

New Insight Into Rumen Methanogen Function: a Barrier Blocking Trimethylamine From Entering the Host Body

Yang Zhou

Nanjing Agricultural University

Jin Wei (✉ jinwei@njau.edu.cn)

Nanjing Agricultural University

Fei Xie

Nanjing Agricultural University

Shengyong Mao

Nanjing Agricultural University

Yanfen Cheng

Nanjing Agricultural University

Weiyun Zhu

Nanjing Agricultural University

Research

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Abstract

Background: Trimethylamine (TMA) is the precursor of trimethylamine N-oxide (TMAO), which has been known to promote human cardiovascular disease. *Methanomassiliicoccales* (Mmc), TMA-utilizing methanogens, may function as a TMA barrier for the host in the rumen. This study aimed to investigate the role of Mmc in rumen TMA elimination and the effect of choline addition in the diet on the rumen TMA and TMAO concentrations in the plasma and milk of dairy cows.

Results: Three experiments, 2 rumen *in vitro* fermentation trials and 1 dairy cow *in vivo* trial, were conducted. Four groups were set in **Experiment 1:** control, nitroglycerin (NG, a methanogen inhibitor), TMA (0.69 mg/mL), and TMA + NG. The methanogenic activity was completely inhibited in the NG group, and no methane production was observed in the NG and TMA + NG groups. The TMA content hardly reduced in the TMA + NG group (0.66 mg/mL) after 2 d of incubation; in contrast, it reduced by 47.2% in the TMA group. Methanogen 16S rRNA gene sequencing showed that the relative abundance of Mmc increased in the TMA group ($P = 0.005$), which was confirmed by real-time polymerase chain reaction testing. Two taxa (Group 9 sp. ISO4-G1 and Group 10 sp.) at the species level mainly contributed to the increase in the relative abundance of Mmc. Four groups were set in **Experiment 2:** control, NG, choline (choline chloride, 1.0 mg/mL), and choline + NG. Choline was completely degraded in 24 h, and the TMA content reached the peak point in the fermentation culture. The TMA content in the choline + NG group did not reduce after the occurrence of the peak point. In contrast, the TMA content in the choline group started to decrease after 24 h, corresponding to the rapid increase in methane production. Eight mid-lactating, rumen-fistulated Holstein cows were randomly assigned to the control ($n = 4$) or choline ($n = 4$) group in **Experiment 3:** In the choline group, the cows were gradually supplemented with 100–250 g/(cow·d) of choline chloride over 4 weeks. In the choline group, TMA accumulated in the rumen fluid, and the abundance of Mmc 16S rRNA gene and choline-degrading bacterial *cutC* gene increased in the rumen content ($P < 0.050$). The TMAO content in the plasma and milk of the dairy cows was approximately 10 times higher in the choline group than in the control at day 28.

Conclusion: Rumen Mmc functioned as a TMA barrier for the host. The increase in dietary choline caused more TMA to enter the host body, resulting in higher TMAO deposition in the milk of the dairy cows. It should be noted that TMAO-rich milk might degrade food safety. Moreover, it should be discreet to mitigate rumen methane emission by inhibiting the rumen methanogen activities, which might destroy the rumen TMA barrier.

Background

It is well known that trimethylamine N-oxide (TMAO) promotes cardiovascular disease [1, 2]. Thus, the metabolism of TMAO is intensively researched. TMAO is derived from the gut microbial degradation of dietary phosphatidylcholine (PC), and choline and trimethylamine (TMA) are the intermediate metabolites. PC is first converted to choline, after which the choline is degraded to TMA by the gut microbiota. Subsequently, TMA is absorbed into the blood from the gut lumen and thereafter converted to

TMAO by flavin-containing monooxygenases (FMOs) in the liver [3]. Therefore, it is crucial to reduce the amount of TMA entering the blood circulation of the host.

Rumen TMA is mainly derived from plant PC [4]. Neill et al. [5] reported that choline is rapidly metabolized to TMA in the rumen, leading to very small quantities in the duodenum [6, 7]. Thus, it is supposed that a considerable amount of TMA is generated in the rumen. Neill et al. [5] reported that the labelled methyl groups of choline eventually turned into methane in the rumen. Moreover, in recent years, a large group of methanogens called *Methanomassiliicoccales* (Mmc) have been discovered to convert TMA to methane in the rumen [8, 9]. It is interesting to analyze if any other metabolic pathway for TMA (besides methane formation) exists in the rumen. Methane is not an expected end metabolite, and it is an important greenhouse gas [10]. Ruminants are estimated to generate 100 million tons of methane, accounting for up to 20% of annual global methane emissions [11]. Therefore, diverting TMA from methane formation is of great significance.

Morgavi et al. [12] observed the presence of TMAO in the urine of lambs, indicating that a part of the rumen TMA was absorbed by the body of host and converted to TMAO in the liver. The harmful effect of TMAO on animal health remains unclear, but this topic requires attention given the risk to human health of TMAO deposition in animal milk, meat, or eggs. It has been reported that orally consumed TMAO could be near completely absorbed by the host body [13].

Morgavi et al. [12] reported a negative interaction between rumen Mmc and urinary TMAO, suggesting that rumen methanogens might play an important role in modulating the amount of TMA entering the host body. It might cause reconsideration of the ruminant methane mitigation strategies. Many such strategies have targeted rumen methanogens [14]. Inhibiting the activity of rumen methanogens may result in more TMA entering the host body, causing harm to animals as well as lowering food safety.

In this work, we hypothesize that (1) the rumen methanogens serve as a TMA barrier for the host, and (2) the increase in dietary choline for dairy cows results in more TMA entering the blood circulation and higher TMAO deposition in the animals' milk. Therefore, in this study, the role of methanogens in the rumen TMA utilization was investigated by inhibiting the activities of these methanogens in the rumen *in vitro* fermentation experiments. Further, the effects of dietary choline on the TMA concentration in the rumen fluid and TMAO concentration in the blood and milk of dairy cows were studied by supplementing choline chloride in the cows' diet. We found that the methanogens were the sole TMA consumers in the rumen. We also observed that increasing the dietary choline content resulted in more TMA entering the blood circulation and higher TMAO deposition in the milk of the dairy cows.

Methods

Animals and diets

Four non-lactating, rumen-fistulated Holstein cows were used as rumen fluid donors in Experiments 1 and 2. The average body weight was 748 ± 59 kg. Throughout the trials, the cows had free access to water

and were fed twice a day (0800 and 1900 h). The diet was composed of 31.0% Chinese wildrye, 27.0% silage corn, 8.0% soybean hull, and 34.0% concentrate.

Eight mid-lactating, rumen-fistulated Holstein cows were used in Experiment 3. The study was conducted from early April to early May 2019 at an experimental farm near Nanjing, China. All the cows were housed in individual tie-stalls during the 4-week trial period. The cows were 186 ± 16 day in milk, with a mean milk yield of 17.2 ± 2.42 kg/d, and the average body weight was 617 ± 45 kg at the beginning of the experiment. The diet was formulated to satisfy the energy requirement of a 620 kg Holstein cow yielding 25 kg/d of milk containing 4.0% milk fat and 3.5% milk protein according to NRC (2001). Approximately 5% feed residual was allowed to minimize sorting. The cows were fed twice in 24 h, at 0800 and 1900 h, and milked twice daily. All the cows had free access to fresh water throughout the experiment.

Experimental design and sampling

Two *in vitro* and one *in vivo* experiment were conducted. In Experiment 1, the effect of methanogen activity inhibition on the TMA utilization by rumen microbiota was investigated by rumen *in vitro* fermentation over 48 h. In Experiment 2, the metabolic pathway from choline to TMA to methane was investigated by rumen *in vitro* fermentation over 72 h. In Experiment 3, the effect of supplementary choline chloride on the TMA concentration in the rumen fluid and TMAO concentration in the blood and milk of dairy cows was studied over 4 weeks.

Experiment 1. Four incubation runs were carried out with 4 bottles per treatment. The treatments were as follows: control (no additive treatment), nitroglycerin (NG, 300 μ mol/L), TMA (0.69 mg/mL), and NG (300 μ mol/L) + TMA (0.69 mg/mL). NG is an inhibitor of rumen methanogens [15]. It was purchased from Beijing Yimin Pharmaceutical Co., Ltd. (Beijing, China). TMA was supplemented in the form of trimethylamine hydrochloride (Sigma-Aldrich, St. Louis, USA). The fermentation substrate was rice straw, which was milled to 1 mm before being weighed into the bottles. The experimental procedure was based on that reported by Martínez-Fernández et al. [16]. The substrate (600 mg, dry matter basis) was weighed into 100 mL serum bottles. Ruminal fluid was obtained from the four cows before the morning feeding, pooled, and strained through four layers of cheesecloth into an insulated flask under anaerobic conditions. The filtered rumen fluid was mixed with the buffer solution devised by Menke and Steingass [17] at a ratio of 1:9 (vol:vol) at 39 °C under anaerobic conditions, and 60 mL was inoculated in each bottle. NG and TMA were directly added to the bottles before inoculation with the buffered rumen fluid. The bottles were sealed with rubber stoppers and aluminum caps, incubated for 48 h with shaking at 80 rpm at 39 °C. The supernatant (2 mL) of the fermentation fluid was sampled for TMA measurement at the beginning and end of the fermentation. Gas production (hydrogen and methane) was measured at the end of the fermentation. The pH value was measured by a pH meter (Ecoscan pH 5, Singapore). The bottles were then immersed in an ice bath to stop the fermentation. The content in each bottle was homogenized and sampled for DNA extraction.

Experiment 2. Four incubation runs were carried out with 4 bottles per treatment. The treatments were as follows: control (no additive treatment), NG (300 μ mol/L), choline (choline chloride, 1.0 g/L), and NG (300

$\mu\text{mol/L}$) + choline (choline chloride 1.0 g/L). Choline was added in the form of choline chloride (Shanghai Ryon Biological Technology CO., Ltd, Shanghai, China). The procedure for this experiment was identical to that of Experiment 1. A serum bottle (180 mL) containing 1.0 g of milled rice straw was filled with 100 mL buffered rumen fluid and incubated for 72 h. The gas production (methane and hydrogen) and TMA were measured at 0, 24, 48, and 72 h. The content in each bottle was homogenized and sampled for DNA extraction at the end of the fermentation.

Experiment 3. Eight cows were randomly assigned to one of two groups: control ($n = 4$) and choline ($n = 4$). The cows in the choline group were gradually supplemented with choline chloride as follows: 100, 150, 200, and 250 g/(cow·d) in weeks 1, 2, 3, and 4, respectively. Dry matter intake (DMI) was recorded daily. Rumen fluid was sampled from the rumen cannula before morning feeding and 4 h after the morning feeding to measure TMA, pH, and volatile fatty acids (VFAs). The rumen digesta were mixed and homogenized for DNA extraction. Blood samples were collected from the tail vein with evacuated tubes containing sodium heparin before the morning feeding at day 28. Upon collection, the blood samples were placed on ice and then centrifuged at $1125\times g$ for 15 min at room temperature for plasma separation within 30 min. The separated plasma was collected and stored in liquid nitrogen to measure the TMAO. Milk samples were collected and stored in liquid nitrogen at day 28. The milk samples from the two time points were mixed in the ratio of 1:1 before measuring the TMAO.

Chemical analyses

Gas production was measured by a pressure transducer and a calibrated syringe [18]. Hydrogen and methane were measured by a gas chromatograph (GC) (Agilent 7890B, Agilent, California, USA) according to Jin et al. [15]. The gases were separated on packed GC columns (Porapak Q and MolSieve 5A packings, Agilent, California, USA) at a column temperature of 80 °C. The injection temperature was 200 °C. The temperature of the thermal conductivity detector was 200 °C. The carrier gas was nitrogen.

The VFAs were measured by the aforementioned GC system according to Jin et al. [15]. The samples were separated on a fused silica capillary column (Supelco, Bellefonte, USA) with a column temperature procedure of 110 °C for 3 min followed by 110–150 °C (40 °C/min). The injection temperature was 200 °C. The temperature of the flame ionization detector (FID) was 220 °C. Nitrogen was used as the carrier gas.

The TMA contents of the rumen fluid and rumen *in vitro* fermentation fluid were analyzed by the GC system. A sample of 1.0 mL was centrifuged at $15000\times g$ for 5 min at 4 °C. An aliquot containing 200 μL of supernatant was thoroughly mixed with 50 μL potassium hydroxide (10 mol/L) and then centrifuged at $15000\times g$ for 2 min at 4 °C. The supernatant was filtered with a 0.22 μm filter. An aliquot of 1 μL filtrate was separated on a WEL-PEG20M capillary column (Welch Technology, Shanghai, China) using a column temperature procedure of 60 °C for 3 min followed by 60–150 °C at 45 °C/min and 150 °C for 2 min. The injection temperature was 220 °C. The temperature of the FID was 220 °C. Nitrogen served as the carrier gas.

The concentrations of the TMAO in the plasma and milk were determined by liquid chromatography–mass spectrometry (LC–MS). The exact molecular weight of the TMAO adduct ion ($[M + H]^+$) is 76.07569 g/mol. The sample (100 μ L) was added to a 1.5 mL microcentrifuge tube, following which 200 μ L of acetonitrile was added to precipitate the protein. The mixture was mechanically vortexed for 2 min and centrifuged at 14000 \times g for 10 min at 4 °C. The supernatant was transferred to vials for the LC-MS. Sample supernatant (2 μ L) was injected into a U3000 dual-gradient LC system (Thermo Fisher Scientific, Waltham, MA, USA) coupled to an Q Exactive mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The analytes were separated on a Waters BEH Amide column (100 mm \times 2.1 mm, 1.8 μ m particle size) at 35 °C. The mobile phase consisted of 90% methanol containing 0.1% acetic acid (solvent A) and water containing 0.1% acetic acid (solvent B) at a flow rate of 0.3 mL/min. The following gradient procedure was utilized: 90% solvent A and 10% solvent B for 0–2 min, followed by a linear gradient to 90% solvent B from 1–15 min, 90% solvent B for 5 min, and the initial conditions from 20–25 min. The compounds were ionized in the electrospray ionization ion source of the MS operated in the positive mode. The ionizing voltage was +3000 V, and the ion source temperature was 350 °C. The sheath gas, auxiliary gas, and capillary temperatures were 30, 10, and 320 °C, respectively. The data were collected by Xcalibur and analyzed using TraceFinder 4.1 software (Thermo Fisher Scientific, Waltham, MA, USA) with the external standard method.

DNA extraction

A representative 1 mL of the rumen digester or rumen *in vitro* fermentation culture was used to extract microbial DNA. A FastPrep-24 Instrument (MP Biomedicals, South Florida, USA) set at 5 for 2 min and cetyltrimethylammonium bromide was used to break up the microbial cell walls [19]. Further extraction was performed with phenol–chloroform–isopentanol [20]. Finally, the DNA in the solution was precipitated with ethanol, and the pellet was suspended in 50 μ L Tris–EDTA buffer. The concentration of the DNA was quantified by a NanoDrop ND-1000 Spectrophotometer (Nyxor Biotech, Paris, France).

Real-Time PCR

Real-time PCR was performed using a QuantStudio™ 7 Flex System Real-Time PCR System (Applied Biosystems, California, USA) with a SYBR® Premix Ex Tag TM kit (TaKaRa, Dalian, China). The 16S rRNA genes of archaea and Mmc were quantified according to Jeyanathan et al. [21]. The primers for the archaea were 915f 5'-AAG AAT TGG CGG GGG AGC AC and 1386r 5'-GCG GTG TGT GCA AGG AGC. The primers for Mmc were 762f 5'-GAC GAA GCC CTG GGT C and 1099r 5'-GAG GGT CTC GTT CGT TAT. The *cutC* genes were quantified with a pair of primers from Campo et al. [22]. The primer sequences were 389-aa-f 5'-TTY GCI GGI TAY CAR CCN TT-3' and 492-aa-r 5'-TGN GGR TCI ACY CAI CCC AT-3'. The reaction mixture (20 μ L) was composed of 10 μ L of SYBR® Premix Ex Tag TM, 0.2 μ mol/L of each primer, and 2 μ L of the template DNA.

The procedure for real-time PCR amplification of the archaea was initiated at 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s, 59 °C for 30 s, and 72 °C for 30 s. The procedure for Mmc was initiated at 95 °C

for 30 s, followed by 40 cycles at 95 °C for 5 s, 56 °C for 30 s, and 72 °C for 30 s. The procedure for the *cutC* genes was initiated at 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s, 60 °C for 30 s, and 72 °C for 30 s. Finally, each of the 3 procedures was followed by a melting curve at 95 °C for 15 s, 95 °C for 1 min, and 95°C for 15 s.

The genomic DNA of the rumen samples was amplified with the three pairs of primers. The amplicons were cloned into *Escherichia coli* JM109 using the pGEM-T Easy Vector (Promega, Madison, USA). The copy number of each standard plasmid was calculated based on its DNA concentration and molecular weight. The standard plasmids were used to construct the standard curves to estimate the 16S rRNA gene or *cutC* gene copy numbers. A 10-fold dilution series of the standard plasmids was run with the samples.

Methanogenic community analysis by Illumina MiSeq sequencing

The 16S rRNA genes of the methanogens were amplified according to Cersosimo et al. [23]. The primer sequences were Met86F 5'-GCT CAG TAA CAC GTG G-3' and Met471R 5'-GWR TTA CCG CGG CKG CTG-3'. The amplicons were sequenced on an Illumina MiSeq PE250 platform (Biozeron, Shanghai, China).

The raw data were processed using the QIIME software package (version 1.9.0) [24]. Sequences were selected such that their lengths exceeded 250 bp, the vague base 'N' was absent, and the average base quality score was higher than 25. Open reference operational taxonomic unit picking was performed at a distance of 0.01 using UPARSE [25]. Alpha and beta diversity analyses were conducted at a sequence depth of 20421. Taxonomic assignment was based on the RIM-DB database [26].

Statistical Analysis

The data from Experiment 1 and the real-time PCR data from Experiment 2 were analyzed by one-way analysis of variance in SPSS (version 20, SPSS, Inc., IBM, Chicago, IL, USA). The DMI data from Experiment 3, and the hydrogen, methane, and TMA data from Experiment 2 were analyzed using a generalized linear model in SPSS. The model included fixed effects for treatment, the week, and their interaction. The data of real-time PCR, TMA, TMAO, pH, and VFAs in Experiment 3 were analyzed using the independent samples *t*-test procedure of SPSS. The nonparametric test in SPSS was used to compare the relative abundance of the methanogenic populations. Significance was declared at *P* values < 0.05.

Results

Rumen *in vitro* fermentation with TMA

As shown in Table 2, the methanogenic activity was completely inhibited by NG, methane production was absent in the NG and TMA + NG groups, and a large amount of hydrogen accumulated at the end of fermentation. Methane production was higher in the TMA group than the control group (*P* < 0.050), which indicated that TMA increased the production of this gas. The amount of TMA remained virtually unchanged in the TMA + NG group, but it reduced considerably in the TMA group (*P* < 0.050), which

indicated that the inhibition of methanogenic activity blocked the TMA utilization and that methanogens were the main consumer of TMA in the rumen. The real-time PCR results showed that the Mmc/archaea ratio increased greatly in the TMA group ($P < 0.050$), which was further confirmed by the 16S rRNA gene sequencing of the methanogens.

The alpha diversity indexes are listed in Supplementary Table S1. As shown in Figure 1, in the TMA group, the relative abundance of Mmc increased greatly at the end of fermentation ($P = 0.005$). Two taxa (Group 9 sp. ISO4-G1 and Group 10 sp.) at the species level were the primary contributors to the increase in the relative abundance of Mmc (Figure 2). These findings indicated that Mmc played a critical role in the rumen TMA utilization.

Rumen *in vitro* fermentation with choline chloride

As shown in Figure 3, methanogenic activity was completely inhibited in the NG group, no methane production occurred in the NG and choline + NG groups, and hydrogen accumulation was observed. The methane production was higher in the choline group than in the control group ($P < 0.050$). The TMA concentration peaked at 24 h in the choline and choline + NG groups, which indicated that the choline was completely degraded to TMA by the rumen microbiota. The choline + NG group did not show a reduction in the TMA concentration after the occurrence of the peak point ($P > 0.050$). The TMA concentration decreased after 24 h ($P < 0.050$) in the choline group, corresponding to the rapid increase in methane production. The results further confirmed that methanogens were the sole consumers of TMA in the rumen. It also suggested that TMA was produced faster than it was consumed in the rumen.

As shown in Table 3, the real-time PCR results proved that choline greatly increased the abundance of archaea and Mmc ($P < 0.050$), as well as the Mmc/archaea ratio ($P < 0.050$), which further confirmed the results presented in experiment 1. The *cutC* gene codes the choline trimethylamine lyase, a key enzyme in the choline-degrading pathway of rumen bacteria. The abundance of the *cutC* gene was higher in the choline group than its choline + NG counterpart ($P < 0.050$), which suggested that TMA accumulation might cause feedback inhibition in the proliferation of choline-degrading bacteria.

***In vivo* experiment using dairy cows**

As shown in Supplementary Table S2, the DMI of the dairy cows did not show a significant difference between the control and choline groups during the experiment ($P < 0.050$).

Table 4 shows that TMA accumulated in the rumen fluid in the choline group at 4 h after the morning feeding on days 7, 14, 21, and 28. On days 7, 14, and 21, the TMA concentration was less than 1.0 mg/L before the morning feeding in both the control and choline groups. On day 28, TMA accumulated in the rumen fluid of the choline group before the morning feeding, suggesting that the TMA produced by the 250 g/(cow·d) choline supplement exceeded the rumen consumption capacity.

As shown in Supplementary Table 3, the rumen pH did not show a significant difference between the control and choline groups at 0 h before and 4 h after the morning feeding ($P > 0.050$), but the pH was

lower at 4 h after the morning feeding than before it in two groups ($P < 0.050$). The acetate ($P = 0.003$), isobutyrate ($P = 0.017$), and total VFA concentrations ($P = 0.041$) and the acetate/propionate ratio ($P = 0.005$) in the rumen fluid were lower in the choline group than the control group before the morning feeding. At 4 h after the morning feeding, the concentrations of acetate, propionate, butyrate, isobutyrate, and total VFAs did not show significant differences between the two groups ($P > 0.050$), but the concentrations of valerate ($P = 0.017$) and isovalerate ($P = 0.040$) were higher in the choline group.

According to Table 5, the real-time PCR results indicated no difference in the abundance of archaea between two groups ($P > 0.050$). The abundance of Mmc and the Mmc/archaea ratio were higher in the choline group at 0 h before and 4 h after the morning feeding ($P < 0.050$). The abundance of the *cutC* gene did not show a difference between the two groups before the morning feeding ($P = 0.420$), but it was significantly higher in the choline group at 4 h after the morning feeding ($P = 0.010$).

As shown in Figure 4, the TMAO concentrations in the plasma (before the morning feeding) and milk of dairy cows at day 28 were approximately 10 times higher in the choline group than its control counterpart. This result indicates that a higher amount of TMA entered the blood circulation of the host in the choline group than in its control counterpart.

Discussion

In this study, we combined *in vitro* and *in vivo* experiments to reveal a novel ecological function of methanogens in the rumen of dairy cows. We found that the rumen methanogens functioned as a TMA barrier for the host.

Experiment 1 inhibited the activities of the methanogens, and the utilization of TMA was found to have been blocked, indicating that the methanogens were responsible for the TMA consumption in the rumen. Among the rumen methanogen community, only the members of Mmc and *Methanomicrococcus blatticola* are capable of using TMA to produce methane [9, 27]; however, *M. blatticola* generally account for a minor component of rumen methanogen populations ($< 0.2\%$). The rumen TMA barrier was thus mainly composed of Mmc. Mmc is a prominent and highly prevalent group of methanogens in the rumen, but the relative abundance of Mmc in different animals is highly variable [28]. In our previous study, the relative abundance of rumen Mmc in 11 dairy cows ranged from 2.5–58.6% (data unpublished), indicating that the rumen TMA barrier capability among individual dairy cows is highly variable. The low abundance of Mmc in the rumen suggested a weak capability for preventing TMA from entering the host body. In this study, the relative abundance of Mmc (1.59–4.09%) was relatively low in the rumens of dairy cows, implying that their TMA barriers were relatively weak. This aspect was confirmed by the relatively high level TMAO contents in the plasma and milk. Morgavi et al. [12] reported a negative interaction between rumen Mmc and urinary TMAO in lambs, supporting the notion that Mmc is the main component of the rumen TMA barrier.

The accumulation of TMA in Experiment 2 suggested that the production of TMA was much more rapid than its utilization. This observation was also confirmed by the *in vivo* study in Experiment 3. The

increase in the dietary choline content raised the rumen TMA concentration. This finding was consistent with that of a previous study. Neill et al. [5] reported that choline was rapidly degraded to TMA, but the following step of converting TMA to methane was more easily saturated by an excess of substrate, resulting in TMA accumulation in the rumen. This accumulation led to more TMA being absorbed by the blood circulation, which was confirmed by the increase in the plasma TMAO content in Experiment 3. Though the accumulation of TMA promoted the proliferation of TMA-consuming methanogens Mmc, choline also increased the abundance of choline-degrading bacteria (the *cutC* gene). Nevertheless, the maximum extent of TMA barrier blocking is unclear and should be clarified in future work.

The results of Experiment 3 showed the deposition of TMAO in milk. It has been reported that oral TMAO could be almost completely absorbed and remain unprocessed by gut microbes [13]. TMAO has been known to promote human cardiovascular disease [1, 2]. Therefore, it should be noted that TMAO-rich milk might cause potential harm to food safety though the level of TMAO harmful to human health is not known. This aspect needs to be clarified in future work. Additionally, the TMAO content in the muscles of dairy cows was not measured in this study; however, it has been reported that the TMAO is deposited in the skeletal muscles of humans [13]. Thus it is possible that the TMAO deposited in the muscles of ruminants and affected the meat quality.

Three potential approaches may be used to reduce the amount of TMA entering the host body. (1) Strengthen the TMA barrier by isolating the rumen Mmc methanogens and selecting optimal isolates as probiotics for ruminants, (2) block the degradation of choline by designing or selecting suitable compounds targeting the key enzymes in the choline degradation pathway, and (3) reduce the choline content in the diet as much as possible. We obtained two isolates of Mmc from the rumens of goat and dairy cows. We are evaluating the potential of these two isolates as probiotics to strengthen the rumen TMA barrier. Moreover, as choline trimethylamine lyase is a key enzyme in the microbial choline degradation pathway, the inhibition of this enzyme could effectively block the degradation of choline. In order to treat TMAO-caused human cardiovascular disease, small molecular compounds targeting the choline trimethylamine lyase of gut microbes have been selected and designed [2, 29].

The inhibition of rumen methanogen activity resulted in TMA accumulation in Experiments 1 and 2, which was consistent with a previous cattle *in vivo* study. Martinez-Fernandez et al. [30] reported that the rumen TMA concentration increased when the activities of the rumen methanogens were inhibited by two inhibitors (3-nitrooxypropanol and chloroform) in cattle fed a tropical forage diet. The accumulation of TMA in the rumen would increase the amount of TMA entering the host body, which was harmful to animals. Rumen methanogens are desirable as a TMA barrier, but they also promote methane emissions, which contribute to global warming. Hence, a balance needs to be struck between their usefulness and this particular disadvantage. A possible approach is to promote the growth of Mmc, meanwhile, inhibit the activity of other rumen methanogen populations, such as *Methanobrevibacter* spp. [28]. *Methanobrevibacter* spp. mainly use hydrogen and carbon dioxide to produce methane [31].

Conclusion

Rumen methanogens, primarily Mmc, serve as a TMA barrier for the host. The degradation of choline was much more rapid than the utilization of TMA, resulting in TMA accumulation in the rumen. Therefore, an increase in dietary choline content might result in more TMA entering the blood circulation and more TMAO deposition in the milk of dairy cows (Fig. 5). Moreover, mitigating rumen methane emissions is possible by inhibiting rumen methanogen activities. However, doing so would destroy the TMA barrier and cause TMA accumulation in the rumen, finally causing all of the produced TMA to enter the host body. It also should be noted that TMAO-rich milk or meat might cause potential food safety issues.

Declarations

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Authors' contributions

WJ conceived and designed the experiments; YZ and SM mainly performed the experiments; FX analyzed the data of methanogen sequencing; WJ and YZ wrote and revised the manuscript. WJ had primary responsibility for final content. SM, WZ and YC advised the experimental design and revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The raw data of methanogen sequencing are available in the Sequence Read Archive of NCBI with the accession numbers SRP271571.

Ethics approval and consent to participate

The animal experimental design and procedures of this study were supported by the Animal Care and Use Committee of Nanjing Agricultural University following the requirements of the Regulations for the Administration of Affairs Concerning Experimental Animals (The State Science and Technology Commission of P. R. China, 1988. No. SYXK (Su) 2017-0007).

Consent for publication

Not applicable.

Competing interests

The authors have declared that no competing interests exist.

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Tables

Table 1
Ingredient and chemical composition of diet (Dry Matter basis)

Ingredient, %	Content	Chemical composition	Content
Corn meal	19.4	NE _L , Mcal/kg	1.57
Soybean meal	13.5	Moisture, % as fed	46.8
DDGS	3.8	CP, %	16.2
Corn silage	12.0	NDF, %	36.1
Alfalfa hay	24.0	NFC, %	35.4
Oaten hay	24.0	Ether extract, %	3.1
Limestone meal	0.8	Starch, %	18.0
CaHPO ₄	1.1	Ash, %	6.0
NaCl	0.4	Ca, %	1.14
Vitamin and mineral mix	1.0	P, %	0.52

Table 2

The hydrogen and methane production at the end of rumen *in vitro* fermentation with or without the addition of TMA and NG

	Control	NG ¹	TMA ²	TMA + NG	SEM	P value
Hydrogen, μmol	0.00 ^c	481.58 ^b	0.00 ^c	562.50 ^a	68.13	< 0.001
Methane, μmol	454.92 ^b	0.00 ^c	792.30 ^a	0.00 ^c	86.24	< 0.001
TMA ³ , mg/mL	ND ⁴	ND	0.38 ^b	0.66 ^a	0.01	< 0.001
Archaea, lg(copies/mL)	9.74	9.40	9.78	9.72	0.92	0.493
Mmc ⁵ , lg(copies/mL)	8.38 ^{ab}	7.64 ^b	8.86 ^a	7.78 ^b	0.18	0.023
Mmc/archaea	4.93 ^b	2.42 ^b	11.36 ^a	3.39 ^b	1.17	0.003
¹ NG, methanogen inhibitor.						
² TMA, trimethylamine.						
³ TMA was provided 0.69 mg/mL before fermentation.						
⁴ ND, not detectable.						
⁵ Mmc, Methanomassiliicoccales.						

Table 3

The abundance of the 16S rRNA gene of archaea and Mmc, and *CutC* genes at the end of rumen *in vitro* fermentation with or without the addition of Choline and NG

	Control	NG ¹	Choline ²	Choline + NG	SEM	P value
Archaea, lg(copies/mL)	9.77 ^c	9.96 ^b	10.60 ^a	9.96 ^b	0.84	< 0.001
Mmc, lg(copies/mL)	8.56 ^b	8.07 ^c	9.78 ^a	8.02 ^c	0.18	< 0.001
Mmc/archaea	6.15 ^b	1.28 ^c	15.12 ^a	1.18 ^c	1.50	< 0.001
<i>CutC</i> gene ³ , lg(copies/mL)	4.36 ^b	4.25 ^b	4.74 ^a	4.37 ^b	0.58	< 0.001
¹ NG, methanogen inhibitor.						
² Choline chloride concentration was 1.0 g/L at the beginning of fermentation.						
³ <i>CutC</i> gene, coding choline trimethylamine lyase.						

Table 4
The TMA concentration (mg/L) in the rumen fluid of dairy cows at day 7, 14, 21 and 28 of experiment.

Day	Time ¹	Control	Choline	<i>P</i> -value
7	0 h	ND ²	ND	---
	4 h	ND	112.2 ± 72.2	---
14	0 h	ND	ND	---
	4 h	ND	53.4 ± 76.2	---
21	0 h	ND	ND	---
	4 h	ND	44.9 ± 57.8	---
28	0 h	ND	14.2 ± 9.3	---
	4 h	6.8 ± 2.0	34.2 ± 16.9	0.046
¹ 0 h, 0 h before morning feeding; 4 h, 4 h after morning feeding.				
² ND, TMA concentration < 1.0 mg/L.				

Table 5
The abundance of the 16S rRNA genes of archaea and Mmc, and *CutC* genes of rumen content of dairy cows at day 28.

	Time	Control	Choline	SEM	<i>P</i> -value
Archaea, lg(copies/mL)	0 h	8.98	8.91	0.03	0.303
	4 h	8.66	8.77	0.05	0.289
Mmc, lg(copies/mL)	0 h	7.17	7.50	0.09	0.049
	4 h	6.93	7.36	0.09	< 0.001
Mmc/archaea, %	0 h	1.59	3.90	0.51	0.006
	4 h	1.98	4.09	0.55	0.040
<i>CutC</i> gene, lg(copies/mL)	0 h	5.99	5.93	0.04	0.420
	4 h	5.91	6.19	0.06	0.010

Figures

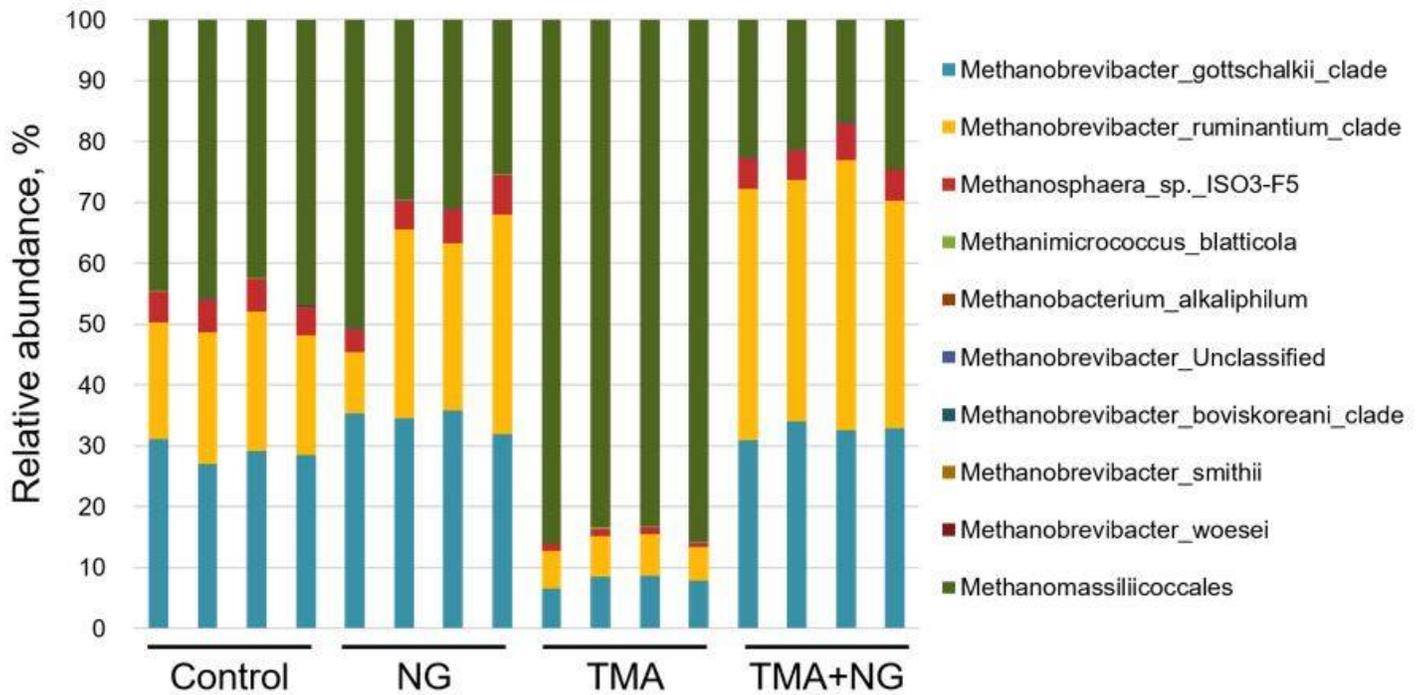


Figure 1

Community composition of methanogens at the end of the rumen in vitro fermentation with or without the addition of TMA and NG. NG, nitroglycerin (a methanogen inhibitor); TMA, trimethylamine.

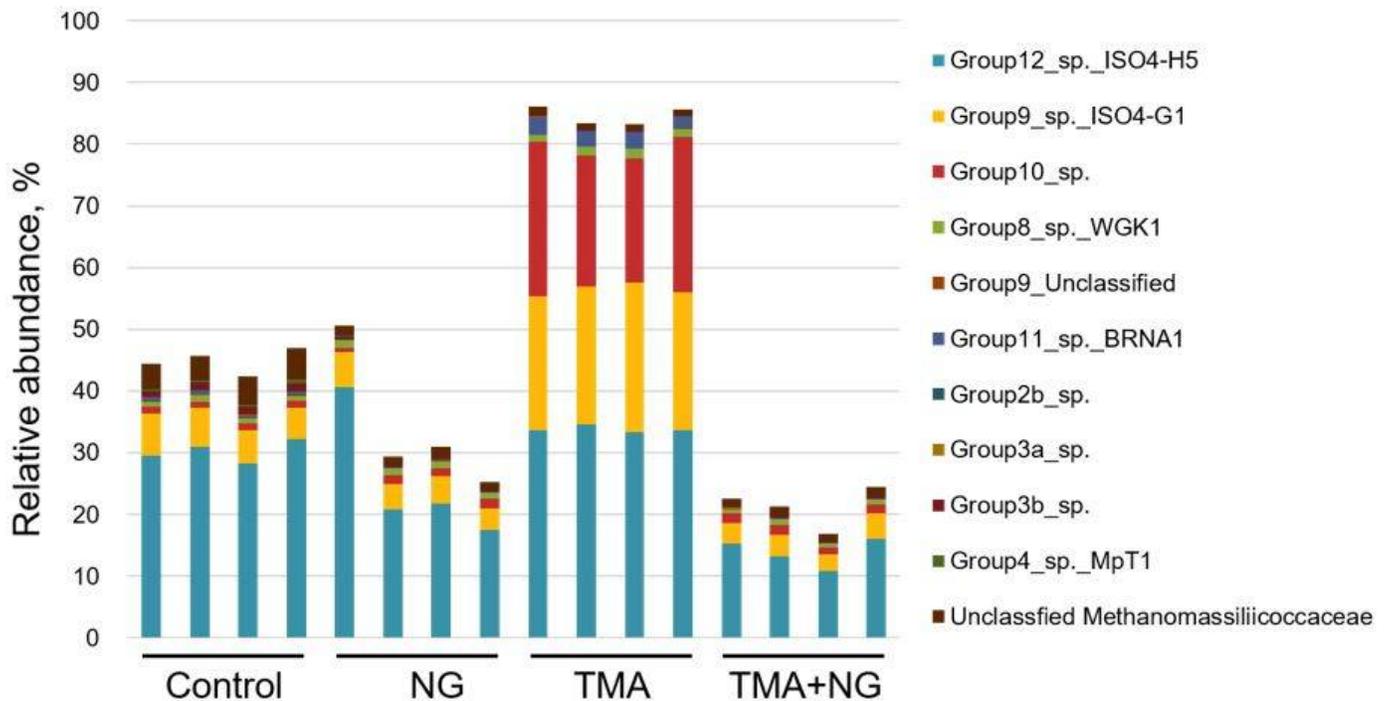


Figure 2

Community composition of Methanomassiliicoccales at the species level at the end of rumen in vitro fermentation with or without the addition of TMA and NG.

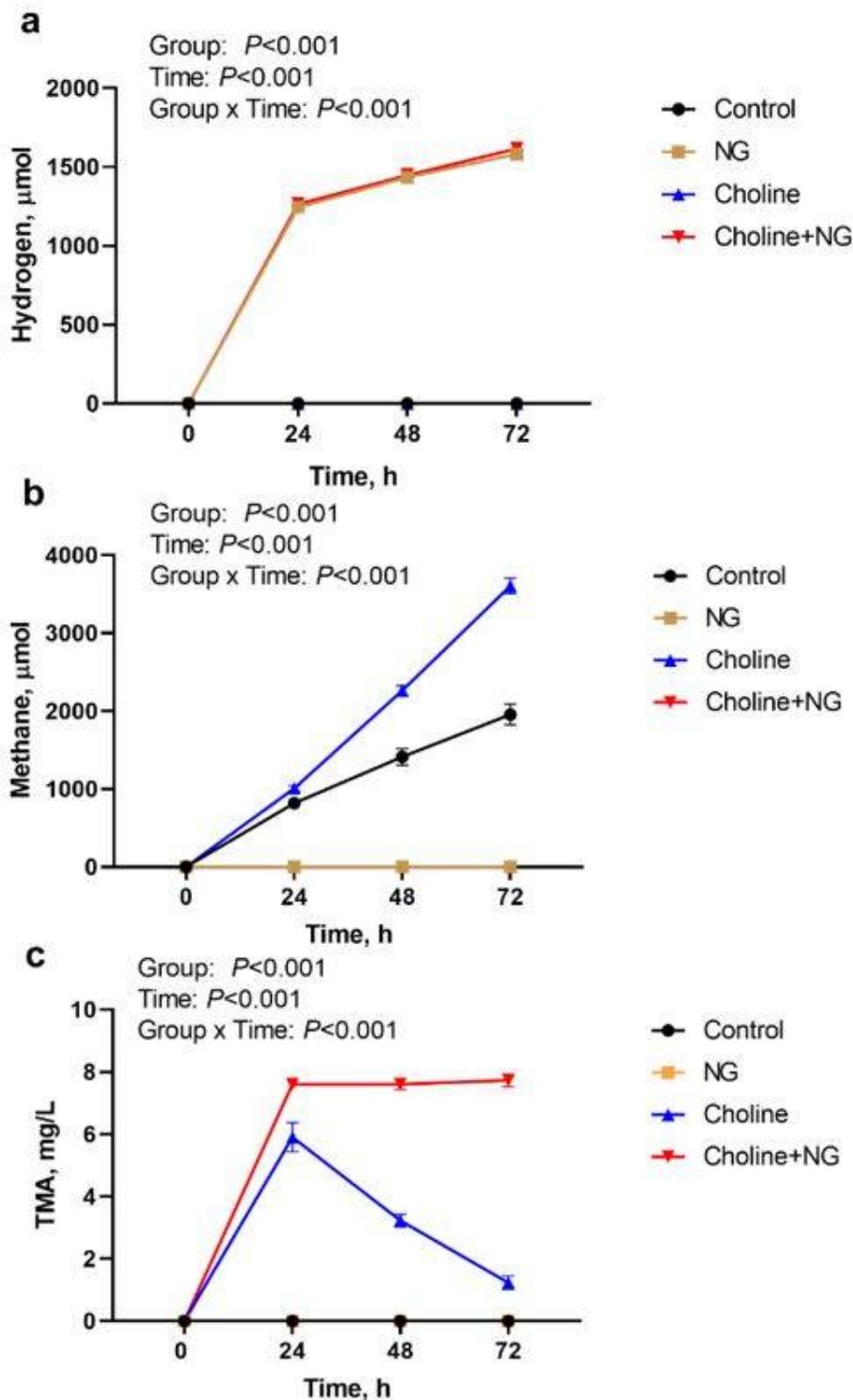


Figure 3

Hydrogen (a), methane (b), and TMA (c) yields during rumen in vitro fermentation with or without the addition of choline and NG. The choline chloride concentration was 1.0 g/L at the beginning of fermentation.

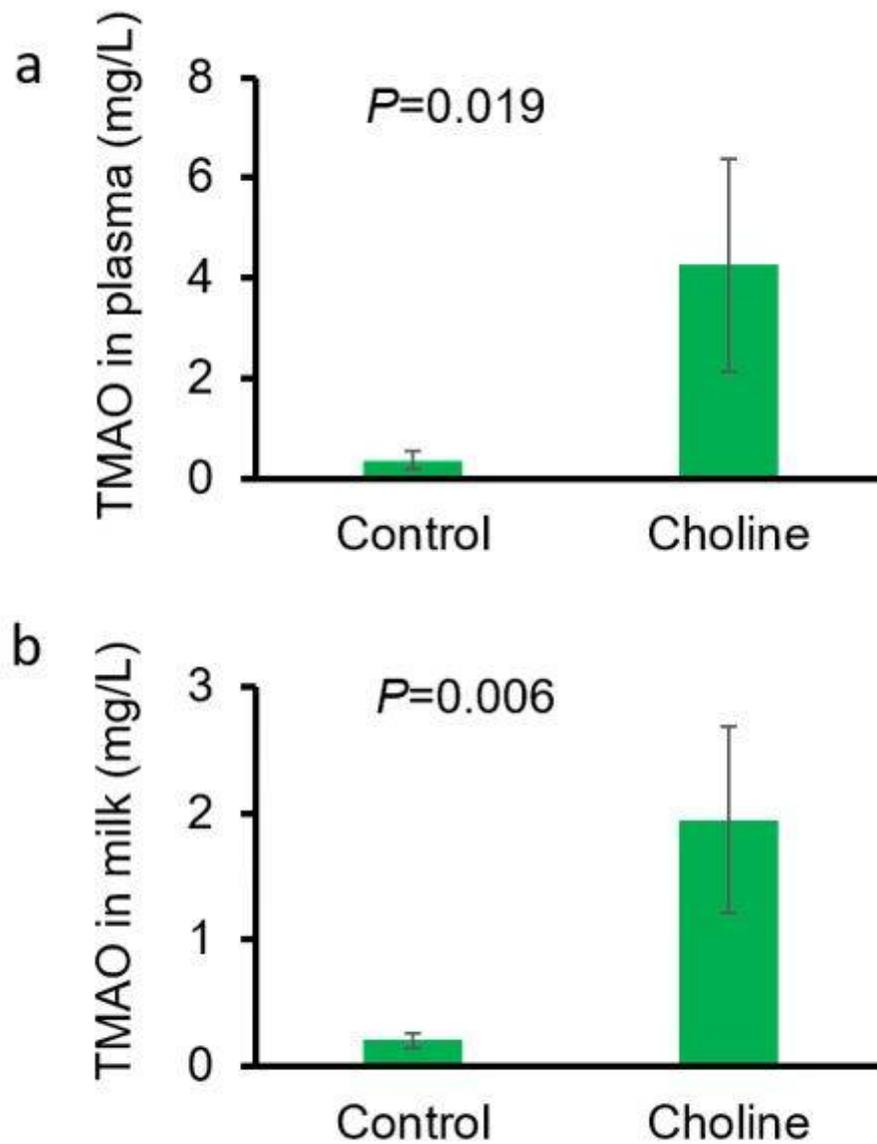


Figure 4

Trimethylamine N-oxide concentrations in the plasma and milk of dairy cows at day 28 of the experiment. Blood samples were collected before the morning feeding. For the choline group, the cows were fed choline chloride. The cows in the control group were not fed choline chloride.

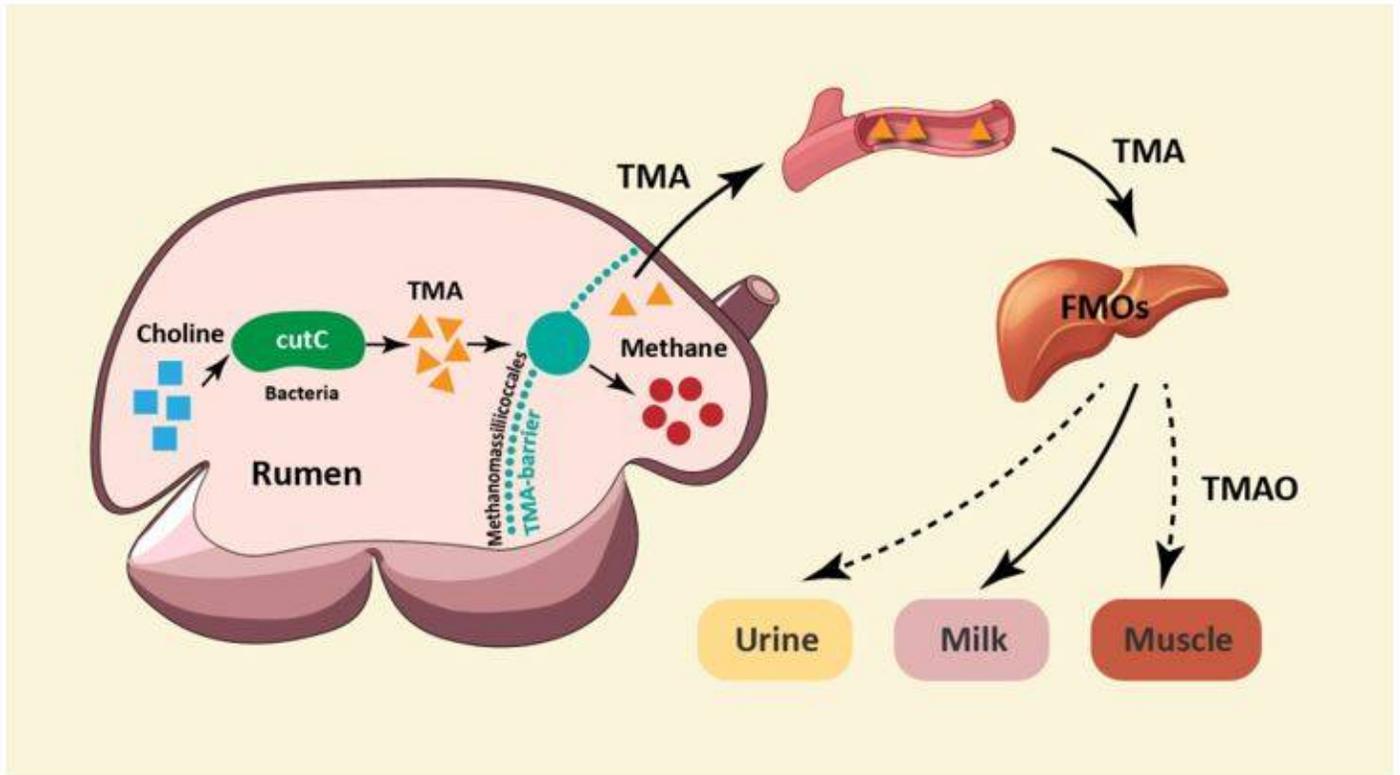


Figure 5

The proposed schematic diagram of dietary choline metabolism in the studied dairy cows. FMOs, flavin monooxygenase; TMA, trimethylamine; TMA barrier, Methanomassiliicoccales; TMAO, Trimethylamine N-oxide.

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