RESEARCH ARTICLE

# Intestinal Microbiota Composition Modulates Choline Bioavailability from Diet and Accumulation of the Proatherogenic Metabolite Trimethylamine-*N*-Oxide

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**ABSTRACT** **Choline is a water-soluble nutrient essential for human life. Gut microbial metabolism of choline results in the production of trimethylamine (TMA), which upon absorption by the host is converted in the liver to trimethylamine-*N*-oxide (TMAO). Recent studies revealed that TMAO exacerbates atherosclerosis in mice and positively correlates with the severity of this disease in humans. However, which microbes contribute to TMA production in the human gut, the extent to which host factors (e.g., genotype) and diet affect TMA production and colonization of these microbes, and the effects TMA-producing microbes have on the bioavailability of dietary choline remain largely unknown. We screened a collection of 79 sequenced human intestinal isolates encompassing the major phyla found in the human gut and identified nine strains capable of producing TMA from choline *in vitro*. Gnotobiotic mouse studies showed that TMAO accumulates in the serum of animals colonized with TMA-producing species, but not in the serum of animals colonized with intestinal isolates that do not generate TMA from choline *in vitro*. Remarkably, low levels of colonization by TMA-producing bacteria significantly reduced choline levels available to the host. This effect was more pronounced as the abundance of TMA-producing bacteria increased. Our findings provide a framework for designing strategies aimed at changing the representation or activity of TMA-producing bacteria in the human gut and suggest that the TMA-producing status of the gut microbiota should be considered when making recommendations about choline intake requirements for humans.**

**IMPORTANCE** **Cardiovascular disease (CVD) is the leading cause of death and disability worldwide, and increased trimethylamine *N*-oxide (TMAO) levels have been causally linked with CVD development. This work identifies members of the human gut microbiota responsible for both the accumulation of trimethylamine (TMA), the precursor of the proatherogenic compound TMAO, and subsequent decreased choline bioavailability to the host. Understanding how to manipulate the representation and function of choline-consuming, TMA-producing species in the intestinal microbiota could potentially lead to novel means for preventing or treating atherosclerosis and choline deficiency-associated diseases.**

**Received** 11 December 2014 **Accepted** 16 February 2015 **Published** 17 March 2015

**Citation** Romano KA, Vivas EI, Amador-Noguez D, Rey FE. 2015. Intestinal microbiota composition modulates choline bioavailability from diet and accumulation of the proatherogenic metabolite trimethylamine-*N*-oxide. mBio 6(2):e02481-14. doi:10.1128/mBio.02481-14.

**Editor** Martin J. Blaser, New York University



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A major role played by the intestinal microbiota is to aid in the harvest of nutrients from the diet (1). Dietary components that are not readily absorbed in the small intestine serve as growth substrates for members of the gut microbiota, which in turn can modify the bioavailability and nutritional properties of those same dietary components (1). For example, many of the beneficial effects associated with consumption of whole grains, vegetables, and fruits are at least in part mediated by end products of microbial metabolism, including short-chain fatty acids (e.g., butyrate) and phenolic acids (e.g., protocatechuic acid) (2–6). Likewise, gut microbes can also convert otherwise beneficial dietary compounds, such as choline, into metabolites that are detrimental to human health (7–10).

Choline is required for a wide range of biological activities, including maintaining the structural integrity of cell membranes, supporting cholinergic neurotransmission, and donating methyl groups in a number of biosynthetic reactions (11). Although choline is synthesized endogenously, this synthesis does not meet the levels necessary for optimal health (11). Previous studies have established that gut microbial metabolism of choline results in the production of trimethylamine (TMA) (12–14). Once TMA is absorbed by the host, it is further metabolized by flavin monooxygenases 1 and 3 (FMO1 and FMO3) in the host liver to generate trimethylamine-*N*-oxide (TMAO) (8, 15, 16).

Recent human studies have established that the levels of TMAO in serum are positively correlated with impaired renal function, colorectal cancer, and cardiovascular disease (CVD) (8, 10, 17, 18). TMAO exacerbates atherosclerosis in a genetic knockout mouse model, in part by promoting forward cholesterol transport and by inhibiting reverse cholesterol transport (8, 10, 19, 20). In addition, TMAO exacerbates impaired glucose tolerance, obstructs hepatic insulin signaling, and promotes adipose tissue inflammation of mice maintained on a high-fat high-sugar diet (21).

**TABLE 1** Bacterial strains used to colonize germ-free mice

| Bacterial strain | Strains introduced into germ-free mice[a] | | | *In vitro* TMA production in the presence of [b]: | |
|---|---|---|---|---|---|
| | "Core" | "Core plus *C. sporogenes*" | "Core plus all" | Choline | L-Carnitine |
| *Bacteroides caccae* ATCC 43185 | √ | √ | √ | − | − |
| *Bacteroides ovatus* ATCC 8483 | √ | √ | √ | − | − |
| *Bacteroides thetaiotaomicron* VPI-5482 | √ | √ | √ | − | − |
| *Collinsella aerofaciens* ATCC 25986 | √ | √ | √ | − | − |
| *Eubacterium rectale* ATCC 33656 | √ | √ | √ | − | − |
| *Anaerococcus hydrogenalis* DSM 7454 | ○ | ○ | √ | + | − |
| *Clostridium asparagiforme* DSM 15981 | ○ | ○ | √ | + | − |
| *Clostridium hathewayi* DSM 13749 | ○ | ○ | √ | + | − |
| *Clostridium sporogenes* ATCC 15579 | ○ | √ | √ | + | − |
| *Edwardsiella tarda* ATCC 23685 | ○ | ○ | √ | + | − |
| *Escherichia fergusonii* ATCC 35469 | ○ | ○ | √ | + | − |
| *Proteus penneri* ATCC 35198 | ○ | ○ | √ | + | − |
| *Providencia rettgeri* DSM 1131 | ○ | ○ | √ | + | − |

[a] Symbols: √, species present in the community; ○, species not present in the community.
[b] Symbols: +, TMA produced; −, TMA not produced.

Subsequent experiments with the choline-degrading sulfate-reducing bacterium *Desulfovibrio desulfuricans* (i) revealed a pathway that involves a radical C–N bond cleavage of choline to generate TMA and acetaldehyde and (ii) identified a gene cluster encoding this activity (22). This cluster includes *cutC*, which encodes a glycyl radical enzyme with choline trimethylamine-lyase activity; *cutD*, which encodes a glycyl radical-activating protein; and genes encoding proteins involved in the assembly of microcompartments which may sequester the acetaldehyde generated as a by-product during TMA production (22, 23). However, the diversity of gut microbes that contribute to TMA production in humans and the impact of these species on serum TMAO levels and choline bioavailability remain unknown.

In this study, we used ultrahigh-pressure liquid chromatography coupled with tandem mass spectrometry (uHPLC-MS/MS) to identify human gut isolates able to convert choline into TMA. Follow-up gnotobiotic mouse experiments characterized the relative contributions of these microbes, host diet, and host factors (e.g., genotype) to choline bioavailability and TMAO accumulation.

## RESULTS AND DISCUSSION

***In vitro* screening reveals human gut isolates able to generate TMA from choline.** Seventy-nine isolates representing six phyla found in the human intestinal tract (i.e., *Bacteroidetes* [21 strains], *Firmicutes* [36 strains], *Actinobacteria* [8 strains], *Proteobacteria* [12 strains], *Verrucomicrobia* [1 strain], and *Lentisphaerae* [1 strain]; see Table S1 in the supplemental material) were tested *in vitro* for choline consumption and TMA production from choline under anaerobic conditions. All strains were inoculated in a diluted gut medium (Table S2) supplemented with 15 mM choline and incubated for 24 h in a 96-well plate at 37°C (24). Cell-free supernatants were derivatized, diluted, and analyzed using uHPLC coupled to mass spectrometry on a high-resolution mass spectrometer (Thermo Scientific Q Exactive) (22). We identified eight species representing two different phyla (*Firmicutes* and *Proteobacteria*) and six genera that showed significant choline consumption and TMA accumulation: *Anaerococcus hydrogenalis*, *Clostridium asparagiforme*, *Clostridium hathewayi*, *Clostridium sporogenes*, *Escherichia fergusonii*, *Proteus penneri*, *Providencia rettgeri*, and *Edwardsiella tarda* (Table S1 and Table 1). These strains generated TMA only if the medium was supplemented with choline. We confirmed that TMA was derived from choline by inoculating cultures of TMA-producing strains with labeled choline [choline chloride-(trimethyl-$d_9$)], which resulted in the appearance of labeled TMA (trimethyl-$d_9$) (see Fig. S1 in the supplemental material). These organisms consumed more than 60% of the choline provided in the growth media, unlike strains that did not make TMA (Table S1). None of the identified TMA-producing strains generated TMA from L-carnitine, another quaternary amine linked to TMAO accumulation, under the same test conditions (Table 1) (10).

Seven of the eight identified species encode components of the choline utilization TMA-producing pathway described above (see Fig. S2 in the supplemental material), including *cutC*, *cutD*, and genes encoding proteins involved in assembly of microcompartments. In contrast, *E. tarda* strain 23685 produces TMA from choline but does not appear to contain these genes in the published draft genome, raising the possibility that it encodes a novel mechanism of choline metabolism. Different strains of the *Edwardsiella tarda* species varied in their ability to consume choline and generate TMA. While *E. tarda* ATCC 23685 strain generated TMA from choline, ATCC 15947 strain did not, suggesting that the ability of microbes to convert choline to TMA is a strain-specific metabolic trait that may be acquired via lateral gene transfer.

We also identified two species—*Providencia alcalifaciens* and *Providencia rustiganii*—predicted to encode key components of the choline utilization TMA-producing pathway that did not generate TMA in our original screen (see Table S1 and Fig. S2 in the supplemental material) (22). Further uHPLC-MS/MS analysis of these two organisms grown individually in Hungate tubes confirmed that they do not accumulate TMA after 24 h of incubation in the medium mentioned above, despite reaching high cell densities; however, *P. rustiganii* showed TMA accumulation after 72 h of incubation, whereas *P. alcalifaciens* did not generate TMA under any of the tested conditions (Table S1). Altogether, these results highlight the importance of functional studies when inquiring about the metabolic activities of a microbe and suggest that

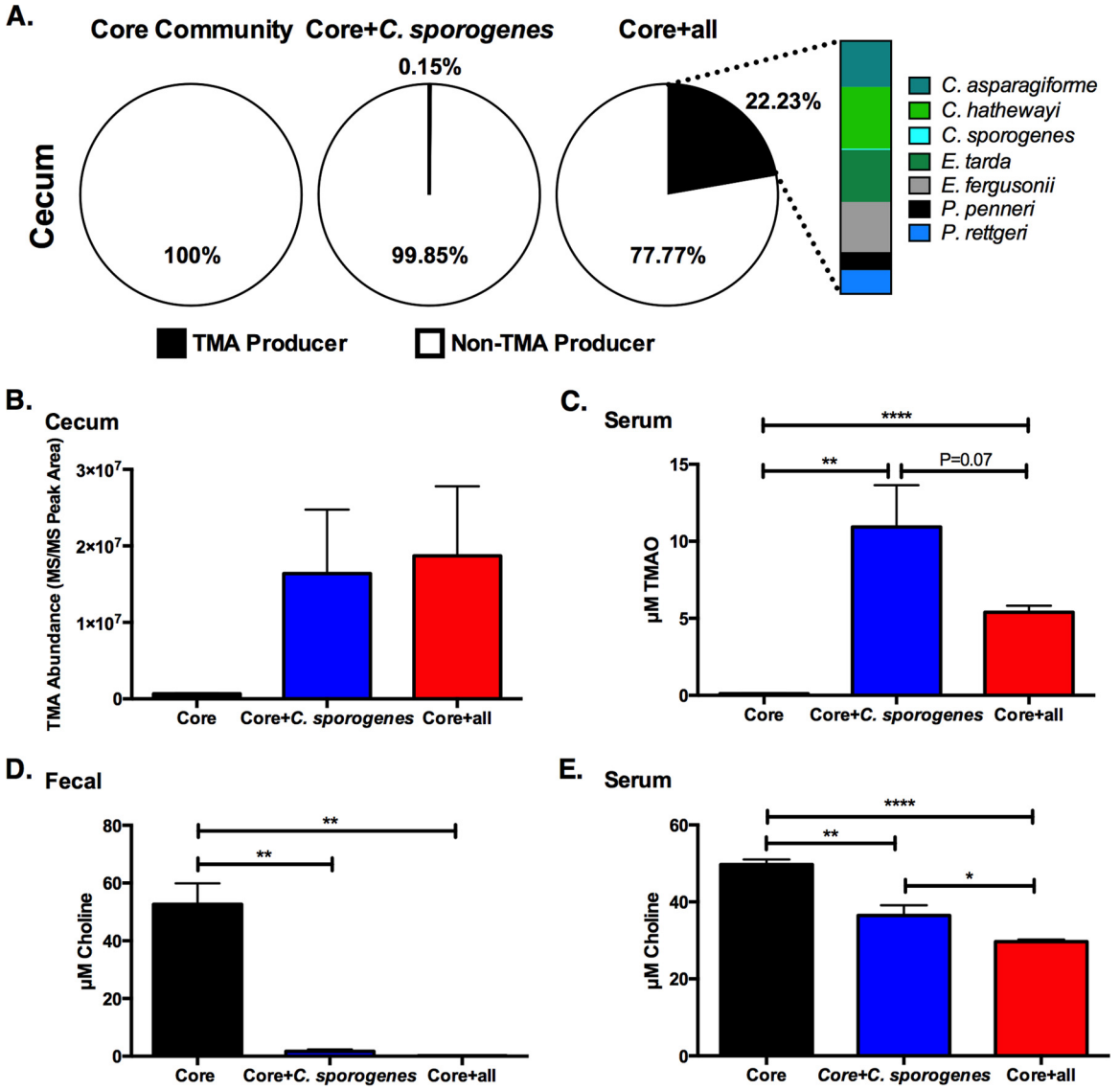


**FIG 1** Colonization with TMA-producing bacteria affects the levels of choline and TMAO in serum. (A) COPRO-Seq (*co*mmunity *pro*filing by *seq*uencing) analysis of cecal contents from male mice colonized with (i) the "core" community, (ii) the "core plus *C. sporogenes*" community, and (iii) the "core plus all" community. The pie charts depict the combined abundance of TMA-producing species and non-TMA-producing species in the community. The color bar chart (right) shows the partial contribution of each TMA producer to the total TMA-producing fraction in "core plus all" community. *A. hydrogenalis* was not detectable in the cecal samples of mice colonized with the "core plus all" community (10,000 to 60,000 reads/sample). (B to E) TMA abundance (in arbitrary units) in cecum (B), serum levels of TMAO (C), fecal levels of choline (D), and (E) serum levels of choline in mice colonized with various communities. Values are averages plus standard errors of the means (SEMs) (error bars) (4 or 5 animals in each experimental group). Values that were significantly different by an unpaired two-tailed Student's *t* test are indicated by a bar and asterisk as follows: *, *P* value of $<0.05$; **, *P* value of $<0.01$; ****, *P* value of $<0.0001$. Similar results were observed in adult female mice (i.e., TMA and TMAO levels were detected only when animals were colonized with TMA-producing bacteria).

phylogeny is a poor predictor of microbial TMA production from choline.

**Colonization with TMA-producing bacteria modulates TMAO accumulation in gnotobiotic mice.** Germ-free mouse models are of critical value for characterizing the properties and functions of gut microbes. We tested whether introducing defined changes in the composition of the gut microbiota can modulate cecal TMA and serum TMAO levels. Three groups of adult germ-free male C57BL/6J mice (5 mice/group) were orally gavaged with the following microbial mixtures: (i) the "core" community which included five species that do not produce TMA from choline *in vitro*, *Collinsella aerofaciens*, *Bacteroides caccae*, *Bacteroides ovatus*, *Bacteroides thetaiotaomicron*, and *Eubacterium rectale* (Table 1); (ii) the "core plus *C. sporogenes*" community that added one TMA producer (*C. sporogenes*) to the "core" community mixture; and (iii) the "core plus all" community that included the "core" community plus the eight TMA-producing species listed in Table 1. All mice were fed a purified diet containing 1% (wt/wt) choline (Harlan TD.140179; see Table S3 for diet composition) for a week before and 2 weeks after colonization. At sacrifice, serum, feces, and cecal contents were collected for analyses of metabolite concentration and microbial community composition.

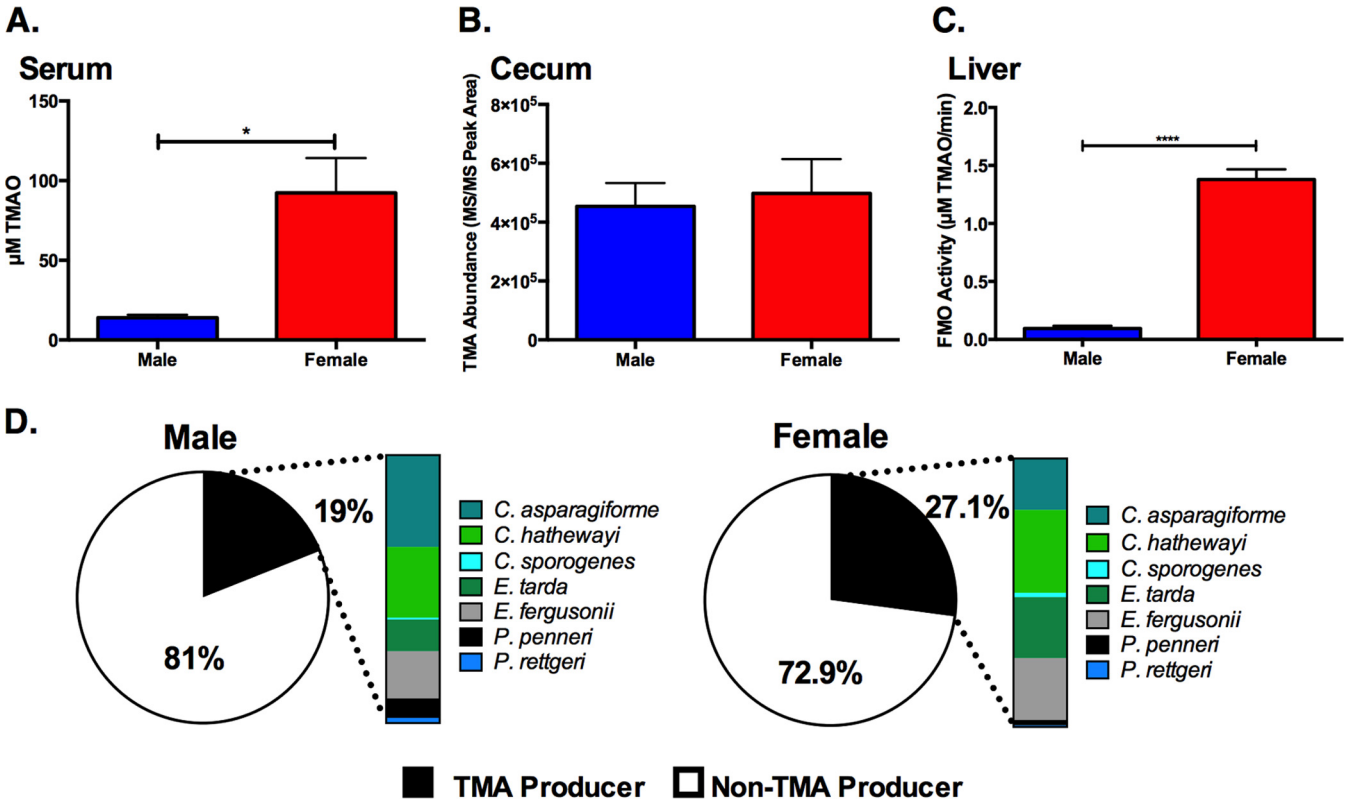


**FIG 2** Gender modulates TMAO accumulation in serum. (A to D) Serum TMAO levels (A), cecal TMA abundance (B), total hepatic FMO enzymatic activity levels (C), and microbial community composition in cecal contents from male and female adult NMRI mice that were colonized with the "core plus all" community (Table 1) (D) and maintained on a choline-supplemented diet (4 or 5 mice in each experimental group). Samples with less than 10,000 reads were not used for analysis. COPRO-Seq results shown in the pie charts are the average abundance of TMA-producing species and non-TMA-producing species in the community. The color bar charts show the partial contribution of each TMA producer to the total TMA-producing fraction. Statistical significance was calculated by an unpaired two-tailed Student's *t* test and indicated by a bar and asterisks as follows: *, *P* value of $<0.05$; ****, *P* value of $<0.0001$.

Figure 1A shows microbial community composition in the three groups of mice as determined by COPRO-Seq (*co*mmunity *pro*filing by *seq*uencing) analysis of cecal contents. With the exception of *A. hydrogenalis*, all species introduced into the mice were detected in cecal samples at a sequencing depth of ≥10,000 reads/sample, which allows us to detect microbes that represent at least 0.1% of the community. uHPLC-MS/MS analysis indicates that colonization with the "core" community did not result in the accumulation of TMA or TMAO (Fig. 1B and C). Addition of the TMA-producing species *C. sporogenes*, which represented only 0.15% ± 0.01% (average ± standard error of the mean [SEM]) of the cecal community, resulted in the significant reduction of fecal choline, accumulation of TMA in the cecum, and appearance of TMAO in serum (Fig. 1B to D). Colonization with the "core plus all" community resulted in >100-fold increase in the relative abundance of TMA-producing bacteria in the distal gut compared to "core plus *C. sporogenes*" community (Fig. 1A). Despite this, cecal levels of TMA, fecal levels of choline, and serum levels of TMAO were not significantly different between these two groups of mice (Fig. 1B to D). These results demonstrate a causal link between gut microbial TMA-producing status and TMAO accumulation *in vivo* and suggest that other factors (e.g., host genotype) besides the abundance of TMA-producing bacteria may account for differences in TMAO accumulation (25).

**Colonization with TMA-producing bacteria decreases levels of choline available to the host.** As mentioned above, colonization with TMA-producing bacteria results in a dramatic decrease in the abundance of choline in feces relative to mice colonized with only the "core" community (Fig. 1D). COPRO-seq analysis revealed that TMA-consuming bacteria were present in the small intestines of mice (the main site of choline absorption) (see Fig. S3, top row, in the supplemental material). We therefore tested whether colonization with TMA-producing species modulates choline bioavailability to the host. We measured serum levels of choline in the three groups of mice described above. Results disclosed a significant decrease in the serum levels of choline as the relative abundance of TMA producers increased (Fig. 1E). Similar trends were also seen in experiments performed in female mice (Fig. S4).

Significant reduction in choline consumption leads to organ dysfunction and failure along with an increased risk of the development of heart disease, cancer, and liver disease (11, 26). Currently, only an estimated 10% of the U.S. population consistently meets or exceeds the daily recommended intake of choline established by the Institute of Medicine (11). Furthermore, the composition of the gut microbiota and the representation of choline-consuming TMA-producing bacteria are not currently considered when developing daily recommended values at the population level. While further experimentation is required to determine whether the reduction in the bioavailability of choline observed here recapitulates the biochemical and pathological manifestations of choline deficiency, our findings suggest that determining

personalized values could be required for optimal health for individuals.

**Host gender affects FMO activity and TMAO accumulation in gnotobiotic mice colonized with the same microbiota.** We examined TMAO accumulation in serum samples obtained from adult male and female gnotobiotic mice colonized with the "core plus all" community. uHPLC-MS/MS analysis of serum revealed that TMAO accumulated at significantly higher levels in females than in their male counterparts (Fig. 2A). Although females harbored increased levels of TMA-producing bacteria, cecal levels of TMA were not significantly different between the two groups (Fig. 2B and D). As mentioned above, microbiota-derived TMA is further metabolized by flavin monooxygenases (FMO) in the host liver to generate TMAO (8, 15, 16). FMO activity measurements in hepatic tissue homogenates indicated significantly higher enzymatic activity in females than in males (Fig. 2C). These data suggest that gender-associated differences in TMAO accumulation are likely not derived from higher levels of microbiota-generated TMA (Fig. 2B), but rather higher FMO activity in females, consistent with previous studies in conventionally raised mice which demonstrated that FMO3 is expressed at higher levels in females than in males (8, 27).

**Host genotype and community composition modulate FMO activity and TMAO accumulation in female mice.** We compared serum TMAO levels in two strains of mice (C57BL/6J and NMRI) colonized with the same "core plus all" community. uHPLC-MS/MS analysis disclosed similar levels of TMAO among males of the two strains ($P > 0.1$) (see Fig. S5 in the supplemental material). In contrast, NMRI females showed a 2-fold increase in serum TMAO levels compared to their C57BL/6J counterparts (Fig. 3A). Similar differences in TMAO levels were observed between C57BL/6J and NMRI adult females colonized with the "core plus *C. sporogenes*" community (Fig. 3A). Consistent with these findings, hepatic FMO enzyme activity assays showed higher FMO activity in NMRI females than in C57BL/6J females (Fig. 3B).

Measurements of serum TMAO levels as a function of microbiota composition revealed that both strains of female mice colonized with the "core plus all" community accumulated lower serum levels of TMAO and exhibited lower hepatic FMO activity than their counterparts colonized with the "core plus *C. sporogenes*" community (Fig. 3A and B). A similar trend in serum TMAO levels was observed in male mice colonized by these two communities (Fig. 1C). These results suggest that gut microbiota composition affects TMAO levels, independently of TMA production, and that at least one species present in the "core plus all" community reduces FMO activity.

Expression of the main FMO enzyme involved in TMAO production from TMA, FMO3, is induced by bile acids via a mechanism that involves the farnesoid X receptor (FXR) (27). Specifically, cholic acid stimulates FMO3 expression (27). Genome analysis for members of the "core plus all" community, using the curated database for metabolic pathways MetaCyc, disclosed that *Clostridium hathewayi* carries genes that encode proteins (3-$\alpha$-hydroxysteroid dehydrogenase/carbonyl reductase and 3-oxocholyl-coenzyme A [CoA] oxidoreductase) predicted to be involved in the metabolism of cholic acid that were not detected in members of the "core plus *C. sporogenes*" community (28). Thus, it is plausible that the decreased levels of TMAO detected in sera of mice colonized with the "core plus all" community (Fig. 1C and Fig. 3A) are caused by increased microbial degradation of cholic acid, which would result in lower levels of *fmo3* expression (27).

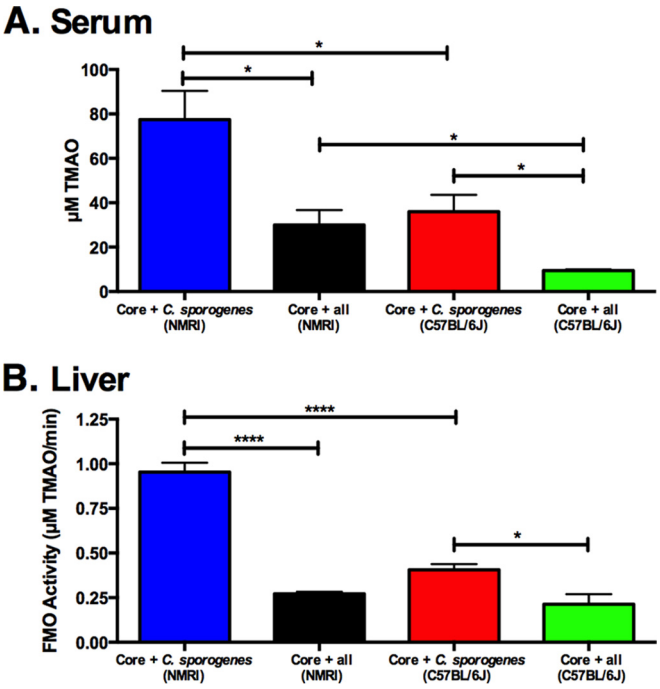


**FIG 3** Host genotype and community composition modulate FMO activity and serum TMAO levels. (A and B) Serum TMAO levels (A) and total hepatic FMO enzymatic activity levels (B) measured in adult female C57BL/6J and NMRI mice colonized with the "core plus *C. sporogenes*" community and with the "core plus all" community(average plus SEM; 3 to 6 animals in each experimental group). Significance was calculated by an unpaired two-tailed Student's *t* test as follows: *, *P* value of <0.05; ****, *P* value of <0.0001.

**Dietary choline is necessary for TMA production but does not impact the abundance of TMA-producing bacteria in a low-complexity gut microbial consortium.** To determine the impact of dietary choline on community composition and serum levels of TMAO, adult male C57BL/6J germ-free mice were inoculated by oral gavage with the "core plus all" community. Mice were maintained for 2 weeks on either the 1% (wt/wt) choline diet described above or the same diet formulated without choline (i.e., choline-deficient diet; see Table S4 in the supplemental material). uHPLC-MS/MS analysis of samples collected at the time of sacrifice showed that mice with choline in their diet showed detectable levels of TMAO in their serum, whereas mice fed the choline-deficient diet did not (Fig. 4A). There were no significant differences in the total abundance of TMA-producing bacteria in the cecum in the two groups of mice (24.7% ± 2.2% for the choline-deficient mice and 24.1% ± 1.8% for the mice given choline [average ± SEM]) despite significant changes in the relative abundance of specific TMA-producing species (*C. hathewayi* and *P. rettgeri*; $P < 0.05$ by Student's *t* test) (Fig. 4B). Both *C. hathewayi* and *P. rettgeri* increased in their abundance in response to dietary choline together with *C. asparagiforme* ($P = 0.10$), whereas *E. tarda* ($P = 0.06$), and *E. fergusonii* ($P = 0.09$) showed decreased abundance, although these changes did not reach statistical significance in our experiments. These results suggest that dietary choline is not necessary for colonization of choline-consuming TMA-producing bacteria and that dietary choline does not seem to

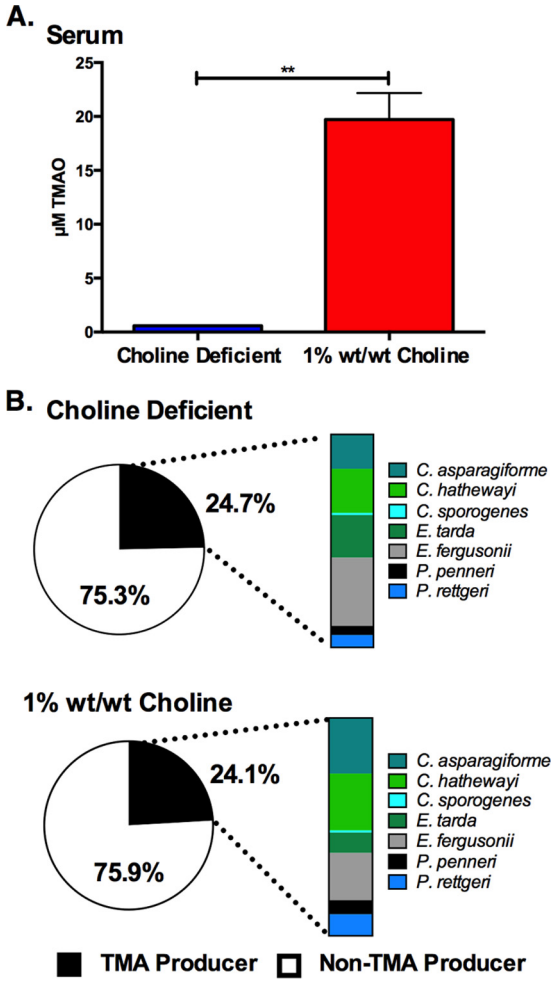


**FIG 4** Dietary choline is required for TMAO accumulation. (A) Levels of TMAO in serum from adult male C57BL/6J mice colonized with the "core plus all" community (Table 1) and fed a 1% (wt/wt) choline-supplemented diet or a choline-deficient diet for 2 weeks after colonization. Data shown are averages plus SEMs (3 mice per group). Similar results were observed when the experiment was conducted in NMRI mice (4 or 5 mice per group; see Fig. S6 in the supplemental material). (B) COPRO-Seq analysis of cecal contents from the mice described above for panel A. Samples included in the analysis have >10,000 reads. The pie charts depict combined abundance of TMA-producing species and non-TMA-producing species in the community. The color bar charts show the partial contribution of each TMA producer to the total TMA-producing fraction. Values that were statistically significant (*P* value of <0.01) by an unpaired two-tailed Student's *t* test are indicated (**).

provide these species with a major fitness advantage, at least in our simplified gnotobiotic mouse model of the human gut ecosystem.

Altogether, the presented results highlight the multiple factors, i.e., microbial, host, and environmental factors, that modulate metabolism of choline to TMAO. Future studies aimed at understanding how to manipulate the representation of choline-consuming TMA-producing bacteria in the gut microbiota or at identifying species that modulate host conversion of TMA to TMAO might lead to novel interventions for preventing or treating atherosclerosis and/or choline deficiency-associated diseases.

## MATERIALS AND METHODS

**Growth medium.** All bacteria were grown on Mega Medium (see Table S2 in the supplemental material) (24). This medium was filter sterilized and stored in a Coy anaerobic chamber (5% $H_2$, 20% $CO_2$, and 75% $N_2$) at least 24 h prior to use.

**Gnotobiotic husbandry.** All experiments involving mice were performed using protocols approved by the University of Wisconsin—Madison Animal Care and Use Committee. Both C57BL/6J and NMRI strains were maintained in a controlled environment in plastic flexible film gnotobiotic isolators under a strict 12-h light cycle and received sterilized water and food *ad libitum*. Experimental diets were sterilized by irradiation. Table S3 and Table S4 in the supplemental material show the compositional information for the choline-supplemented and choline-deficient diets used in our experiments. Sterility of germ-free animals was assessed by incubating freshly collected fecal samples under aerobic and anaerobic conditions using standard microbiology methods.

**Gnotobiotic mouse colonization.** Strains used to colonize mice were grown as monocultures on Mega Medium agar plates anaerobically for 48 to 72 h at 37°C. Single colonies were then inoculated into 3 ml of Mega Medium and grown anaerobically for 36 h at 37°C. After 36 h, strains belonging to the same treatment group were combined in an equal volume ratio in a Hungate tube. Germ-free 6- to 16-week-old mice were inoculated by oral gavage with ~0.2 ml of mixed bacterial culture inside the gnotobiotic isolator, using a mix of 5, 6, or 13 strains as shown in Table 1. Mice were maintained on the experimental diet for a week before and for 2 weeks after colonization. The mice were then sacrificed, and their intestinal contents were immediately collected, frozen, and stored at −80°C.

**uHPLC-MS/MS analysis of metabolites.** Twenty-five microliters of frozen bacterial cultures were inoculated into 1 ml of Mega Medium supplemented with 15 mM choline chloride (Sigma-Aldrich) in a 96-well deep-well plate sealed with sterile foil, and incubated anaerobically for 48 h at 37°C. Cell culture supernatants were harvested by centrifugation at 4°C and then derivatized according to published methods with minor modifications to accommodate the large number of samples being run in parallel (22). Samples were filtered through a 0.2-μm filter (Millipore) and diluted with uHPLC-grade $H_2O$. Supernatant used to measure choline was not derivatized before being filtered with a 0.2-μm filter and diluted 1:10,000 using uHPLC-grade $H_2O$. Samples were analyzed using a uHPLC coupled to a high-resolution mass spectrometer (Thermo Scientific Q Exactive) (see "uHPLC-MS/MS parameters" below).

Serum samples were prepared for analysis by precipitating proteins with 4 volumes of ice-cold methanol spiked with 2.5 μM deuterium-labeled choline and TMAO internal standards. Samples were centrifuged at 18,213 × *g* at 4°C for 3 min. The recovered supernatants were diluted 1:1 in uHPLC-grade water prior to screening. Feces and intestinal contents were homogenized with a 40:40:20 mixture of ice-cold acetonitrile, methanol, and water (20 μl/mg of sample). Samples were centrifuged for 5 min at 4°C at 7,227 × *g*, and supernatants were prepared as described above for TMA or diluted 1:10,000 for choline quantification.

Liver homogenate samples were prepared and incubated according to previously published methods with minor modifications (27). Briefly, protein was extracted from liver samples in radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) spiked with a protease inhibitor cocktail (catalog no. 97036-010; VWR) and quantified using a Bradford assay kit (Bio-Rad) after a 1:100 dilution in double-distilled water ($ddH_2O$). Determination of FMO enzymatic activity was conducted in 250-μl reaction mixtures containing 1 mg protein homogenate, 100 μM TMA, and 100 μM NADPH in 10 mM HEPES (pH 7.4). The reactions were quenched with 100 μl acetonitrile after 1, 5, 15, 60, and 120 min of incubation at 37°C. FMO activity was determined by calculating the conversion rate of TMA to TMAO during the first 5 min of incubation.

**uHPLC-MS/MS parameters.** After sample preparation, identification and quantitation of TMA, TMAO, and choline was performed using a uHPLC (Dionex 3000) coupled to a high-resolution mass spectrometer (Thermo Scientific Q Exactive). Liquid chromatography separation was achieved on a Dikma Bio-Bond $C_4$ column (150 mm by 2.1 mm; 3-μm

particle size) using a 7-min isocratic gradient (50:50 methanol [MeOH]–water, 5 mM ammonium formate, and 0.1% formic acid). A heated electrospray ionization interface, working in positive mode, was used to direct column eluent to the mass spectrometer. Quantitation of TMA, $D_9$-TMA, TMAO, and $D_9$-TMAO was performed via targeted MS/MS using the following paired masses of parent ions and fragments: TMA (146.118 and 118.0865), $D_9$-TMA (155.1740 and 127.1434), TMAO (76.0762 and 58.0659), and $D_9$-TMAO (85.1318 and 68.1301). Quantitation of choline and $d_9$-choline was performed in full-MS scan mode by monitoring their exact masses: 104.1075 and 113.1631, respectively.

**COPRO-Seq analysis.** Bacterial communities resulting from inoculation of germ-free animals were analyzed using Illumina sequencing according to the COPRO-Seq (*co*mmunity *pro*filing by *seq*uencing) method (29). In short, DNA isolated from contents of the intestine via bead beating was used to prepare libraries for shotgun Illumina sequencing. Five hundred nanograms of DNA from each sample was fragmented by sonication and subjected to enzymatic blunting and adenine tailing. Customized Illumina adapters containing maximally distant 8-bp bar codes were ligated to the poly(A)-tailed DNA. Gel-extracted DNA (size selection ~250 to 300 bp) was amplified by PCR using primers and cycling conditions recommended by Illumina. Purified PCR products were submitted to the UW-Madison Biotechnology Center for a single end 50-bp Illumina MiSeq run. Results were processed using the software pipeline detailed by McNulty et al. (29).

**Data deposition.** The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database (www.ncbi.nlm.nih.gov/geo) under accession number GSE63461.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.02481-14/-/DCSupplemental.

Figure S1, TIF file, 0.2 MB.
Figure S2, TIF file, 0.6 MB.
Figure S3, TIF file, 0.3 MB.
Figure S4, TIF file, 0.1 MB.
Figure S5, TIF file, 0.1 MB.
Figure S6, TIF file, 0.3 MB.
Table S1, PDF file, 0.1 MB.
Table S2, PDF file, 0.04 MB.
Table S3, PDF file, 0.04 MB.
Table S4, PDF file, 0.04 MB.

## ACKNOWLEDGMENTS

We thank David Stevenson for technical support and Nate McNulty for assistance with COPRO-Seq analysis pipeline. We are indebted to Jeffrey I. Gordon, Dave O'Donnell, Maria Karlson, Janaki Guruge, and Sabrina Wagoner.

The project described was supported by the Clinical and Translational Science Award (CTSA) program through the NIH National Center for Advancing Translational Sciences (NCATS) grants UL1TR000427 and KL2TR000428 (F.E.R.).

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