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Enterobacter aerogenes ZDY01 Attenuates Choline-Induced Trimethylamine *N*-Oxide Levels by Remodeling Gut Microbiota in Mice

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Trimethylamine *N*-oxide (TMAO), which is transformed from trimethylamine (TMA) through hepatic flavin-containing monooxygenases, can promote atherosclerosis. TMA is produced from dietary carnitine, phosphatidylcholine, and choline via the gut microbes. Previous works have shown that some small molecules, such as allicin, resveratrol, and 3,3-dimethyl-1-butanol, are used to reduce circulating TMAO levels. However, the use of bacteria as an effective therapy to reduce TMAO levels has not been reported. In the present study, 82 isolates were screened from healthy Chinese fecal samples on a basal salt medium supplemented with TMA as the sole carbon source. The isolates belonged to the family Enterobacteriaceae, particularly to genera *Klebsiella*, *Escherichia*, *Cronobacter*, and *Enterobacter*. Serum TMAO and cecal TMA levels were significantly decreased in choline-fed mice treated with *Enterobacter aerogenes* ZDY01 compared with those in choline-fed mice treated with phosphate-buffered saline. The proportions of Bacteroidales family S24-7 were significantly increased, whereas the proportions of Helicobacteraceae and Prevotellaceae were significantly decreased through the administration of *E. aerogenes* ZDY01. Results indicated that the use of probiotics to act directly on the TMA in the gut might be an alternative approach to reduce serum TMAO levels and to prevent the development of atherosclerosis and "fish odor syndrome" through the effect of TMA on the gut microbiota.

Keywords: Trimethylamine *N*-oxide, trimethylamine, *Enterobacter aerogenes*, gut microbiota

Introduction

The gut microbiota is an assortment of microorganisms inhabiting the mammalian gastrointestinal tract and is intimately involved in many aspects of normal host physiology, from nutritional status to behavioral responses [1]. In recent years, the gut microbiota and its external factors causing obesity and related diseases have received increasing attention. Many studies have reported that metabolites generated by the gut microbiota are correlated with host physiology and metabolic diseases [2, 3]. Specifically, trimethylamine *N*-oxide (TMAO), which is oxidized from trimethylamine (TMA) through hepatic flavin-containing monooxygenases (FMOs) (particularly FMO3 and FMO1) [4], can promote atherosclerosis through enhanced accumulation of macrophage cholesterol and foam cell formation in animal models [5]. TMA is a volatile low-molecular-weight tertiary aliphatic amine that can be produced from dietary carnitine, phosphatidylcholine, and choline by gut microbiota [5–7]. Mutations in the FMO3 gene result in accumulation of TMA in sweat and urine, which is known as "fish odor syndrome" [8].

Therefore, interventions reducing plasma TMAO and cecal TMA levels are considered to be health benefits [9]. Treatment with meldonium significantly decreases intestinal microbiota-dependent formation of TMA/TMAO by shifting carnitine microbial degradation [10]. Dietary allicin, a potent antimicrobial compound found in garlic, also reduces the

transformation of TMAO from L-carnitine through the effect of allicin on the gut microbiota [11]. In addition, 3,3-dimethyl-1-butanol (DMB), a structural analog of choline prevalent in wine, olive oil, and grapeseed oil, inhibits TMA production from gut microbes by inhibiting distinct microbial TMA lyases. DMB further reduces TMAO levels in mice [12]. Recently, resveratrol, a natural phytoalexin with prebiotic benefits, has been found to attenuate TMAO-induced atherosclerosis by decreasing plasma TMAO levels and increasing hepatic bile acid neosynthesis by changing the intestinal flora [13]. All measures mentioned above mainly target the process (choline or L-carnitine converted into TMA by gut microbes) with a new small molecule inhibitor. Recent works also target another process, which is that the host hepatic FMO3 oxidizes TMA into TMAO. An antisense oligonucleotide is recruited to suppress FMO3, and the circulating TMAO levels are reduced, thereby decreasing the risk of diet-enhanced atherosclerosis [14, 15].

However, no study has employed such therapeutic strategy in metabolizing and decreasing the TMA contents in the cecum in vivo before these contents enter into the bloodstream. Brugère *et al*. [9] proposed that archaea *Methanomassiliicoccus luminyensis* strain B10 should be used to prevent trimethylaminuria and cardiovascular diseases because this strain depletes TMA by reducing it with H_2 for methanogenesis in vitro [9]. Recently, Gregory *et al*. [16] determined the causality in the susceptibility of transmission of TMAO-associated atherosclerosis via transplantation of gut microbiota in a murine model. As opportunistic pathogens, the family Enterobacteriaceae, normal gut flora in animal intestines, is involved in the production of TMA from TMAO. The Enterobacteriaceae served as an electron acceptor during anaerobic metabolism to support oxidative phosphorylation [17]. However, whether the Enterobacteriaceae can further metabolize TMA and influence the plasma TMAO levels in vivo remains unknown.

Therefore, the current study aimed to investigate the effect of TMA-metabolizing bacterium on cecal TMA and plasma TMAO levels in mice and provide an alternative approach to prevent diet-induced atherosclerosis with addition of probiotics.

Materials and Methods

Growth Media and Isolation of TMA-Metabolizing Microorganisms

Basic salt medium supplemented with 20 mM TMA (BSMT) as the sole carbon and energy source was used to isolate the bacteria. BSMT contained 1 g/l KH_2PO_4, 2.5 g/l K_2HPO_4, 2 g/l$(NH_4)_2SO_4$, 0.1 g/l $MgSO_4$, and 1.911 g/l TMA·HCl. The medium without TMA was adjusted to pH 7.0 and wet autoclaved at 121°C for 20 min. The prepared TMA was sterilized through filtration and added into sterilized BSM. The solid medium was prepared by adding 20 g/l agarose into the liquid medium.

Fresh fecal samples were obtained from three healthy humans (25–35 years old). The fecal samples were dispersed and suspended in 17.5 g/l brain–heart infusion (BHI) medium, 10 g/l peptone, 5 g/l NaCl, 2 g/l glucose, and 2.5 g/l Na_2HPO_4. The samples were then serially diluted with 0.1 M phosphate-buffered saline (PBS). The diluted samples (10^{-6} and 10^{-7}) were placed on BSMT plates. The medium without TMA was used as a negative control. The plates were incubated in anaerobic condition at 37°C for 24 h. Isolates on the BSMT plate were inoculated in liquid medium and identified by amplifying and sequencing the 16S rRNA with primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) in a 50 µl polymerase chain reaction (PCR) assay, which consisted of 25 µl of 2×Taq Master mix (Tiangen, China), 1 µl of DNA template, 0.5 µl of each primer (10 µM), and 23 µl of dH_2O. The reaction was initiated at 94°C for 5 min; 94°C for 30 sec, 55°C for 30 sec, and 72°C for 2 min for 30 cycles; and 72°C for 10 min. PCR products were purified according to the instructions of SanPrep Column DNA Gel Extraction Kit, and sequenced using an Applied Biosystems 3730xl DNA Analyzer by Sangon Biotech (China). Basic Local Alignment Search Tool (BLAST) nucleotide analyses in the National Center for Biotechnology Information server (NCBI) (http://www.ncbi.nlm.nih.gov/) were used to find the highly similar sequences (megablast). Chimeric 16S rRNA gene sequences were identified by Chimera Check of Ribosomal Database Project II. The isolates identified were stored in 15% (v/v) glycerol at −80°C.

Fermentation of TMA by TMA-Metabolizing Microorganisms In Vitro

To determine the TMA-metabolizing capability of the bacteria in vitro, isolated bacteria were cultured in the BHI liquid medium for 24 h. After the precipitates were centrifuged at 12,000 ×*g* for 15 min and washed three times with PBS (pH 7.0), the cell precipitates were suspended in 25 ml of BSM liquid medium with TMA or TMA plus 0.1% glucose. The cell precipitates were then cultured under anaerobic condition at 37°C for 48 h. Supernatants were harvested through centrifugation at 12,000 ×*g* for 15 min, filtered through a 0.2 µm sieve, diluted with uHPLC-grade methanol, and analyzed by ultra-high performance liquid chromatography-tandem mass spectrometry (uHPLC-MS/MS). The medium without bacteria was used as a negative control.

TMA Reduction by TMA-Metabolizing Microorganisms In Vivo

To examine whether TMA-metabolizing microorganisms can influence the serum level of TMAO by metabolizing TMA in the cecum, a TMA-metabolizing microorganism was grown in BSMT, centrifuged, frozen in PBS, and orally gavaged at a dose of 1×10^9 CFU/mouse/day. BALB/c mice ($n = 19$, 6–7 weeks old) were obtained from the Experimental Animal Center, Nanchang

University (China) and housed for 1 week under standard conditions (21–25°C, 12 h light/dark cycle, and 45%–65% relative humidity) before treatment. The protocol for the animal experiment was approved by the Nanchang University Animal Ethical Committee, and all the ethical requirements to conduct the experiment were met (Approval No. 0064257).

BALB/c mice were randomly classified into three groups: control group (n = 7), mice fed a normal chow diet and treated with PBS; PBS-treated group (n = 5), mice fed a normal chow diet supplemented with 1.3% choline chloride (Sigma-Aldrich, USA) and treated with PBS; and single TMA-metabolizing microorganism-treated group (n = 7), mice fed a normal chow diet supplemented with 1.3% choline chloride (Sigma-Aldrich) and treated with *Enterobacter aerogenes* ZDY01, which demonstrated the strongest metabolic capacity of TMA in vitro. Mice were fed a normal chow diet supplemented with 1.3% choline chloride for 4 weeks before colonization. Mice were orally administered with the strain once daily for 4 weeks and then sacrificed. Whole blood was collected, and serum TMA/TMAO was determined through uHPLC-MS/MS. The intestinal contents from the cecum and tissues were immediately collected, frozen, and stored at −80°C.

uHPLC-MS/MS Analysis

uHPLC-MS/MS was used for quantification of plasma TMAO, cecal TMA, and TMA in the fermentation supernatant as previously reported with minor modifications [18]. Briefly, liquid chromatography separation was performed on a Waters Acquity BEH Amide (2.1 mm × 50 mm, 1.7 μm) analytical column in gradient mode, with 100% water/0.1% formic acid as mobile phase A and acetonitrile as mobile phase B. The gradient consisted of 15% solvent A and 85% solvent B held for 0.0 to 1.0 min, followed by a linear gradient of 40% B held from 1.0 to 2.5 min. The 40% B was then held for another 0.5 min and set to initial conditions from 3.0 to 5.0 min. The total run time was 5 min. The ion transitions (m/z 60 → 44 for TMA, m/z 69 → 44 for d9-TMA, m/z 76.2 → 58.3 for TMAO, and m/z 85.1 → 66.2 for d9-TMAO) were used to quantify TMA, d9-TMA, TMAO, and d9-TMAO.

Serum samples spiked with 2.5 μM deuterium-labeled TMA (Toronto Research Chemicals, Canada) and deuterium-labeled TMAO (Cambridge Isotope Laboratories, USA) as internal standards were treated with seven volumes of ice-cold methanol to precipitate proteins and then incubated at 4°C for 2 h. Afterward, samples were centrifuged at 12,000 ×g at 4°C for 15 min. The recovered supernatants were analyzed through uHPLC-MS/MS in positive-ion electrospray mode (Applied Biosystems, USA). Different concentrations of TMAO and TMA standards and fixed amounts of internal standards were spiked into the control serum to prepare the calibration curves for quantification of serum TMAO and TMA.

The cecal content was collected in sterile tubes, homogenized with a mixture of acetonitrile, methanol, and water (40:40:20 (v/v)), and then centrifuged at 12,000 ×g for 10 min at 4°C. The supernatants were used to quantify TMA through uHPLC-MS/MS, whereas the precipitations used in the analysis of microbiota by amplifying the V4 domain of bacterial 16S ribosomal DNA were stored at −80°C until analysis.

Total proteins from mice liver were extracted and placed in radio immunoprecipitation assay buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) supplemented with a final concentration of 1 mM PMSF and quantified using a BCA protein assay kit (Solarbio, China). FMO enzymatic activity was determined in accordance with previously published methods [19] and detected in 250 μl reaction mixtures containing 1 mg of total protein, 100 μM d9-TMA, and 100 μM NADPH in 10 mM HEPES (pH 7.4), incubated at 37°C for 8 h; the FMO enzymatic activity was then stopped with 0.2 M formic acid. Hepatic FMOs were determined as oxidized products of d9-TMAO through uHPLC-MS/MS.

Analysis of Cecal Microbiota in Different Groups

DNA isolation from cecal samples. To determine the effect of the TMA-metabolizing microorganism on the microbial composition in the cecum, which is the main site of TMA production [19], the compositions of cecal microbiota were also analyzed in the PBS (n = 3) and ZDY01 groups (n = 4).

Metagenomic DNA was extracted from the precipitation of cecal samples by using the CTAB method. On the basis of the quality and quantity of DNA from the cecal samples, the DNA was diluted to 1 ng/μl with sterile ddH_2O and sent to the Novogene Corporation (Beijing, China) for the V4 domain of bacterial 16S rDNA gene amplification, product purification, library construction, and sequencing.

Gene sequencing. Primers 515F (5′-GTGCCAGCMGCCGCG GTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) with barcodes were used to amplify the V4 domain of bacterial 16S rDNA. PCR amplification was performed in a 50 μl reactant containing 25 μl of 2× Phusion High-Fidelity PCR Master Mix with GC Buffer (New England Biolabs, USA), 0.2 μM each of 515F and 806R, and 20 ng DNA templates. The reaction conditions were as follows: an initial 98°C for 1 min, followed by 30 cycles of 98°C for 10 sec, 50°C for 30 sec, and 72°C for 1 min, as well as a final extension of 72°C for 5 min. The PCR products were assessed through 2% agarose gel electrophoresis and ethidium bromide staining and then purified using a QIAquick Gel Extraction Kit (Qiagen, Germany). The V4 domain library was constructed using the TruSeq DNA PCR-Free Sample Preparation Kit (Illumina, USA), assessed on the Qubit@2.0 Fluorometer (Thermo Scientific, USA) and Agilent Bioanalyzer 2100 system, and then sequenced on an Illumina HiSeq 2500 platform (250 bp paired-end reads).

Data Processing and Analysis

Paired-end reads were assigned to samples on the basis of their unique barcode, truncated by cutting off the barcode and primer sequence, and merged using Fast Length Adjustment of Short reads (ver. 1.2.7) [20]. Sequences with ambiguous or low quality bases (containing the character "N") were filtered by QIIME

quality filters [21], and sequence analyses were performed with Uparse software (ver. 7.0.1001) [22]. Sequences with ≥97% similarity were assigned to the same operational taxonomic units (OTUs). A representative sequence for each OTU was screened for further annotation. For each representative sequence, the GreenGene Database was used in accordance with the RDP classifier algorithm to annotate taxonomic information [23]. Chao1 was used to identify the species abundance. The observed species identifying the amount of unique OTUs found in each sample and Shannon index identifying community diversity were calculated with Quantitative Insights Into Microbial Ecology (ver. 1.7.0) [21] and displayed with R software (ver. 2.15.3). Principal coordinate analysis (PCoA) was performed to obtain the principal coordinates and to visualize complex, multidimensional data. A distance matrix of weighted UniFrac among the obtained samples was transformed to a new set of orthogonal axes, by which the first maximum variation factor was demonstrated by the first principal coordinate, the second maximum variation factor was shown by the second principal coordinate, and so on. PCoA analysis was displayed through the WGCNA package, stat packages, and ggplot2 package in R software (ver. 2.15.3). The linear discriminant analysis (LDA) effect size (LEfSe) was used to elucidate the differences of bacterial taxa. An LDA score of ≥2 was considered to be an important contributor to the model [24]; LEfSe was used online in the Galaxy workflow framework (http://huttenhower.sph.harvard.edu/galaxy).

Statistical Analysis

All values were expressed as the mean ± SEM. Statistical differences between the two groups were calculated through one-way ANOVA, followed by Newman–Keuls multiple comparison test. Data were analyzed using GraphPad Prism 5.0 software (GraphPad Software, USA). A value of $p < 0.05$ was considered statistically significant.

Results

Isolation of TMA-Metabolizing Microorganisms from Human Gut In Vitro

Fecal samples from three healthy Chinese adults were targeted for isolation of TMA-degradation strains by using TMA·HCl as the sole carbon source for the culture medium because it is nonvolatile. As presented in Table 1, a total of 82 isolates were screened for potential degradation of TMA. Phylogenetic analysis through 16S rRNA sequencing showed that all the isolates belong to Enterobacteriaceae, and they were categorized into the four genera *Cronobacter*, *Enterbacter*, *Klebsiella*, and *Escherichia*, including six species of *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *E. aerogenes*, *Enterobacter ludwigii*, *Escherichia coli*, and *Cronobacter sakazakii*. Among them, *E. aerogenes* ZDY01, *K. pneumoniae* ZDYA1, *K. oxytoca* ZDY8D, *E. ludwigii* ZDY3D, *E. coli* ZDYD4, and *C. sakazakii* ZDY5G were selected as representative strains for the following test because of their relative fast growth in solid medium containing TMA·HCl (data not shown).

Isolates Can Utilize TMA in Liquid Medium In Vitro

To screen the high-capacity species for TMA degradation, the utilization of TMA in liquid medium among six representative strains growing relatively fast in BSMT was compared. Unexpectedly, all six strains did not grow well anaerobically in the liquid medium, and the TMA content was almost constant as indicated by uHPLC-MS/MS analysis (Table S1). Nevertheless, the six strains grew well in the presence of 0.1% glucose, and the TMA content decreased. As shown in Fig. 1, the TMA content from *E. aerogenes* ZDY01 supernatant decreased by 36.3% compared with the others, in contrast with the negative control; this finding indicated that the capability of TMA utilization of these bacteria may be dependent on the bacterial amount and strain specificity.

TMA-Metabolizing Bacteria Can Decrease TMAO Serum Levels In Vivo

A comparison test between *E. aerogenes* ZDY01 and PBS was performed to investigate the efficacy of *E. aerogenes* ZDY01 in modulating the cecal TMA and serum TMAO levels in female BALB/c mice. This study found that the

Table 1. Gut bacteria screened from human feces for their ability to utilize trimethylamine.

Family	Genus	Species included	Isolates
Enterobacteriaceae	*Klebsiella*	*pneumoniae*	ZDY A1, A2, A3, A4, A5, A6, A8, B1, B2, B3, B4, B5, B6, B7, B8, D1, F3, 1B, 3B, 4B, 5B, 6B, 7B, 8B, 9B, 1D, 2D, 6D, 7D, 9D, 2C, 3C, 5C, 6C, 7C, 8C, 1E, 4E, 5E, 9E, 4F
		oxytoca	ZDY 8D, 7E, 1F, 2F, 3F
	Enterobacter	*aerogenes*	ZDY 01, D7, E3, E4, E8, F1, F2, F6, F7, F8
		ludwigii	ZDY 3D
	Escherichia	*coli*	ZDY D4, E1, F4, F5, G1, G2, G5, G6, G7, H1, H2, H3, H4, H5, H6, 1G, 2G, 3G, 4G, 7G, 8G, 9G, 3E
	Cronobacter	*sakazakii*	ZDY 5G, 6G

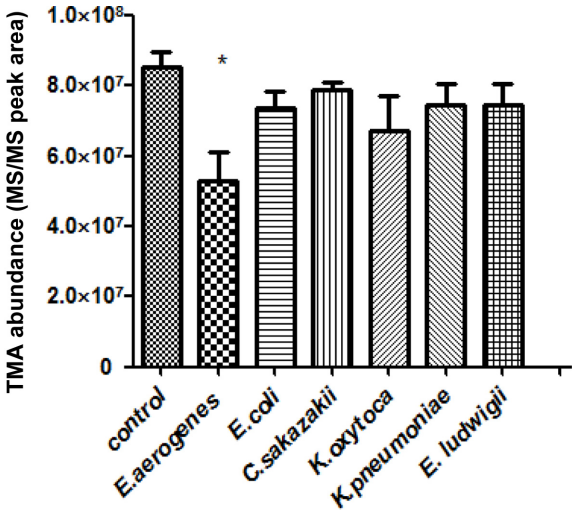

Fig. 1. Effect of trimethylamine (TMA)-metabolizing bacteria on TMA contents in the basal salt medium (BSM) in vitro.
The strains were cultured in BSM with 20 mM TMA•HCl in anaerobic condition at 37°C for 48 h, and the TMA content was significantly reduced by *E. aerogenes* ZDY01 ($p = 0.037$). Data are expressed as the mean ± SD of three replicates.

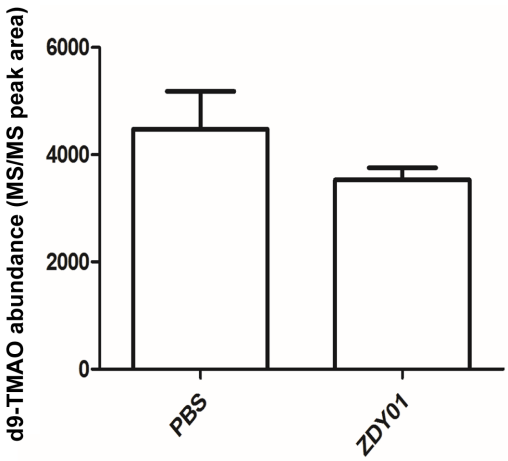

Fig. 3. Effect of trimethylamine (TMA)-metabolizing bacteria on hepatic flavin-containing monooxygenase (FMO) activity.
TMA-metabolizing bacteria had no effect on hepatic FMO activity in BALB/c mice. PBS, 1% choline-fed mice treated with PBS ($n = 5$). ZDY01, 1% choline-fed mice treated with TMA-metabolizing strain ($n = 7$). Data are shown as the means ± SEM. Statistical significance was calculated by the Newman–Keuls multiple comparison test.

ZDY01 group gained significantly ($p < 0.05$) less serum level of TMAO, with a decrease of 43.3% of TMAO, compared with the PBS group (Fig. 2A), probably because of the significant reduction of cecal TMA level in the ZDY01 group compared with that in the PBS group (Fig. 2B).

This study also determined the hepatic FMO3 activity to confirm whether it was regulated by the colonization of TMA-metabolizing bacteria. The results showed no significant difference in FMO3 activity between the ZDY01 and PBS groups; hence, FMO3 was not affected by the TMA-metabolizing bacteria (Fig. 3).

TMA-Metabolizing Bacteria Can Affect Cecal Bacteria Partially Related to Serum TMAO Levels

The formation of TMA and TMAO from dietary choline is dependent on the gut microbiota [6]. To explore whether the decrease of serum TMAO levels was caused by the change in gut microbial community, this study examined

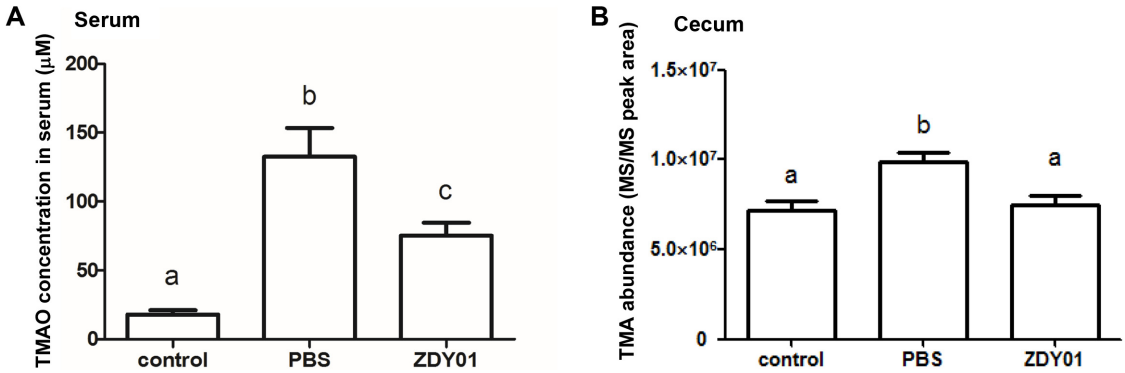

Fig. 2. Effect of trimethylamine (TMA)-metabolizing bacteria on serum trimethylamine *N*-oxide (TMAO) levels and cecal TMA levels in BALB/c mice.
TMA-metabolizing bacteria significantly inhibited TMAO synthesis (**A**) and cecal TMA (**B**) in BALB/c mice. Control, conventionally raised mice treated with PBS ($n = 7$). PBS, 1% choline-fed mice treated with PBS ($n = 5$). ZDY01, 1% choline-fed mice treated with TMA-metabolizing strain ($n = 7$). Data are shown as the means ± SEM. Statistical significance was calculated by the Newman–Keuls multiple comparison test and indicated with a letter. Means with the same letter are not statistically different (one-way ANOVA; $p = 0.0086$).

the composition of cecal bacteria of mice in two groups through bioinformatics analysis. To compare the overall microbiota structure in the cecum, the weighted UniFrac distance matrix was calculated using the OTUs of each sample. The results of PCoA based on distance exhibited a significant difference in the bacterial structure in the cecum (Fig. S1).

Further analysis revealed that there were significant variations in the composition of intestinal flora between the PBS and ZDY01 groups at different bacterial levels. In the ZDY01 treatment group, the relative abundance of order Bacteroidales and family S24-7 (an abundant family from Bacterioidetes) was significantly increased by 17.3% (67.02% ± 4.94% vs. 57.1% ± 4.68%) and by 39.6% (58.65% ± 6.26% vs. 42.01 ± 7.8%), and the relative abundance of order Clostridiales, families Helicobacteraceae and Prevotellaceae was decreased by 37%, 25.3%, and 64.3% compared with that in the PBS group, respectively. In addition, the relative abundance of order Burkholderiales was profoundly increased by approximately five times and Flavobacteriales was almost eliminated by ZDY01 treatment (Figs. 4A and 4B).

In the examination of which specific genera were associated with changes in TMAO levels, LEfSe analysis showed the greatest differences in genera *Sutterella*, *Rummeliibacillus*, *Oligella*, *Allobaculum*, *Facklamia*, *Aerococcus*, and *Corynebacterium*, which presented relatively higher abundance in the ZDY01 group, whereas genera *Clostridium*, *Odoribacter*, and *Acinetobacter* were relatively more abundant in the PBS group than in the ZDY01 group (Fig. 4C).

Discussion

TMA depletion in the gut may be beneficial for reducing serum TMAO levels related to early atherosclerosis in

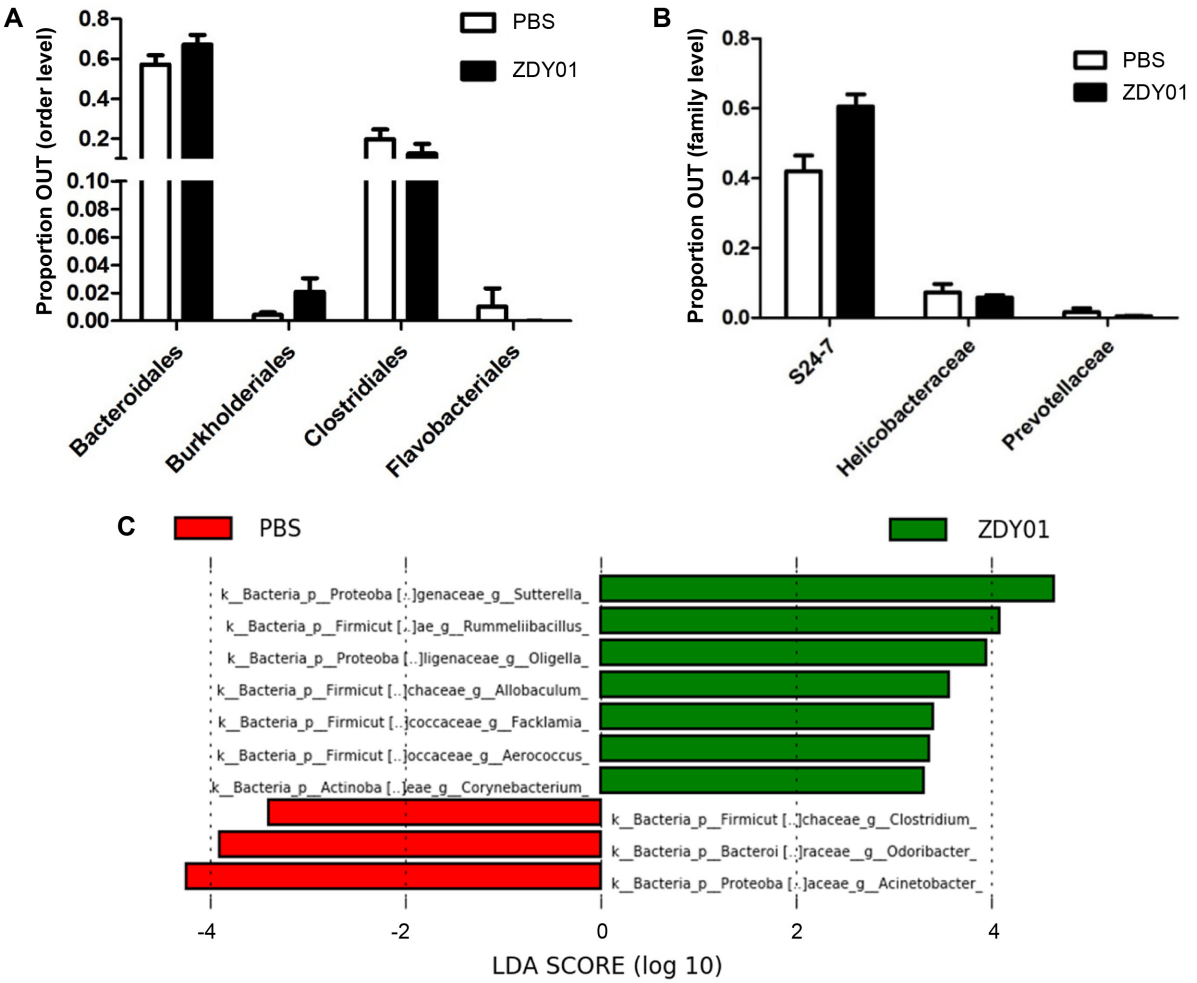

Fig. 4. Relative abundance of different taxa of intestinal microbiota between the PBS and ZDY01 treatment groups.
Order-level differing between the two groups (**A**), family-level differing between the two groups, (**B**) and histogram of the linear discriminant analysis (LDA) scores for differentially abundant genus level (**C**).

humans [25]. Although few scientific reports have proved such hypothesis, only Brugère *et al*. [9] deliberately proposed that archaea should be used to prevent trimethylaminuria and cardiovascular diseases; however, their proposal presented no experimental data in vivo. In the current study, a series of in vitro and in vivo tests were performed to determine the relationship of TMA-metabolizing bacteria with cecal TMA and serum TMAO levels and compare the efficiency of respective single bacteria in the reduction of serum TMAO levels with that of the PBS group. In particular, *E. aerogenes* ZDY01 with TMA-metabolizing capability was screened out from healthy human feces. Through gavage for 4 weeks, the serum TMAO levels decreased significantly in 1.3% choline-fed mice treated with *E. aerogenes* ZDY01 relative to PBS treatment, probably because of the alteration of compositions of the gut microbiota.

A variety of aerobic, anaerobic, and facultative anaerobic bacteria, including *Paracoccus*, *Methylophilus*, *Pseudomonas*, and *Hyphomicrobium* [26–29], are employed to decrease the TMA levels to improve the environmental conditions around us. These bacteria can utilize TMA as their energy source to yield formaldehyde and ammonium. In the present study, to diminish the TMA content in the cecum, which is the main site of gut microbial production of TMA from carnitine and choline [30], TMA-metabolizing bacteria were screened from human gut on basic salt medium supplemented with 20 mM TMA as the sole carbon source and energy source. TMA-metabolizing bacteria, which belong to the family Enterobacteriaceae, were successfully isolated. To our best knowledge, *K. pneumoniae*, *K. oxytoca*, *E. aerogenes*, *E. ludwigii*, *E. coli*, and *C. sakazakii*, which belong to the family Enterobacteriaceae, are reported for the first time to be capable of utilizing TMA in vivo. Although the metabolites transformed from TMA need to be further studied, various studies suggest that Enterobacteriaceae can produce dimethylamine (DMA) and monomethylamine (MMA) from TMA [31, 32], which may indicate that family Enterobacteriaceae harbors a pathway of methylamines so it can grow in the presence of TMAO, TMA, or DMA. Although other bacteria such as *Methylocella sylvestris* and *Aminobacter aminovorans* are lacking known pathways for TMA utilization that can convert TMA to TMAO, both formaldehyde and ammonium are further produced via the intermediate DMA [33].

Previous reports [4–7, 34] indicated that two factors primarily influence the serum TMAO levels: one is the cecal TMA formed by gut bacterial conversion of dietary ingredients, such as choline and L-carnitine; and the other is hepatic FMO3 activity, which can oxidize >95% TMA into TMAO in the liver. According to our results, *E. aerogenes* ZDY01 did not affect the activity of hepatic FMO3.

The gut microbiota played an important role in TMAO formation from dietary sources of methylamines, such as choline and carnitine [5]. Several bacterial taxa are significantly associated with plasma TMAO levels. The order Clostridiales [6] and genera *Prevotella, Clostridium, Fusibacter*, and *Ruminococcus* are positively associated with plasma TMAO levels, whereas the genus *Bacteroides* and proportions of S24-7, which is an abundant family from Bacteroidetes, are negatively associated with TMAO levels [12]. In our experiments, 16S rRNA sequencing of cecal bacteria showed that the richness (number of OTUs/species present in a sample) and composition (species diversity present in a sample) were completely different between the two groups at the family level (Fig. S2). The serum TMAO levels were related to the composition of cecal microbiota. Similar to previous reports, the positive correlation between Prevotellaceae and the TMAO levels as well as the negative correlation between the abundance of S24-7 and TMAO levels were confirmed. Furthermore, a novel finding was disclosed, indicating that the reduced abundance of Helicobacteraceae was associated with low TMAO levels. Additionally, ZDY01 significantly reduced the relative abundance of genera *Clostridium* and *Acinetobacter* (Fig. 4C), both of which demonstrate TMA lyase activity, cleaving choline or carnitine to form TMA [35, 36]. Therefore, the reduced TMA lyase activity from genera *Clostridium* and *Acinetobacter* caused by ZDY01 is another potential factor contributing to the reduced TMA content from choline or carnitine in the cecum.

Previous data showed that intake of dietary phosphatidylcholine and choline may change the composition of the gut microbiota and disturb the balance between protective and harmful intestinal bacteria [5]. Enterobacteriaceae, which is traditionally regarded as a bacterial family of opportunistic pathogens in humans and animals, may revert the disrupted structure of the gut microbiota to stabilize diversity. However, the specific mechanism by which Enterobacteriaceae affects the gut microbiota still needs further studies.

Based on these findings, addition of Enterobacteriaceae was demonstrated for the first time to change the community composition in vivo, and this change may provide other microbiota with a competitive advantage, leading to reduction of cecal TMA and serum TMAO levels.

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