a</sup> | 3.82<sup>b</sup> | 5.08<sup>b</sup> | 7.03<sup>d</sup> | 2.25<sup>b</sup> | 3.06<sup>a</sup> | 4.17<sup>b</sup> | 5.06<sup>b</sup> | 0.48 | 0.01 | <0.01<sup>1</sup> | 0.03 |
| Met                 | 1.56 | 1.15 | 1.01 | 1.17 | 1.22 | 1.24 | 1.14 | 1.14 | 0.91 | 1.01 | 0.87 | 1.04 | 1.04 | 0.26 | 0.53 | 0.30 | 0.42 |
| PCho                | 229 | 253 | 262 | 267 | 238 | 281 | 273 | 317 | 261 | 263 | 376 | 294 | 37.7 | 0.19 | 0.05<sup>1</sup> | 0.50 |
| GPC                 | 1,748 | 1,710 | 1,678 | 1,574 | 1,757 | 1,801 | 1,652 | 1,767 | 1,855 | 1,832 | 1,728 | 1,755 | 147 | 0.82 | 0.03<sup>a</sup> | 0.61 |
| PC                  | 585 | 605 | 657 | 627 | 726 | 754 | 703 | 702 | 618 | 621 | 665 | 718 | 55.8 | 0.29 | 0.43 | 0.18 |
| SM                  | 1,194 | 1,206 | 1,332 | 1,264 | 1,376 | 1,368 | 1,305 | 1,274 | 1,174 | 1,262 | 1,273 | 1,264 | 126 | 0.81 | 0.70 | 0.34 |
| Total choline       | 4,406 | 4,182 | 4,324 | 4,294 | 4,328 | 4,434 | 4,249 | 4,287 | 4,251 | 4,272 | 4,426 | 4,267 | 316 | 0.90 | 0.96 | 0.77 |

<sup>a</sup>Differences in superscript within prototype represent significance at $P < 0.05$.  
<sup>b</sup>Trimethylamine N-oxide (TMAO), phosphocholine (PCho), glycerophosphorylcholine (GPC), phosphatidylcholine (PC), sphingomyelin (SM). Total choline is the sum of choline, PCho, GPC, PC, and SM. 
<sup>1</sup>P1 = prototype 1 of RPC; P2 = prototype 2 of RPC; P3 = prototype 3 of RPC (containing 59, 56, and 30% choline chloride, respectively), with 0, 18, 36, and 54 g of choline chloride provided as the dose within each prototype. 
<sup>2</sup>Standard error of the mean represented as the highest SEM for each variable. 
<sup>3</sup>Effect of RPC prototype. 
<sup>4</sup>Effect of choline chloride dose, 0, 18, 36, or 54 g/d. 
<sup>5</sup>Effect of the interaction between RPC prototype and dose. 
<sup>6</sup>Linear dose effect as a contrast statement. 
<sup>7</sup>Quadratic dose effect as a contrast statement.
cows over time (Figure 3). The RPC feeding increased TMAO AUC (control vs. rest, \( P < 0.01 \); Figure 3). We were interested to determine whether individual cow variation within prototype was observed for plasma TMAO AUC (Figure 3). Within each prototype, we observed a 2-fold variation between the top and bottom quartiles (3 cows/quartile) for plasma TMAO AUC.

**DISCUSSION**

In dairy cows, studies have evaluated changes in plasma choline (total or free) concentrations when feeding RPC (Elek et al., 2008; Zenobi et al., 2018; Potts, 2019) or abomasally infusing unprotected choline chloride (de Veth et al., 2016; Myers et al., 2019). de Veth et al. (2016) observed an increase in arterial plasma choline concentrations from 4.54 \( \mu M \) in control cows to 5.34 or 13.09 \( \mu M \) in cows that were fed 25 g/d of choline ion as RPC or abomasally infused 25 g/d of unprotected choline ion in late-lactation cows, respectively. Myers et al. (2019) abomasally infused 37.3 g/d of choline ion to increase plasma choline concentrations from 14.0 to 57.1 nmol/mL (SEM = 1.16). Milk choline concentrations and yields are also responsive to increases in postruminal choline chloride supply (Sharma and Erdman, 1989). However, some studies...
have reported no change in plasma or milk choline concentrations with RPC feeding [e.g., feed-restricted dry cows (Zenobi et al., 2018) or fresh cows (Potts, 2019)]. These discrepancies may be due to differences in gastrointestinal degradation, intestinal release, and bioavailability of choline in rumen-protected form. Alternatively, stage of lactation may influence choline and choline metabolite outcomes. These uncertainties will require clarity considering that choline has not yet been defined as an essential nutrient for milk production in dairy cattle (NASEM, 2021).

In both mid- and late-lactation cows, we observed limited increases in plasma choline concentrations with RPC provision. The magnitude of these responses was dependent on prototype. However, we only observed increases in milk choline yields in mid-lactation cows provided RPC (i.e., a 26% increase for P2 at 54 g vs. 0 g of choline chloride). Milk choline concentrations and yields were comparable when comparing results derived from experiment 1 and 2 (i.e., mid- vs. late-lactation cows); however, in the present study, milk PCho concentrations and yields were ~35% greater in mid-lactation cows as compared with those in late lactation, which is supported by Artegoitia et al. (2014). Milk PCho is a key micronutrient for the development of the neonatal calf. In our study, in mid-lactation cows (experiment 1), the observed increase in milk choline yields with P2 provision supports that choline escaped ruminal degradation, was released in the intestine for absorption, and was bioavailable for use by the mammary gland. In experiment 2, P2 with a ruminal stability of 75% did not influence milk choline concentrations or yields when compared with P4 or P5 with 60 and 79% ruminal stability, respectively. This lack of a response could be due to the later stage of lactation. Alternatively, the negligible difference in ruminal stability of RPC was insufficient to elicit a biological response. Rumen-protected choline, regardless of prototype, increased milk yields of PCho with increasing dose in mid-lactation cows (i.e., experiment 1). This PCho response could potentially indicate that choline was used by the cow to activate the cytidine diphosphate-choline pathway. Whether this occurred in the mammary gland or other tissue (e.g., the liver) is uncertain. It deserves to be mentioned that Coleman et al. (2019) abomasally infused choline chloride to mid-lactation dairy cows in negative energy balance and observed no difference in the hepatic expression of choline kinase A or B, which suggests that an increase in choline supply did not enhance PCho production; however, changes in plasma or milk PCho concentrations were not measured.

![Figure 2](image-url)
Figure 3. Observed changes in plasma choline (A) and trimethylamine N-oxide (TMAO; B) concentrations in 12 late-lactation Holstein cows provided prototypes of rumen-protected choline (RPC) area under the curve (AUC) concentrations (C), and top and bottom quartile response for plasma TMAO AUC (D) when cows were provided different RPC prototypes. Data are represented as LSM with SEM minimum and maximum. For plasma choline concentrations: period, $P = 0.03$; prototype, $P = 0.21$; time, $P = 0.01$; square, $P = 0.89$; time × prototype, $P = 0.39$. For plasma TMAO concentrations: period, $P < 0.01$; prototype, $P = 0.01$; time, $P = 0.01$; square, $P = 0.62$; time × prototype, $P < 0.01$. For plasma TMAO AUC concentrations: period, $P < 0.01$; prototype, $P = 0.01$; square, $P = 0.71$. Contrast statement control versus rest had $P$-values of $P = 0.04$, $P < 0.01$, and $P < 0.01$ for panels A, B, and C, respectively. Contrast statement P2 versus P4 and P5 had $P$-values of $P = 0.66$, $P = 0.49$, and $P = 0.22$ for panels A, B, and C, respectively. Mean separations between prototypes ($P < 0.05$) within a given time point were evaluated: control versus rest (*); P2 versus P4 (†); and P2 versus P5 (‡).
Mixed plasma or milk betaine responses have been observed with RPC supplementation in nonlactating and lactating dairy cattle (de Veth et al., 2016; Zenobi et al., 2018; Potts, 2019). de Veth et al. (2016) observed a plasma betaine response to abomasal choline chloride infusion or dietary RPC feeding. Potts (2019) reported no change in milk or plasma betaine concentrations with dietary RPC supplementation. Similarly, plasma betaine concentrations were not modified by dose of RPC in nonlactating, feed-restricted cows (Zenobi et al., 2018). Conversion of choline to betaine supports labeling choline as a methyl donor because betaine homocysteine S-methyltransferase converts betaine to dimethylglycine and Met to support transmethylation. We observed that RPC increased milk betaine yields in a dose-dependent manner in mid- and late-lactation cows. A similar response was observed for plasma betaine concentrations in mid-lactation cows but not late-lactation cows. Interestingly, milk betaine yields were ~35% greater in late-lactation than mid-lactation cows. It is possible that the increased yields of and demand for milk betaine during late lactation prevented a plasma betaine response to RPC provision. This is supported by the absence of an RPC response in milk choline yields during late lactation. We hypothesize that choline was oxidized more so to betaine during late lactation. Moreover, the absence of a milk PCho response to RPC during late lactation is suggestive that choline was not used by the cytidine diphosphate-choline pathway but rather oxidized to betaine; however, we don’t know whether this is specific for the mammary gland or alternative tissues. These uncertainties need to be addressed in a future study comparing effects of RPC in various stages of lactation within a single experiment with similar conditions.

We observed a limited response to RPC prototype or dose with regard to plasma and milk complex lipids containing the choline moiety. In support, the apparent majority of published findings don’t detect a response in plasma or milk total PC, LPC, or SM concentrations or yields to RPC supplementation (de Veth et al., 2016; Zhou et al., 2016; Myers et al., 2019; Potts, 2019). This is likely due to the dependence for choline but also one or 2, predominantly unsaturated, fatty acyl chains for the synthesis of these phospholipids. We have hypothesized that co-supplementation of RPC and omega-3 fatty acids may activate the phosphatidylethanolamine N-methyltransferase pathway and enhance the incorporation of choline into PC (Bernhard et al., 2020; McFadden et al., 2020). It could be

Table 5. Experiment 2 concentration and yield of choline and choline metabolites in milk when late-lactation dairy cows were provided different prototypes of rumen-protected choline (RPC)

| Metabolite1 | Prototype2 | SEM3 | P-value | Control vs. rest4 | P2 vs. P4 and P55 |
|-------------|------------|------|---------|-------------------|------------------|
| Choline     | Control    | 268  | 17.0    | 0.77              | 0.11             |
|             | P2         | 278  |         | 0.08              | 0.70             |
|             | P4         | 262  |         | 0.08              | 0.70             |
|             | P5         | 259  |         | 0.08              | 0.70             |
| Betaine     | Control    | 51.3 | 6.13    | 0.05              | 0.77             |
|             | P2         | 55.3 |         | 0.05              | 0.77             |
|             | P4         | 53.7 |         | 0.05              | 0.77             |
|             | P5         | 59.1 |         | 0.05              | 0.77             |
| TMAO        | Control    | 1.16 | 1.14    | 0.07              | 0.18             |
|             | P2         | 1.25 |         | 0.07              | 0.18             |
|             | P4         | 1.21 |         | 0.07              | 0.18             |
|             | P5         | 1.14 |         | 0.07              | 0.18             |
| Met         | Control    | 71.2 | 70.6    | 0.48              | 0.88             |
|             | P2         | 68.6 |         | 0.48              | 0.88             |
|             | P4         | 65.3 |         | 0.48              | 0.88             |
|             | P5         | 65.3 |         | 0.48              | 0.88             |
| PCho        | Control    | 641  | 657     | 0.54              | 0.77             |
|             | P2         | 645  | 657     | 0.54              | 0.77             |
|             | P4         | 638  | 657     | 0.54              | 0.77             |
|             | P5         | 638  | 657     | 0.54              | 0.77             |
| GPC         | Control    | 96.7 | 102     | 0.19              | 0.76             |
|             | P2         | 101  | 102     | 0.19              | 0.76             |
|             | P4         | 97.5 | 102     | 0.19              | 0.76             |
|             | P5         | 97.5 | 102     | 0.19              | 0.76             |
| PC          | Control    | 148  | 154     | 0.07              | 0.85             |
|             | P2         | 154  | 154     | 0.07              | 0.85             |
|             | P4         | 153  | 154     | 0.07              | 0.85             |
|             | P5         | 154  | 154     | 0.07              | 0.85             |
| SM          | Control    | 1,226| 1,227   | 0.32              | 0.31             |
|             | P2         | 1,253| 1,227   | 0.32              | 0.31             |
|             | P4         | 1,227| 1,227   | 0.32              | 0.31             |
|             | P5         | 1,227| 1,227   | 0.32              | 0.31             |
| Total choline | Control | 1,226| 1,227 | 0.32              | 0.31             |

1Trimethylamine N-oxide (TMAO), phosphocholine (PCho), glycerophosphorylcholine (GPC), phosphatidylcholine (PC), sphingomyelin (SM).
2Control = no supplemental RPC provided; P2 = prototype 2 from experiment 1; P4 = prototype 4 of RPC; P5 = prototype 5 of RPC (containing 0, 56, 60, and 62% choline chloride, respectively).
3Standard error of the mean represented as the highest SEM for each variable.
4Contrast statement to determine a difference between the control and different prototypes (P2, P4, and P5).
5Contrast statement to examine difference between P2 versus P4 and P5.
6Total choline is the sum of choline, PCho, GPC, PC, and SM.
argued that dietary fatty acid intake, digestibility, and absorption, as well as endogenous fatty acid status in relation to changes in body fat mobilization and energy balance are likely determinants of whether RPC supplementation enhances the synthesis of PC, LPC, or SM. For transparency, Zenobi et al. (2018) was able to observe increases in plasma total PC, LPC, and SM concentrations with RPC feeding in feed-restricted dry cows experiencing negative energy balance; however, they did not observe changes in plasma choline or betaine concentrations. The increased hepatic uptake of adipose-tissue derived fatty acids, which occurs during negative energy balance, could influence activation of the phosphatidylcholine-N-methyltransferase and cytidine diphosphate-choline pathways because of their required utilization of diacylglycerol for PC synthesis; however, this remains to be evaluated in dairy cattle experiencing shifts in energy balance and hepatic fatty acid supply. We did detect an ~7% decrease in plasma PC and LPC concentrations in mid and late-lactation cows provided RPC, respectively; however, milk PC and LPC concentrations and yields were either not responsive or undetectable. Decreases in plasma PC and LPC concentrations would suggest a downregulation in choline utilization for anabolic processes (i.e., phospholipid synthesis) but an upregulation in choline oxidation (i.e., betaine synthesis; Li and Vance, 2008).

Choline, phospholipids with the choline moiety, betaine, and l-carnitine are precursors for microbial trimethylamine formation in the gut, and in turn, TMAO synthesis in the liver (Al-Waiz et al., 1987). We recently confirmed that the abomasal infusion of unprotected choline chloride increases plasma choline and TMAO concentrations in late-lactation dairy cows (Myers et

Figure 4. Observed changes in milk trimethylamine N-oxide (TMAO; A–B) and betaine (C–D) concentrations and yields in 12 late-lactation Holstein cows provided different prototypes of rumen-protected choline (RPC; control, P2, P4, or P5 containing 0, 56, 60, and 62% choline chloride, respectively) in experiment 2. Data are represented as LSM with SEM minimum and maximum. For milk TMAO concentrations: period, \( P = 0.87; \) prototype, \( P < 0.01; \) time, \( P < 0.01; \) square, \( P = 0.60; \) time × prototype, \( P < 0.01. \) For milk TMAO yields: period, \( P = 0.27; \) prototype, \( P < 0.01; \) time, \( P < 0.01; \) square, \( P = 0.72; \) time × prototype, \( P < 0.01. \) For milk betaine concentrations: period, \( P = 0.69; \) prototype, \( P = 0.12; \) time, \( P < 0.01; \) square, \( P = 0.70; \) time × prototype, \( P = 0.82. \) For milk betaine yields: period, \( P = 0.45; \) prototype, \( P = 0.15; \) time, \( P = 0.03; \) square, \( P = 0.64; \) time × prototype, \( P = 0.42. \) Contrast statement control versus rest had \( P \)-values of \( P < 0.01, P < 0.01, P = 0.08, \) and \( P = 0.05 \) for panels A, B, C, and D, respectively. Contrast statement P2 versus P4 and P5 had \( P \)-values of \( P = 0.37, P = 0.78, P = 0.69, \) and \( P = 0.62 \) for panels A, B, C, and D, respectively. Mean separations between prototypes (\( P < 0.05 \)) within a given time point were evaluated: control versus rest (\( * \)); P2 versus P4 (\( † \)); and P2 versus P5 (\( ‡ \)).
al., 2019). These data demonstrate that postruminal choline degradation to trimethylamine occurs in dairy cattle, which decreases choline available for absorption and metabolic use by the cow. In the present study, we hypothesized that plasma TMAO concentrations would be elevated 8 to 16 h after RPC delivery to align with the potential intestinal arrival and release of choline. However, we observed limited or no TMAO response at this time. These findings suggest that postruminal choline release is negligible or the RPC prototypes tested did not release choline at the small intestine but rather escaped in feces. Regarding this matter, it is timely to reconsider the use of in vitro intestinal digestibility assays that don’t account for the role of intestinal bacteria trimethylamine lyase because such approaches may overestimate metabolizable choline supply.

In contrast to our hypothesis, we observed a robust plasma TMAO response at h 2 to 6 following the provision of RPC in mid- and late-lactation cows. In vitro studies of RPC stability, with consideration of rate of passage in the rumen, found that 86% of choline chloride escaped intact from the rumen after 8 to 24 h in 1 of 4 RPC products tested (Elek and Husveth, 2007). The 4 h timeframe in which we observed peak plasma TMAO concentrations suggests that the RPC studied is partially degraded in the rumen. In turn, trimethylamine was likely absorbed across the rumen epithelium for conversion to TMAO in the liver. These findings demonstrate that changes in plasma TMAO concentrations immediately postcholine feeding may potentially be used as a surrogate marker for in vivo ruminal choline degradation; however, future studies that simultaneously investigate ruminal choline degradation are needed to confirm. In our study, the observation that P2 had the greatest TMAO AUC in mid-lactation cows is suggestive that P2 released choline in the foregut more so than P1 or P3. Although this could have limited the available choline for intestinal absorption, this degradation proved necessary to degrade the encapsulation for intestinal release considering the plasma and milk choline and betaine responses were greatest for P2 as compared with P1 and P3. A nonexistent or low plasma TMAO response is indicative of a high degree of rumen protection for encapsulated choline, but the protection may be excessive to limit its intestinal release. Similar to our previous abomasal infusion trial (Myers et al., 2021), our study confirms that a plasma and milk choline response can develop with increases in TMAO production. However, our data suggests that the plasma and milk TMAO response precedes the choline and betaine response at low doses. Future studies will need to examine whether the conversion of choline to trimethylamine (i.e., the bacterial expression and activity of trimethylamine lyase and substrate-product relationship) is influenced by level and duration of choline feeding.

It appears that individual variation in microbial choline or l-carnitine degradation exists in the human population (Miller et al., 2014; Koeth et al., 2019). Whether an individual is TMAO-responsive or not depends on their microbiome [i.e., do they carry bacteria that express the cutC gene (responsible for converting choline to trimethylamine) and at what abundance]. For instance, Miller et al. (2014) performed a randomized, controlled, dose-response study to investigate the effects of egg yolk consumption on plasma TMAO response in humans. They discovered that increasing egg yolk intake, which contains PC, increased plasma TMAO in a dose-dependent manner. But what was intriguing was that the magnitude of the TMAO response depended on the individual, which differed by 4-fold depending on the participant and likely their microbiome.

In the present study (experiment 2), we performed a preliminary evaluation to determine whether such variation might exist in our population of dairy cows. We observed a 2-fold difference in the plasma TMAO AUC response between the top and bottom quartile of late-lactation cows. Although we cannot rule out the possibility that the source of variation might be attributed to differences in ruminal digestion, which could cause variable rates of ruminal choline release, we should consider the likelihood that gastrointestinal choline degradation, and thus choline bioavailability, is potentially variable across individual dairy cattle. Our findings are supported by the observation that Methanomassiliicoccales, a highly prevalent group of methanogens in the rumen, has a relative abundance among 11 dairy cows ranging from 3 to 59%, which could influence trimethylamine synthesis (Zhou et al., 2021). Such information is important to consider for future studies that aim to quantify choline bioavailability in dairy cattle.

**CONCLUSIONS**

Plasma or milk TMAO concentrations or yields are highly responsive to the provision of RPC as a ruminal bolus or as part of TMR premeal; albeit, the magnitude of the response is dependent upon RPC type, dose, and stage of lactation evaluated. In mid-lactation cows, plasma choline and betaine concentrations, and milk choline and betaine yields were responsive to RPC dose. In late-lactation cows, plasma choline concentrations and milk betaine yields were responsive to the provision of RPC; however, plasma betaine concentrations and milk choline yields were not modified. In our experimental conditions, a lack of a response in plasma or milk choline or betaine when feeding RPC is potential...
tially suggestive of inadequate choline bioavailability or an outcome dependent upon stage of lactation. We determined that increases in plasma TMAO concentrations and milk TMAO concentrations and yields can develop with an increase in circulating choline concentrations in mid- and late-lactation cows. Future work should evaluate changes in circulating and milk TMAO concentrations as a means to optimize the ruminal protection and intestinal release of choline for use by the cow, and determine whether changes in circulating and milk TMAO concentrations can be used as a surrogate measure of total gastrointestinal tract choline degradation.

ACKNOWLEDGMENTS

We acknowledge funding support from the Cornell Institute of Biotechnology Center for Advanced Technology and Balchem Corporation. We acknowledge the following author responsibilities: T. F. and J. M. designed the experiments; T. F., W. M., A. J., I. F., and J. M. conducted research; T. F. analyzed data. T. F. and J. M. wrote the manuscript. J. M. conceptualized the idea and was primary responsibility for final content. We thank the Cornell University Dairy Research Center farm staff for assisting with animal care. We also acknowledge Marie Caudill and Olga Malysheva (Cornell University) for their contributions pertaining to the analyses of choline and choline metabolites in plasma and milk. The authors have not stated any conflicts of interest.

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