

Article

Metagenomics and Machine Learning Identify TMA-Producing *Serratia* Induced by High-Fat/Choline Diet: A Novel Obesity Target for TMA

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Abstract

Background: High-fat diet-induced metabolic disorders are associated with trimethylamine (TMA)/trimethylamine *N*-oxide (TMAO), whose production is linked to gut microbial choline metabolism. However, changes in specific gut microbiota under a high-fat diet and the relationship between these changes and choline in TMA/TMAO production remain unclear. **Methods:** A total of 48 7-week-old male C57BL/6J mice were subjected to one-week acclimatization feeding, and then randomly divided into four groups (12 mice per group) to establish a 2 × 2 factorial design animal experiment: the control group (CON, basal diet), the choline-supplemented control group (CON + C, basal diet supplemented with 1% choline), the high-fat diet group (HF, high-fat diet), and the high-fat plus choline group (HF + C, high-fat diet supplemented with 1% choline). The experiment lasted for 9 weeks, during which dynamic monitoring of TMAO levels in mice was performed in the first 4 weeks. At the ninth week, the mice were sacrificed and samples were collected for subsequent assays, including the concentrations of TMA and TMAO in serum, colonic contents and feces; the pathological morphology of liver tissue, adipocyte staining characteristics and serum biochemical parameters; and the expression levels of key genes and proteins in liver, small intestine and colon tissues. Meanwhile, metagenomic analysis was conducted on colonic contents, combined with machine learning to predict the correlation between gut microbiota and TMA. In addition, gene cloning, multiple sequence alignment, molecular simulation and in vitro culture experiments were carried out to verify the TMA-producing function of the target strain. **Results:** This study elucidated that high-fat diet and high choline exert a significant interaction in TMA/TMAO production through a 2 × 2 animal experiment; meanwhile, the significantly increased TMA/TMAO levels co-induced by the two factors further exacerbate metabolic disorders. Notably, through combined metagenomics and machine learning, we identified *Serratia marcescens* as the primary TMA-producing microorganism under high-fat/choline diet induction. In vitro cultures simulating the intestinal environment revealed that the TMA conversion ability of *Serratia marcescens* is time-dependent, reaching 60 ± 2.49% after 24 h of anaerobic culture with choline chloride. Multiple sequence alignment and molecular simulation further demonstrated that the CutC enzyme of *Serratia marcescens* has a conserved amino acid sequence and high affinity for choline. **Conclusions:** We uncovered a two-factor synergistic effect of a high-fat/choline diet on TMA/TMAO, and for the first time identified the genus *Serratia* as a TMA-producing bacterium. These findings provide a new potential target for intervening in metabolic disorders mediated by high-fat diet-induced TMAO elevation.



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Keywords: high-fat diet; obesity; TMAO; metagenomics; machine learning; *Serratia marcescens*

1. Introduction

With the acceleration of global industrialization and profound shifts in dietary patterns, high-fat diets have emerged as a defining feature of modern dietary structures [1]. Typically, such diets in humans derive 52–60% of energy from fat [2]. Fueled by the proliferation of processed foods, the rise of fast-food culture, and increasing dietary refinement, high-fat diets have not only become normalized in developed European and American countries but also spread rapidly among urban populations in developing nations [3]. According to the World Health Organization, as of 2022, over 2.5 billion adults worldwide are overweight, including 890 million with obesity. High-fat diets are widely recognized as a key environmental driver of this global health crisis, with their adverse impacts on metabolic health posing a cross-population, cross-regional public health challenge [4]. Short-term high-fat intake causes abnormal body fat accumulation via energy metabolism imbalance [5]; long-term exposure induces intestinal barrier impairment (e.g., downregulated expression of tight junction proteins), triggering endotoxemia and subsequently driving systemic low-grade inflammation [6]. In recent years, studies have revealed that the pathogenic mechanisms of high-fat diets are not limited to host metabolic disorders but are closely linked to gut microbiota structure [7]. Research confirms that high-fat intake significantly reduces gut microbiota α -diversity and alters the niche distribution of core genera (e.g., imbalanced ratio of *Bacteroidetes* to *Firmicutes*) [8]. It also leads to significant enrichment of opportunistic pathogens such as *Proteobacteria* [9], *Escherichia coli* [10], *Desulfovibrionaceae* [11], and *Lachnospirillum* [12].

Research has revealed that the microbial metabolite trimethylamine (TMA) and its host-derived product trimethylamine N-oxide (TMAO) are significantly associated with various metabolic diseases [13]. Clinical research has confirmed that TMAO is an independent risk marker for obesity [14]. TMA is primarily produced by gut microbiota through metabolizing dietary substrates such as choline (e.g., eggs, liver), carnitine (e.g., red meat), and phosphatidylcholine. After intestinal absorption, it is catalytically converted to TMAO by host hepatic flavin-containing monooxygenases (FMOs, predominantly FMO3) [15]. Western diets, which are high in fat, are generally rich in methylamine-containing nutrients such as choline. The observation that long-term consumption of Western diets significantly increases the risk of obesity and neurological diseases suggests that the close association between high-fat diets and obesity-related metabolic diseases may be mediated by high levels of choline [16].

At the molecular level, TMA production depends on gut microbiota-encoded choline-TMA lyase (CutC) and its activating enzyme (CutD) [17]. As a specific glyceryl radical enzyme (GRE), CutC catalyzes the cleavage of the carbon–nitrogen bond in choline molecules, while CutD regulates its function by mediating the radical activation of CutC [18]. This enzyme system was first identified in anaerobic sulfate-reducing bacteria, and several metagenomic and proteomic studies have found that GREs are among the most abundant protein families in the human gut microbiota [19]. Notably, *Lachnospirillum saccharolyticum* has been identified as a dominant TMA-producing strain, with in vitro experiments showing it can convert choline to TMA with a 98.7% conversion rate [20]. Another study demonstrated that long-term exposure to a high-fat diet enhances choline catabolism in *Escherichia coli* by altering intestinal epithelial physiology [21]. These findings reveal a high overlap between previously reported TMA-producing bacteria and microbiota with increased abundance

under high-fat diets, suggesting that specific microbiota may participate in metabolic disorders by enhancing TMA synthesis capacity under high-fat conditions.

High fat diet-induced metabolic diseases are closely linked to the production of TMA/TMAO. Yet, while existing studies have demonstrated that high-fat diets and choline can induce elevated TMA/TMAO levels in vivo, their individual effects and interactive relationships remain to be elucidated. Moreover, under high-fat diet conditions, the core bacterial genera contributing to TMA elevation, as well as the association between their TMA conversion efficiency and choline, await further investigation.

To address these questions, the present study employed a 2×2 factorial experiment to systematically investigate the effects of two levels of high fat and choline on TMA/TMAO levels, clarifying the impacts of these factors and their interaction on TMA/TMAO in mice. Correlation analysis was used to determine the strength of association between elevated TMA/TMAO and metabolic disorder indices. Using metagenomics, combined with functional gene annotation to identify microbiota carriers of *CutC/D* genes and machine learning algorithms, we screened core genera significantly associated with TMA levels under high-fat diet. In vitro pure culture experiments quantified their choline-to-TMA conversion efficiency, providing direct evidence for their functional core role. This study helps clarify the synergistic regulatory mechanism of high fat and choline in the TMA/TMAO metabolic pathway, precisely identifies functional TMA-producing genera under specific dietary conditions, and offers potential targets and theoretical basis for specifically inhibiting key TMA-producing bacteria via gut microbiota targeting to prevent obesity-related metabolic diseases.

2. Materials and Methods

2.1. Animal Experimental Design

Forty-eight specific-pathogen-free male C57BL/6J mice (7 weeks old) were purchased from SiPeiFu Biotechnology Co., Ltd. (Beijing, China). Male mice were selected primarily to avoid the potential complex effects of hormone fluctuations associated with the estrous cycle in female mice on lipid metabolism, inflammatory responses, and intestinal flora composition. Mice were housed in the animal facility of Sichuan Agricultural University (Ya'an, China) under controlled environmental conditions: temperature maintained at 25 ± 1 °C, relative humidity at 50–60%, and a 12 h light/dark cycle. Food and water were provided ad libitum throughout the experiment. After 1 week of acclimatization, mice were randomly allocated to 4 experimental groups ($n = 12$ per group): control (CON, basal diet), control + choline (CON + C, basal diet supplemented with 1% choline), high fat (HF, high-fat diet), and high fat + choline (HF + C, high-fat diet supplemented with 1% choline; Figure 1A). All mouse diets were sterile (BiotechHD Co., Ltd., Beijing, China), with detailed formulations provided in Table S1. The experiment lasted 9 weeks, after which mice were euthanized and sampled. Body weight of each mouse was monitored weekly, and daily food intake was recorded throughout the trial. The experimental protocol was approved by the Animal Ethics and Welfare Committee of Sichuan Agricultural University (approval No. 20230038, approval date 8 March 2023).

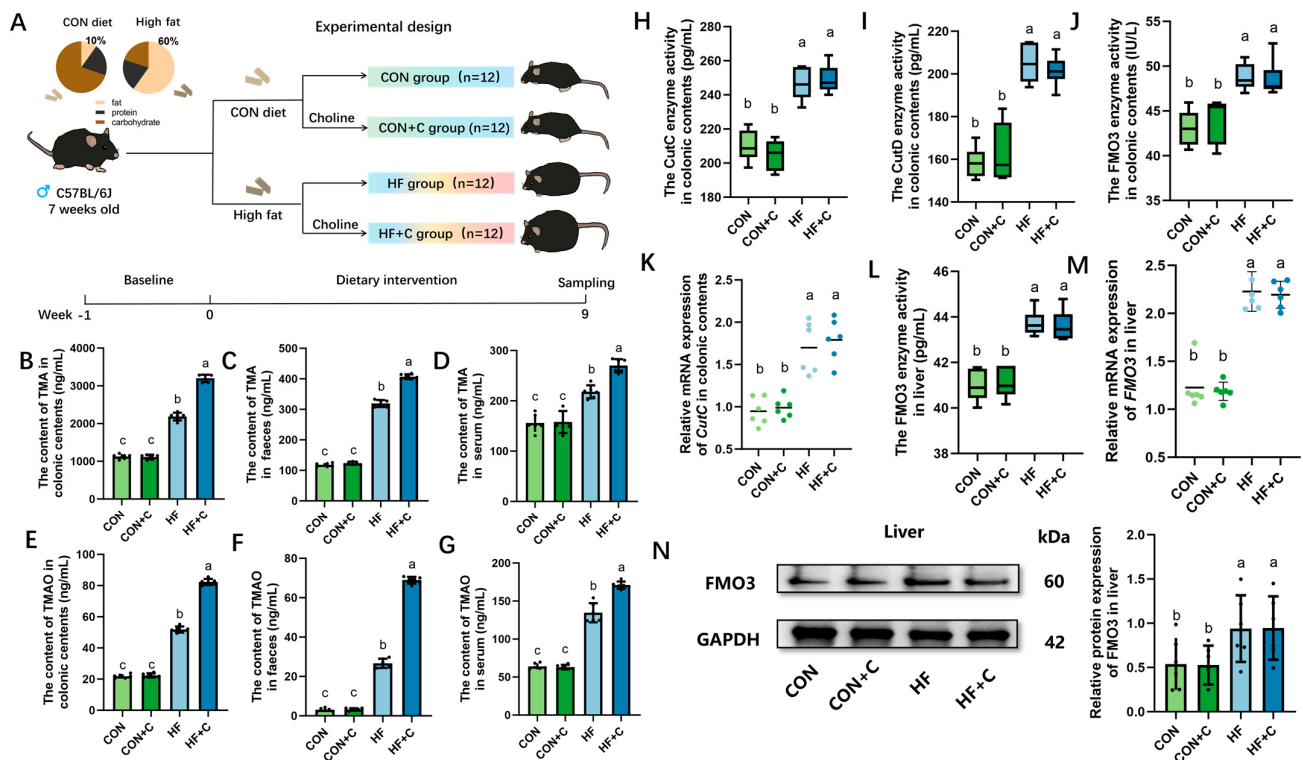


Figure 1. High-fat/choline diet increases TMA/TMAO level via the choline-CutC/D-FMO3 pathway. (A) Animal experimental design; quantification of TMA in (B) colonic contents, (C) faeces and (D) serum for 9 weeks ($n = 6$); quantification of TMAO in (E) colonic contents, (F) faeces and (G) serum for 9 weeks ($n = 6$); (H) CutC enzyme activity, (I) CutD enzyme activity and (J) FMO3 enzyme activity in colonic contents for 9 weeks ($n = 6$); (K) *CutC* mRNA expression level in colonic contents for 9 weeks ($n = 6$); (L) FMO3 enzyme activity, (M) *FMO3* mRNA expression level and (N) FMO3 protein expression level in liver for 9 weeks ($n = 6$). Light green indicates the CON group, dark green represents the CON+C group, light blue denotes the HF group, and dark blue corresponds to the HF + C group. All data are presented as mean $\pm$ SD. One-way ANOVA with Tukey's range test was used. a–c represent one-way ANOVA conducted amongst the groups.

2.2. Morphological Analysis of Mouse Liver and Adipose Cells by H&E Staining

Mouse liver tissues were fixed in 10% buffered formalin for 24 h, followed by dehydration through a graded ethanol series (70%, 80%, 90%, 95%, and 100%). Tissues were cleared in xylene, infiltrated with molten paraffin, and embedded. Paraffin-embedded tissues were sectioned at 5 μ m using a Leica CM3050S rotary microtome (Leica Biosystems, Nürnberg, Germany), mounted on glass slides, oven-dried at 56 $^{\circ}$ C for 24 h, and stored at room temperature until use. Abdominal adipose tissue sections were prepared identically. For H&E staining, sections were deparaffinized in xylene, rehydrated through graded ethanol to distilled water, stained with hematoxylin, rinsed, differentiated in 1% acid alcohol, counterstained with eosin, dehydrated through graded ethanol, cleared in xylene, and mounted with neutral balsam. Images were acquired using a Leica Aperio CS2 slide scanner (Leica Biosystems, Nürnberg, Germany). White adipocyte diameters were quantified using Fiji software (v2.15.0).

2.3. Serum and Tissue Biochemical Index Assays

Serum samples were obtained via retro-orbital bleeding followed by centrifugation. Serum total cholesterol (TC), triglycerides (TG), high-density lipoprotein (HDL) cholesterol, and low-density lipoprotein (LDL) cholesterol were quantified using a Hitachi High-Tech automatic biochemical analyzer (Hitachi High-Tech Corporation, Tokyo, Japan).

Inflammatory factors in serum, such as tumor necrosis factor- α (TNF- α), interleukin (IL)-1 β , and IL-6, were detected using an Enzyme-Linked Immunosorbent Assay (ELISA) kit (Jingmei Biotechnology Co., Ltd., Yancheng, China) according to the manufacturer's protocol. Serum lipopolysaccharide (LPS) levels were also quantified with the same ELISA kit. For assessing serum antioxidant capacity, malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GPx) contents were measured using commercial assay kits (Jingmei Biotechnology Co., Ltd., Yancheng, China).

Liver tissue, colon contents, and fecal samples were homogenized with 0.9% saline (1:9, *w/v*), centrifuged at $12,000 \times g$ for 15 min at 4 °C to collect supernatants (all operations on ice). Total bile acid (TBA) in serum, liver, colon contents, and feces was determined using a TBA Assay Kit (Jingmei Biotechnology) per protocol.

Enzymatic activities of choline trimethylamine-lyase (CutC) and its activating enzyme (CutD) in colonic contents, and hepatic flavin-containing monooxygenase 3 (FMO3), were quantified using colorimetric kits (Jingmei Biotechnology) per manufacturer instructions.

2.4. Quantitative Real-Time PCR (qRT-PCR) Assay

Total RNA was isolated from mouse liver, small intestine, and colon tissues using a TRIzol kit (Tiangen Biotechnology, Beijing, China) per manufacturer protocol. RNA concentration was determined using a Nanodrop ND2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA was reverse-transcribed into cDNA using a reverse transcription kit (Tiangen Biotechnology). qRT-PCR was performed with the SYBR Green qPCR kit (Tiangen Biotechnology). Primer sequences are listed in Table S2. GAPDH served as an internal reference, and the relative expression levels of target genes were calculated and quantified by the $2^{-\Delta\Delta CT}$ method.

2.5. Western Blot Assay

Liver, small intestine, and colon tissues were collected; total proteins were extracted using RIPA lysis buffer containing protease/phosphatase inhibitors. Homogenates were centrifuged at $12,000 \times g$ for 5 min at 4 °C, and supernatants were boiled at 100 °C for denaturation. Protein concentration was measured using a BCA kit (Conviviality, CW0014). Equal amounts of protein were separated by SDS-PAGE, transferred to PVDF membranes, blocked with TBST containing 5% skimmed milk for 1 h at room temperature, and incubated with primary antibodies at 4 °C overnight. After TBST washes, membranes were incubated with secondary antibodies for 1 h at room temperature. Bands were visualized using an ECL kit (Beyotime Biotech, Yancheng, China) and analyzed with a Gel Doc XR+ system; band intensities were quantified using ImageJ (version 1.53t, National Institutes of Health, Bethesda, MD, USA). All antibodies employed in this study (including GAPDH, PPAR γ , LPL, PPAR α , FXR, Tgr5, CYP7A1, CYP27A1, Cldn1, Ocln, and ZO-1) were sourced from Proteintech Group (Wuhan, China) and were validated.

2.6. TMA and TMAO Quantification

TMA and TMAO were quantified using a stable isotope-labeled internal standard method. Briefly, samples were mixed with 4 volumes of 0.2% formic acid in water, followed by addition of known amounts of internal standards (d9-TMA and d9-TMAO). The mixture was centrifuged at $12,000 \times g$ for 15 min at 4 °C. The supernatants were then mixed with cold acetonitrile, re-centrifuged, filtered through a 0.22 μ m membrane, and subjected to LC-MS/MS analysis. LC-MS/MS was performed on an InfinityLab Poroshell 120 EC-C18 column (50 $\times$ 2.1 mm, 1.9 μ m) coupled with an Agilent 6470 triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) in positive electrospray ionization (ESI+) mode. Chromatographic conditions were as follows: column temperature: 30 °C; flow rate: 0.1 mL/min; mobile phase A: 0.1% formic acid in water, B: acetonitrile.

A gradient elution program was used: 0–2.0 min, 95% A; 2.1–3.0 min, 70% A; 4.0–5.0 min, 95% A. Mass spectrometric parameters included electrospray voltage: 3500 V; ion source temperature: 350 °C; sheath gas temperature: 400 °C; sheath gas flow: 12 L/min; nebulizer pressure: 45 psi. Analytes were monitored via multiple reaction monitoring (MRM) with the following transitions: TMA (m/z 60→44 and 60→45), TMAO (m/z 75.9→58 and 75.9→59.1), d9-TMA (m/z 69→49), and d9-TMAO (m/z 85.1→68.2). For quantification, calibration curves were constructed by spiking control samples with standard solutions of TMA and TMAO at varying concentrations, along with fixed amounts of internal standards. The peak area ratios of analytes to their corresponding internal standards were used to generate linear calibration curves, which were then applied to quantify TMA and TMAO levels in experimental samples.

2.7. qPCR Analysis of Colonic Contents

Total genomic DNA was extracted from mouse colonic contents using the CTAB method. PCR amplified bacterial 16S rRNA (internal reference; primers: 16S_q515F: 5'-GTGCCAGCMGCCGCGG-3'; 16S_q806R: 5'-GGACTACHVGGGTWTCTAAT-3') and *cutC* (primers: *cutC*_qF: 5'-TTYGCIGGITAYCARCCNTT-3'; *cutC*_qR: 5'-TGNGGYTCIACRCAICCCAT-3'). Reaction mixtures (20 µL) and cycling conditions are detailed in Table S3. Amplicons were verified by melting curve analysis and 1% agarose gel electrophoresis (Figure S1; 1 µL PCR product + 5 µL 6× loading buffer; 80 V for 50 min; visualized via gel documentation). Relative *CutC* abundance was calculated using $2^{-\Delta\Delta CT}$ (vs. 16S rRNA).

2.8. Metagenomic Analysis

Total genomic DNA was extracted via CTAB. Concentration/purity were assessed by 1% agarose gel electrophoresis and Qubit fluorometry. DNA was sheared to 300 bp using a Covaris ultrasonicator. Libraries were constructed with the Truseq® DNA PCR-Free Sample Preparation Kit (Illumina, San Diego, CA, USA) and sequenced on an Illumina NovaSeq platform (250 bp paired-end reads). Raw data were quality-filtered using FASTP (v0.22.0) to generate clean reads. De novo assembly was performed with FLASH (version 1.2.11, Johns Hopkins University, Baltimore, MD, USA) (Magoc and Salzberg 2011) to generate contigs, which were subjected to coding sequence prediction (short fragments excluded). A non-redundant unigene set was clustered using Uparse (v7.0.1001).

2.9. Blast Comparison of CutC/D Sequences

The amino acid sequences of CutC/D were queried using the National Center for Biotechnology Information (NCBI) RefSeq database. The query for CutC employed the keyword “choline trimethylamine-lyase,” while the query for CutD used the keyword “choline TMA-lyase-activating enzyme.” Subsequently, the FASTA formats of these sequences were downloaded. The metagenomic sequencing results were then aligned against the CutC/D enzyme sequences retrieved from the NCBI RefSeq database using BLASTP (2.7.1) with alignment parameters set to an E-value of 1×10^{-5} and a sequence identity of 45%.

2.10. Machine Learning

Intestinal flora data were filtered to exclude redundant taxa (total abundance < number of samples). The TMA score was generated by normalizing TMA content. Microbial data (X) were modeled against TMA and *cutC* scores (Y) using the PyCaret toolkit (Python 3.8, Anaconda). Data were split into training (80%) and test (20%) sets; 10-fold cross-validation was applied to training data. Top models (by cross-validation) were tested on

the independent set. Model performance was evaluated by R^2 (goodness of fit), mean absolute error (MAE), and root mean squared error (RMSE):

$$R^2 = 1 - \frac{SS_{\text{res}}}{SS_{\text{tot}}} = 1 - \frac{\sum_i (y_i - f_i)^2}{\sum_i (y_i - \bar{y})^2}$$

$$\text{MAE} = \frac{1}{n} \sum_{i=1}^n |(y_i - f_i)|$$

$$\text{RMSE} = \sqrt{\frac{1}{n} \sum_{i=1}^n (y_i - f_i)^2}$$

where y_i , f_i and $\bar{y}$ are the true, predicted, and mean values, respectively; SS_{res} is the sum of squares of the residuals; SS_{tot} is the total sum of squares; and n is the number of samples.

SHAP analysis interpreted model results to identify key bacteria affecting CutC enzyme activities and TMA content.

2.11. Growth Curves and MIC of *Serratia marcescens* Under Anaerobiosis

Serratia marcescens (CICC 10187) was cultured in nutrient broth (pH 7). MIC of choline chloride (0–12.5 mg/mL) was determined via broth microdilution under anaerobiosis (80% N_2 , 20% CO_2); MIC = lowest concentration preventing turbidity in two adjacent tubes after 48 h (triplicate experiments).

Initial bacterial concentration: 1×10^4 CFU/mL. Choline chloride was added at 1/2 and 1/4 MIC. Cultures were incubated at 37 °C under anaerobiosis (BX-180CB incubator, Shanghai Boxun, Shanghai, China). OD_{600} was measured every 4 h using a UV-1200 spectrophotometer (BFRL, Beijing, China).

2.12. TMA Quantification in *Serratia marcescens* Cultures

Serratia marcescens was cultured under pH 7 to logarithmic phase; 1 mM choline chloride was added. Samples were collected at 0, 8, 16, and 24 h, and centrifuged at $12,000 \times g$ for 15 min. Supernatants were mixed with 4 volumes of 0.2% formic acid in water, centrifuged at $12,000 \times g$ for 15 min at 4 °C, then mixed with cold acetonitrile, re-centrifuged, and filtered. TMA content was determined as in Section 2.6.

2.13. PCR Amplification and Sequencing of *Serratia marcescens* CutC/D

Genomic DNA of *Serratia marcescens* was extracted using a bacterial genome kit (Tiangen Biotechnology) per protocol. Concentration/purity were determined via Nanodrop 2000. *cutC/D* primers were designed based on *Serratia marcescens* genome NZ_CP127881.1 (Sangon Biotech, Shanghai, China; Table S4).

PCR reactions (25 μ L): 12.5 μ L $2 \times$ Taq PCR Master Mix, 1 μ L each primer (10 μ M), 1 μ L template DNA (50–100 ng), ddH₂O to volume. Cycling: 94 °C for 2 min; 35 cycles of 94 °C for 30 s, 55–65 °C (gradient-optimized) for 30 s, 72 °C for 60 s; final extension at 72 °C for 5 min.

Amplicons were analyzed by 1% agarose gel electrophoresis (0.5 μ g/mL GoldView; 120 V for 30 min; visualized via Bio-Rad gel documentation). Validated amplicons were gel-extracted (Tiangen Biotechnology) and Sanger-sequenced (Sangon Biotech) with forward/reverse primers. Contigs were aligned to NCBI *Serratia marcescens* sequences via BLASTx; identity/coverage were calculated, and alignments visualized with BioEdit 7.2.6.

The FASTA sequences of CutC enzymes expressed by previously reported microorganisms (Table S10) were downloaded from the NCBI Protein Database and integrated with the CutC enzyme sequence of *Serratia marcescens*. Multiple sequence alignment was

performed using the ClustalW algorithm in BioEdit software. The unaligned sequences at both the head and tail ends were trimmed to obtain the alignment results.

2.14. Molecular Dynamics Simulation

Simulations were performed with GROMACS (v2024.03)⁴. Protein parameters: Amber14SB force field⁵; water: TIP3P model⁶; ligand topology: GAFF force field (generated via ACPYPE). The ligand–protein complex was placed in a periodic octahedral box with TIP3P water; Na⁺/Cl[−] was adjusted to 0.150 mol/L (neutral pH).

System preparation: 50,000 steps of steepest descent energy minimization; 100 ps NVT (constant particles, volume, temperature) and 100 ps NPT (constant particles, pressure, temperature) equilibration (position restraints on protein backbone). Temperature: 300 K (V-rescale thermostat); pressure: 1 bar (Parrinello–Rahman). Production simulation: 100 ns (no restraints); trajectory integrated at 2 fs, long-range electrostatics via PME. Trajectories were saved every 10 ps (10,000 frames). Binding free energy (90–100 ns) was calculated using gmx_MMPBSA⁷ (van der Waals, charge, polar/non-polar solvation energies).

2.15. Statistical Analysis

Graphical representations were generated using GraphPad Prism v9.0 (GraphPad Software, San Diego, CA, USA). Normality distribution was verified through Shapiro–Wilk testing, with variance homogeneity assessed via Brown–Forsythe test for multi-group comparisons. Parametric datasets underwent one-way ANOVA with Tukey’s post hoc analysis, while non-parametric distributions were evaluated using the Kruskal–Wallis test with Dunn’s correction. Comparisons between two datasets were conducted via unpaired t-test or Welch’s correction for parametric data, whereas the Mann–Whitney U test was employed for non-normally distributed data. Significance thresholds were designated as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns = non-significant ($p \geq 0.05$). Results are presented as the mean $\pm$ standard deviation (SD). Pearson correlation analysis was performed to assess the correlations between TMA/TMAO levels and other measured parameters. For gut microbiota profiling, raw sequencing data were processed through Metware Cloud (Metware Biotech Co., Ltd., Wuhan, China). Operational taxonomic units (OTUs) were clustered at 97% sequence similarity using USEARCH v11.

3. Results

3.1. High-Fat/Choline Diet Increases TMA/TMAO Level via the Choline–CutC/D–FMO3 Pathway

To identify factors driving diet-induced TMA/TMAO elevation, dynamic monitoring was conducted. From Figure S1, it can be seen that in the third week, there was no significant difference in TMAO content between the HF group and the control diet group ($p > 0.05$), but the TMAO content in the HF + C group was already significantly higher than that in the HF group ($p < 0.05$). By the fourth week, the TMAO content in the HF group began to be significantly higher than that in the control diet group ($p < 0.05$), and the TMAO content in the HF + C group remained significantly higher than that in the HF group ($p < 0.05$). Quantifying the levels of TMA/TMAO in colonic contents, feces, and serum at week 9 (Figure 1B–G), the HF and HF + C groups had significantly higher TMA and TMAO levels than the CON and CON + C groups across all sample types ($p < 0.01$), with the HF + C group showing even higher levels than the HF group ($p < 0.05$). By contrast, TMA/TMAO levels did not differ significantly between CON and CON + C groups ($p > 0.05$). The 2×2 two-way ANOVA (Table 1) revealed a significant interaction between fat and choline ($p < 0.0001$), identifying their combination as the primary driver of TMA/TMAO elevation. Effect size analysis (η^2) showed that this interaction was most pronounced in the gut, with $\eta^2 > 90\%$ in colonic contents and feces, indicating the gut is

the primary site of TMA/TMAO induction under high-fat/choline diets in mice. Dynamic monitoring of TMAO levels demonstrated that the HF + C group exhibited an earlier onset and greater magnitude of TMAO elevation, while two-way ANOVA confirmed the significant synergistic effect between fat and choline. This combined action enhanced the efficiency of TMAO production, leading to more pronounced increases in the HF + C group relative to the HF group throughout the experimental period.

Table 1. 2 × 2 interaction analysis of TMA and TMAO contents.

	Fat			Choline			Fat * Choline		
	F (DFn, DFd)	η^2 (%)	<i>p</i>	F (DFn, DFd)	η^2 (%)	<i>p</i>	F (DFn, DFd)	η^2 (%)	<i>p</i>
TMA in colonic contents	F (1, 20) = 2112	99.10	0.001	F (1, 20) = 214.4	91.5	0.0001	F (1, 20) = 224.1	91.8	0.001
TMAO in colonic contents	F (1, 20) = 4244	99.53	0.001	F (1, 20) = 504.1	96.19	0.0001	F (1, 20) = 472.2	95.93	0.001
TMA in faeces	F (1, 20) = 5623	96.64	0.001	F (1, 20) = 207.2	91.19	0.0001	F (1, 20) = 162.1	89.01	0.001
TMAO in faeces	F (1, 20) = 5085	99.61	0.001	F (1, 20) = 1148	98.29	0.0001	F (1, 20) = 1134	98.26	0.001
TMA in serum	F (1, 10) = 162.6	94.21	0.001	F (1, 10) = 15.42	60.67	0.0028	F (1, 10) = 13.24	56.98	0.0045
TMAO in serum	F (1, 20) = 936.2	97.91	0.001	F (1, 20) = 36.80	64.79	0.0001	F (1, 20) = 41.16	67.30	0.001

The canonical TMA/TMAO production pathway involves choline metabolism via the CutC/D-FMO3 axis. We thus examined key components of this pathway (Figure 1H–N and Figure S2). CutC/D enzymatic activity and gene expression were significantly higher in the HF and HF + C groups than in the CON and CON + C groups ($p < 0.05$). Consistent with TMAO levels in colonic contents and feces, FMO3 activity was also significantly elevated in these samples from the HF and HF + C groups ($p < 0.05$), mirroring trends in liver tissues. These findings confirm that a high-fat/high-choline diet promotes TMA/TMAO production through the choline-CutC/D-FMO3 pathway.

3.2. High-Fat/Choline Diet Induces Weight Gain and Lipid Metabolism Disorders

To assess the impact of a high-fat/choline diet on body weight and lipid metabolism, key parameters were analyzed. By week 9, mice in the HF + C group had significantly higher body weight than those in the HF group ($p < 0.05$; Figure 2A), with no significant differences in food intake across groups ($p > 0.05$; Figure S3). For serum lipids (Figure 2B–E), TG and TC levels were significantly higher in the HF + C group than in the HF group, whereas LDL and HDL levels showed non-significant numerical increases in the HF + C group ($p > 0.05$). H&E staining of abdominal adipocytes (Figure 2F) showed significantly larger adipocyte diameters in the HF + C group compared to the HF group ($p < 0.05$). At both transcriptional and translational levels (Figure 2G,H), lipid synthesis-related genes/proteins (LPL, PPAR γ) were significantly upregulated in the HF + C group relative to the HF group ($p < 0.05$), while the lipolysis-associated gene/protein PPAR α was significantly downregulated ($p < 0.05$). Collectively, these findings indicate that a high-fat/choline diet elicits greater alterations in body weight, adipocyte size, and serum lipid profiles than a high-fat diet alone.

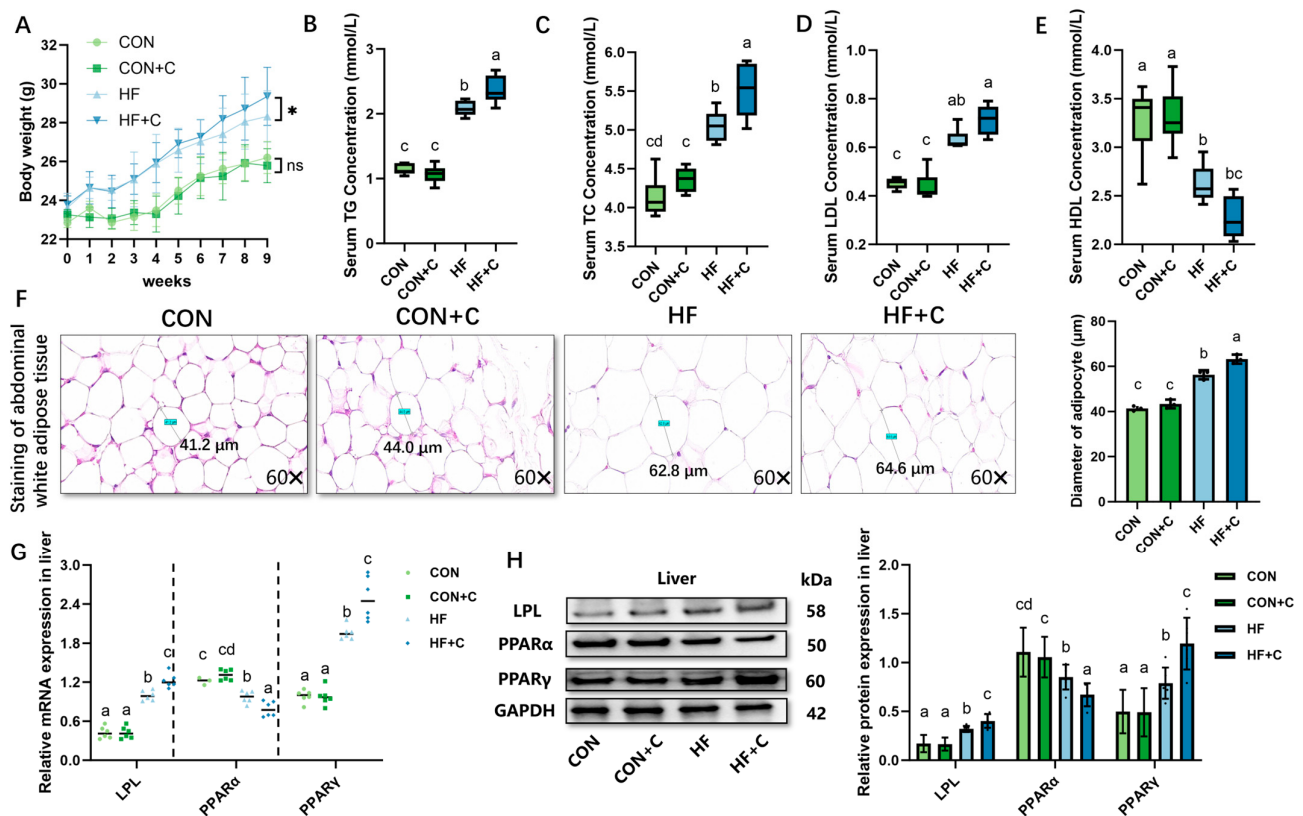


Figure 2. High-fat/choline diet induces weight gain and lipid metabolism disorders. (A) Average body weight ($n = 12$ over 9 weeks); levels of (B) TG, (C) TC, (D) LDL, and (E) HDL in serum over 9 weeks ($n = 6$); (F) H&E staining of abdominal white adipocytes and analysis of cell diameter ($n = 3$); (G) mRNA expression levels of *LPL*, *PPAR α* , and *PPAR γ* in the liver over 9 weeks ($n = 6$); (H) protein expression levels of *LPL*, *PPAR α* , and *PPAR γ* in the liver over 9 weeks ($n = 6$). Light green indicates the CON group, dark green represents the CON+C group, light blue denotes the HF group, and dark blue corresponds to the HF + C group. All data are presented as mean $\pm$ SD. One-way ANOVA followed by Tukey's range test was used, where ns denotes no significance and * indicates $p \leq 0.05$. a–d indicate results of one-way ANOVA among groups.

3.3. High-Fat/Choline Diet Induces Liver Injury and Inflammatory Response

Liver pathological sections (Figure 3A) showed intact hepatocyte structure with no obvious lesions in the CON and CON + C groups. In contrast, the HF and HF + C groups exhibited significant lipid vacuole accumulation (blue arrows). Serum liver injury markers (ALT, AST) were significantly higher in the HF + C group than in the HF group ($p < 0.05$; Figure 3B,C). Inflammatory factors (TNF- α , IL-1 β , IL-6) were also significantly upregulated in the HF + C group ($p < 0.05$; Figure 3G–I), indicating exacerbated inflammation. Antioxidant capacity assessment showed significantly lower SOD activity in the HF + C group compared to the HF group ($p < 0.05$; Figure 3E). These results demonstrate that a high-fat/choline diet exacerbates liver injury, enhances inflammatory responses, and impairs antioxidant defense function relative to a high-fat diet alone.

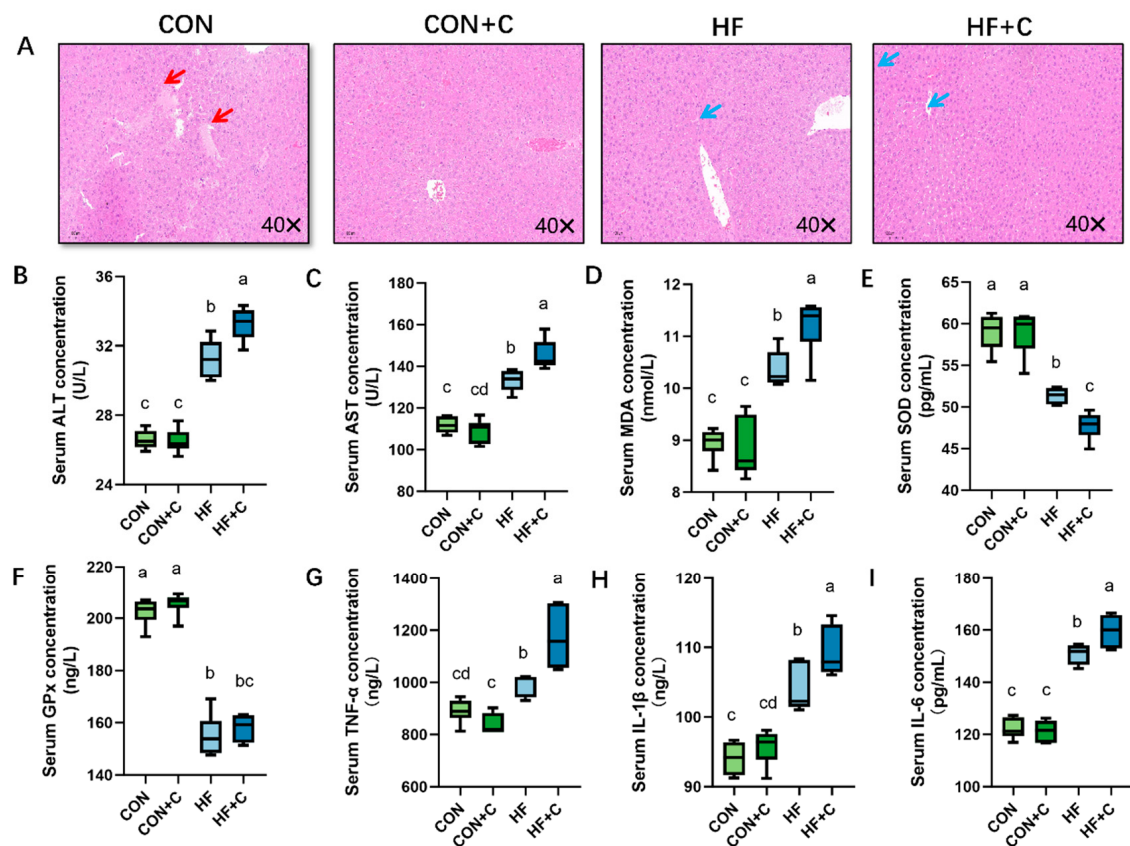


Figure 3. High-fat/choline diet induces liver injury and inflammatory response. (A) H&E staining of hepatocytes, the red arrows indicate the sites of inflammatory foci, while the blue arrows point to the locations of fat vacuoles; levels of (B) ALT, (C) AST, (D) MDA, (E) SOD, (F) GPx, (G) TNF- α , (H) IL-1 β , (I) IL-6 in serum over 9 weeks ($n = 6$). Light green indicates the CON group, dark green represents the CON+C group, light blue denotes the HF group, and dark blue corresponds to the HF + C group. All data are presented as mean $\pm$ SD. One-way ANOVA followed by Tukey's range test was used. a–d indicate results of one-way ANOVA among groups.

3.4. High-Fat/Choline Diet Induces Disruption of Bile Acid Metabolism

TBA levels in systemic circulation and related tissues were evaluated. Compared to the HF group, the HF + C group had significantly higher TBA in serum and liver ($p < 0.05$; Figure 4A,B) but significantly lower TBA in colonic contents ($p < 0.05$; Figure 4C,D). For bile acid regulatory receptors, mRNA and protein levels of the nuclear receptor FXR and membrane receptor Tgr5 in liver and small intestine were significantly lower in the HF + C group than in the HF group ($p < 0.05$; Figure 4E–H). Additionally, mRNA and protein levels of key bile acid synthesis enzymes (CYP7A1, CYP27A1) were significantly higher in the HF + C group ($p < 0.05$). These findings indicate that a high-fat/choline diet exacerbates bile acid metabolic disorders compared to a high-fat diet.

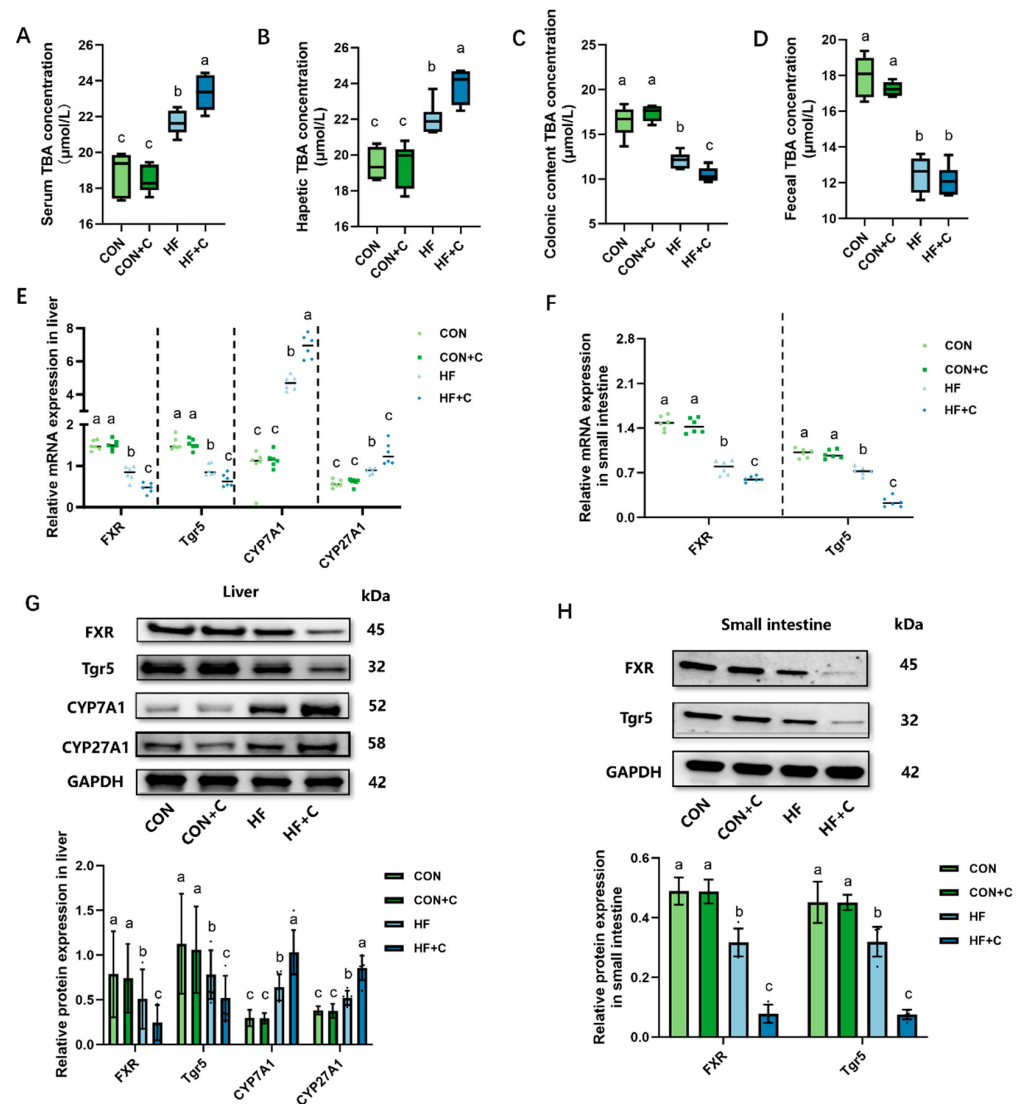


Figure 4. High-fat/choline diet disrupts bile acid metabolism. Level of TBA in (A) serum, (B) hepatic, (C) colonic contents and (D) feces for 9 weeks ($n = 6$); (E) *FXR*, *Tgr5*, *CYP7A1* and *CYP27A1* mRNA expression levels in hepatic for 9 weeks ($n = 6$); (F) *FXR* and *Tgr5* mRNA expression in small intestines for 9 weeks ($n = 6$); (G) *FXR*, *Tgr5*, *CYP7A1* and *CYP27A1* protein expression levels in hepatic for 9 weeks ($n = 6$); (H) *FXR* and *Tgr5* protein expression in small intestines for 9 weeks ($n = 6$). Light green indicates the CON group, dark green represents the CON+C group, light blue denotes the HF group, and dark blue corresponds to the HF + C group. All data are presented as mean $\pm$ SD. One-way ANOVA followed by Tukey's range test was used. a–c indicate results of one-way ANOVA among groups.

3.5. High-Fat/Choline Diet Impairs Intestinal Barrier Function and Alters Gut Microbiota Composition

Serum LPS (a major component of Gram-negative bacterial outer membranes) was significantly higher in the HF + C group than in the HF group ($p < 0.05$; Figure 5A), indicating exacerbated LPS leakage due to colonic barrier impairment. Analysis of tight junction proteins in colonic tissues showed significantly lower mRNA and protein levels of *Cldn1*, *Ocln*, and *ZO-1* in the HF + C group compared to the HF group ($p < 0.05$; Figure 5B,C), confirming more severe disruption of intestinal epithelial barrier integrity in the HF + C group.

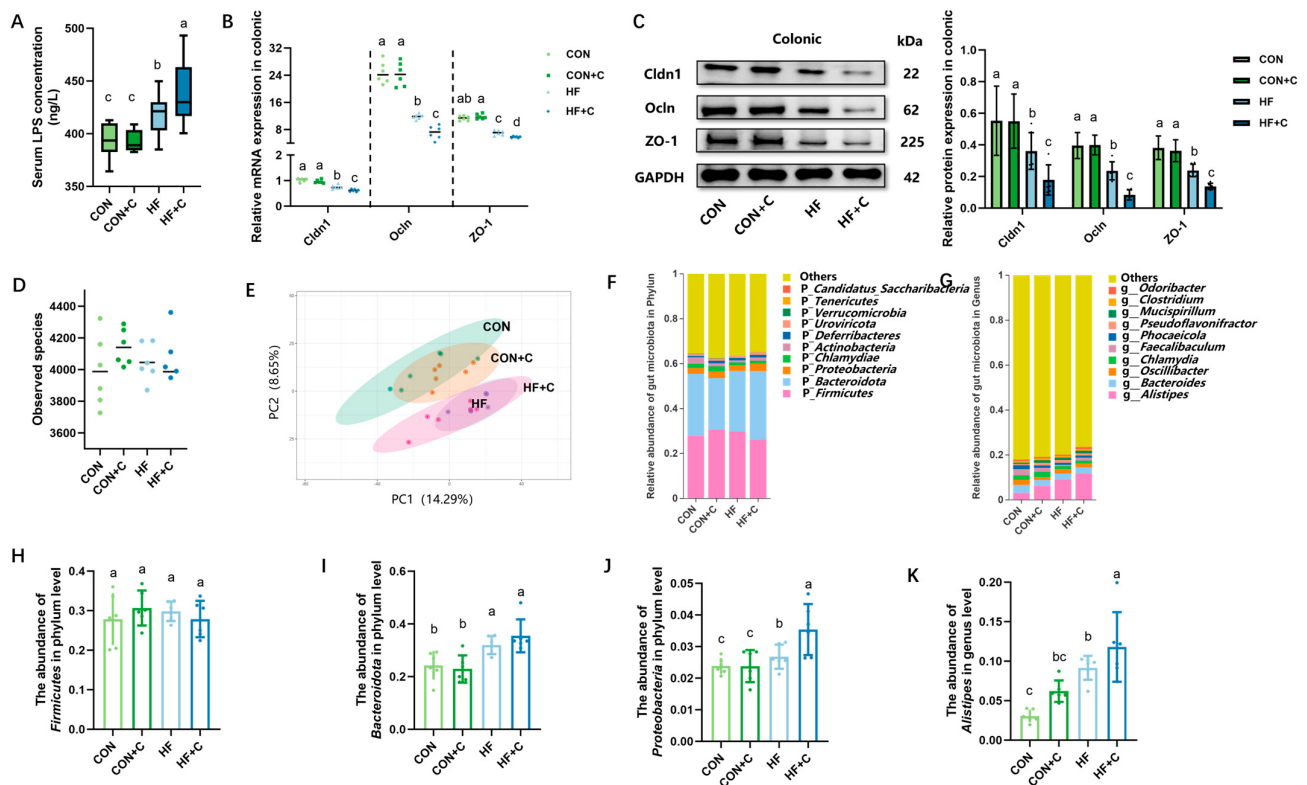


Figure 5. High-fat/choline diet impairs intestinal barrier function and alters gut microbiota composition. (A) Level of LPS in serum over 9 weeks ($n = 6$); (B) *Cldn1*, *Ocln* and *ZO-1* mRNA expression levels in colonic for 9 weeks ($n = 6$); (C) *Cldn1*, *Ocln* and *ZO-1* protein expression levels in colonic for 9 weeks ($n = 6$); (D) observed species in colonic contents; (E) PCA plot of gut microbiota; (F) stacked bar plot of species at the phylum level; (G) stacked bar plot of species at the genus level; the abundance of (H) *Firmicutes*; (I) *Bacteroidota*; (J) *Proteobacteria*; (K) *Alistipes*. Light green indicates the CON group, dark green represents the CON+C group, light blue denotes the HF group, and dark blue corresponds to the HF + C group. All data are presented as mean $\pm$ SD. One-way ANOVA followed by Tukey's range test was used. a–d indicate results of one-way ANOVA among groups.

Additionally, as shown in Figure 5D,E, there was no significant difference in the number of observed species among the four groups ($p > 0.05$), but distinct differences were observed in species composition—specifically, the CON and CON + C groups shared similar microbial species, as did the HF and HF + C groups. Figure 5D,E illustrate the differences in species composition at the phylum and genus levels. At the phylum level, the intestinal microbiota of all four groups were predominantly composed of *Firmicutes* and *Bacteroidota*. At the genus level, the abundance of *Alistipes* (a genus of Gram-negative bacteria) was significantly increased in the HF and HF + C groups ($p < 0.05$, Figure 5K), which also explains the significant elevation of LPS.

3.6. Elevated TMA/TMAO Induced by High-Fat/Choline Diet Exacerbates Metabolic Disorders and Associated with Intestinal Microbiota

TMA/TMAO levels were significantly higher in the HF + C group than in the HF group ($p < 0.05$), alongside exacerbated weight gain, lipid dysfunction, liver injury, inflammation, bile acid disorders, and intestinal barrier impairment. Correlation analysis (Figure S4) showed that TMA/TMAO levels were significantly positively correlated with body weight, adipocyte diameter, TG, TC, LDL, ALT, AST, serum/hepatic TBA, LPS, MDA, TNF- α , IL-1 β , IL-6, LPL, PPAR γ , CYP7A1, and CYP27A1. Conversely, TMA/TMAO levels were significantly negatively correlated with HDL, colonic/fecal TBA, SOD, GPx, PPAR α , liver/small intestine FXR and TGR5, OCLN, CLDN1, and ZO-1. Mantel tests confirmed sig-

nificant associations between TMA/TMAO and these indices ($p < 0.01$, $r \geq 0.4$), indicating strong links between elevated TMA/TMAO and exacerbated metabolic disorders.

Correlation analysis (Figure S5) explored associations between TMA levels and intestinal microbiota. No significant correlation was found with *Firmicutes* ($r^2 = 0.053$, $p > 0.05$), but significant correlations were observed with *Bacteroidetes* and *Proteobacteria* (strongest with *Proteobacteria*, $r^2 = 0.65$), highlighting the need to identify specific *Proteobacteria* taxa involved.

3.7. Metagenomic Identification of Bacteria Associated with TMA Elevation Under High-Fat/Choline Diet

Using the NCBI RefSeq database, 2916 CutC-related and 1636 CutD-related sequences were retrieved with keywords “choline trimethylamine-lyase” and “choline TMA-lyase-activating enzyme,” respectively. A local database of CutC/D-expressing microbes was constructed, and BLASTP alignment (E-value $\leq 1 \times 10^{-5}$, sequence identity $\geq 45\%$) against metagenomic data identified 1084 potential CutC/D-encoding species, distributed across 631 bacterial species from 113 genera (Table S5). CutC was present in 585 species (7 *Actinomycetota*, 53 *Bacillota*, 34 *Proteobacteria*, 2 *Fusobacteriota*, 17 *Thermodesulfobacteriota* genera), and CutD in 499 species (7 *Actinomycetota*, 50 *Bacillota*, 29 *Proteobacteria*, 2 *Fusobacteriota*, 17 *Thermodesulfobacteriota* genera), showing high overlap in distribution. *Bacillota* accounted for 50% of both, including common gut genera like *Clostridium* and *Enterococcus*, suggesting a key role in choline-dependent TMA production. Multiple *Proteobacteria* genera were also involved, indicating diverse TMA-synthesizing potential with likely synergistic effects across phyla. ANOVA analysis of CutC/D-encoding microbes (Table S6) showed that 65 CutC-expressing and 30 CutD-expressing strains were significantly enriched in the HF and HF + C groups compared to the CON group. Most belonged to *Bacillota*, followed by *Proteobacteria*, including *Lacrimispora aerotolerans*, *Clostridium scatologenes*, and *Serratia marcescens*. Their increased abundance indicates high-fat/choline diets specifically induce proliferation of CutC/D-carrying microbes, enhancing gut TMA production potential. These changes were diet-dependent, reflecting adaptive responses of the gut microbiota to specific nutritional conditions, likely linked to efficient choline utilization. A Venn diagram (Figure 6A) identified nine strains expressing CutC/D: *Lactococcus lactis*, *Clostridium aminobutyricum*, uncultured *Clostridium* sp., *Desulfosporosinus* sp. FKB, *Desulfosporosinus* sp. HMP52, *Anaerovorax odorimutans*, *Cetobacterium* sp. ZOR0034, *Desulfovibrio* sp. MES5, and *Serratia marcescens*. Their significantly increased abundance under HF and HF + C diets suggests core roles in TMA metabolism. *Serratia marcescens* (*Proteobacteria*) exhibits strong metabolic adaptability, while *Desulfosporosinus* sp. (*Thermodesulfobacteriota*) may act in anaerobic gut niches, warranting further study. Phylogenetic analysis (maximum likelihood) of CutC/D from these nine strains revealed CutC of *Serratia marcescens* clustered with *Desulfosporosinus* sp. HMP52 and *Desulfosporosinus* sp. FKB (91% bootstrap support; Figure 6B), indicating high sequence similarity and potential functional conservation. CutD of *Serratia marcescens* showed close genetic distance to *Lactococcus lactis* (Figure 6C), suggesting protein-level functional similarity. These relationships provide molecular evidence for further validation of TMA-producing capacity in CutC/D-expressing gut strains.

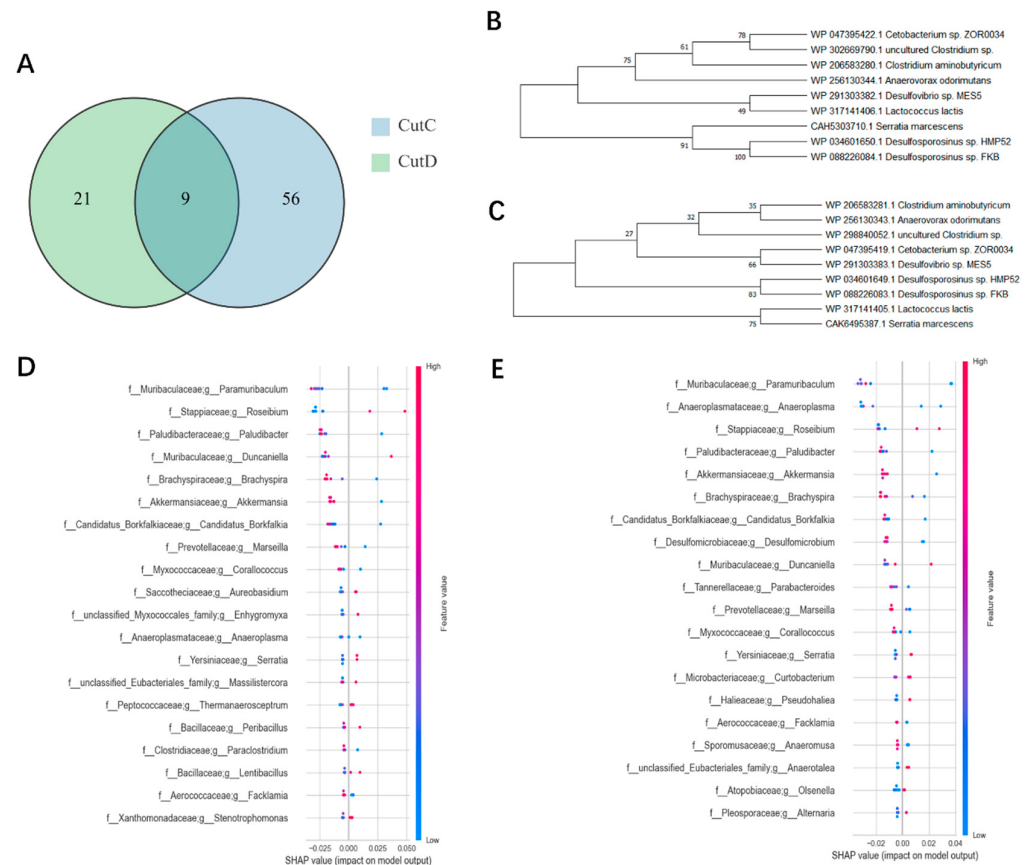


Figure 6. Metagenomics combined with machine learning identifies high-fat/choline diet-induced TMA-producing gut microbiota. (A) Venn diagram analysis of CutC/D-co-occurring species; (B) phylogenetic tree analysis of CutC enzyme in 9 CutC/D-co-occurring species; (C) phylogenetic tree analysis of CutD enzyme in 9 CutC/D-co-occurring species; (D) SHAP analysis for predicting TMA content using gut microbiota based on the random forest model; (E) SHAP analysis for predicting CutC enzyme activity using gut microbiota based on the random forest model.

3.8. Machine Learning Identifies TMA-Producing Bacteria Induced by High-Fat/Choline Diet

Sixty machine learning models were compared to analyze TMA levels and gut microbiota abundance, using R^2 , MAE, and RMSE for evaluation. The random forest regressor ($R^2 = 0.8810$, MAE = 0.0910, RMSE = 0.0989) outperformed others in fitting microbiota abundance to TMA levels, best revealing their underlying relationships (Table S8). Validation on the independent test set confirmed strong predictive performance: The training set R^2 was 0.942, and the test set R^2 was 0.854 (residual plot, Figure S6A). Most residuals clustered near the zero line, indicating small fitting deviations and good generalization. The prediction error plot (Figure S6B) showed a strong linear correlation between actual and predicted values ($R^2 = 0.854$), with data points near the best-fit line. SHAP analysis interpreted random forest regressor results, visualizing microbial influences on TMA levels. Genera with large positive SHAP values and high feature abundances—including *f__Stappiaceae*, *g__Roseibium*, *f__Muribaculaceae*, *g__Duncaniella*, *f__Yersiniaceae*, *g__Serratia*, *f__Bacillaceae*, *g__Peribacillus*, and *g__Lentibacillus*—were associated with elevated TMA (Figure 6D). Conversely, genera like *f__Muribaculaceae*, *g__Paramuribaculum*, and *g__Akkermansia* had negative SHAP values, indicating their increased abundance was linked to lower TMA levels. A separate model predicting CutC activity identified the random forest regressor as optimal (training set $R^2 = 0.958$, test set $R^2 = 0.856$; Table S9). Residuals clustered near zero (Figure S6C), and actual vs. predicted values showed strong correlation ($R^2 = 0.856$; Figure S6D). SHAP analysis (Figure 6E) highlighted key genera influencing CutC enzyme activity: *Roseibium*,

with choline chloride under anaerobic conditions for different durations; (C) multiple sequence alignment of CutC enzyme; (D) TOTAL energy trajectory plot of the CutC-Choline complex over the entire 100 ns simulation time; (E) extraction and superposition of the complex trajectory during the last 10 ns (the small molecule is shown in orange, and the A and B chains of CutC are shown in light blue and light yellow, respectively); (F) 2D interaction diagram of CutC and choline.

4. Discussion

This study employed a 2×2 in vivo factorial experiment combined with interaction effect analysis to elucidate the synergistic induction of TMA/TMAO production by high-fat and high-choline diets. Results showed that the combined high-fat and high-choline diet significantly upregulated TMA/TMAO levels in mice via the choline-CutC/D-FMO3 metabolic axis, with this elevation exhibiting a strong association with exacerbated lipid metabolism disorders, amplified inflammatory responses, abnormal bile acid metabolism, and colonic barrier dysfunction. Further integrated metagenomics and machine learning analyses precisely identified *Serratia marcescens* as the key functional strain driving TMA production under high-fat/choline conditions. In vitro pure culture experiments verified its 24 h choline-to-TMA conversion rate as $60 \pm 2.49\%$. Gene amplification, multiple sequence alignment, and molecular simulation experiments confirmed that the CutC enzyme of *Serratia marcescens* possesses direct catalytic activity for choline-to-TMA conversion.

This study demonstrates that a high-fat diet alone can induce a significant increase in in vivo TMA/TMAO levels. Extensive research has established that high-fat diets elevate TMAO levels and promote obesity-related metabolic diseases [21–24]. More importantly, using a two-factor model, we first showed that when high-fat and high-choline diets coexist, TMA/TMAO levels are significantly higher than those induced by a high-fat diet alone, indicating a synergistic effect in TMA/TMAO production. High choline provides sufficient substrates for the choline-CutC/D-FMO3 pathway of TMA/TMAO biosynthesis, while high-fat diets may enhance metabolic efficiency by regulating gut microbiota function or the intestinal microenvironment, collectively facilitating TMA/TMAO accumulation. Meanwhile, the study results showed that TMA/TMAO levels exhibited no significant changes after adding high choline to a normal diet, which suggests that a high-fat diet is the main factor in TMA/TMAO production. Phosphatidylcholine, the most abundant dietary source of choline in humans, is present in nutritious foods such as eggs, milk, liver, red meat, poultry, shellfish, and fish [25], with unavoidable daily intake. One study identified TMAO, a metabolite of dietary phosphatidylcholine, as a biomarker for increased metabolic disease risk and defined the metabolic pathway as phosphatidylcholine—choline—TMA [26]. Additionally, multiple studies have observed significant TMAO elevation in combined high-fat and high-choline diet models [21,27–29]. Another study showed that adding 0.5% carnitine (a TMA precursor, though not choline) to a high-fat diet resulted in significantly higher serum TMAO levels in mice than a high-fat diet alone [30], providing indirect evidence that sufficient substrates promote TMAO production. As our results show, the TMA content in the colonic contents in our study was significantly higher than the TMAO content, whereas in that study, the TMAO content was significantly higher than the TMA content. This is because that study used a different dietary formulation, with carnitine as the substrate. It has been reported that carnitine is metabolized by carnitine oxygenase (CntA) and carnitine reductase (CntB), which may differ in conversion efficiency compared to the choline–TMA lyase (CutC/D) pathway that is central to our model. These differences in dietary composition, substrate type, and underlying metabolic pathways could all contribute to the variations in the absolute concentrations reported. Additionally, the 60% high-fat diet used in our study may substantially alter gut microbiota composition and metabolic capacity. As our results indicate, TMA levels were notably elevated in

colonic contents. It is plausible that under this specific dietary condition, the rate of TMA production could transiently exceed the liver's capacity for oxidation, leading to an accumulation of TMA in the bloodstream. This aligns with our observation of concurrently high levels of both TMA and TMAO in the HF and HF + C groups, suggesting that ample substrate (choline) coupled with enhanced microbial conversion capacity led to substantial TMA generation, partially overwhelming its immediate hepatic clearance.

We found that high-fat/choline diet-induced metabolic disorders were exacerbated and positively correlated with TMA/TMAO levels. Mechanistically, the pleiotropic molecular and physiological effects of TMAO likely serve as a key mediator of the exacerbated metabolic burden under high-fat/choline diets. Regarding inflammatory responses, mice in the high-fat/high-choline group exhibited the most severe inflammation, which correlated positively with TMAO levels. Studies have demonstrated that TMAO promotes reactive oxygen species (ROS) production, activates the NLRP3 inflammasome in endothelial cells, and increases proinflammatory cytokine IL-1 β production [31]. TMAO also activates mitogen-activated protein kinase (MAPK) [32] and NF- κ B pathways [33], upregulating inflammatory genes such as *IL-6* and *TNF- α* . In terms of lipid metabolism, our results align with clinical findings: a clinical study reported significant associations between TMAO and blood lipids, specifically positive correlation with TG ($r = 0.303$) and negative correlation with HDL-c ($r = -0.405$, both $p < 0.05$) [34]. Consistent with this, mice in the high-fat/high-choline group developed obvious dyslipidemia and lipid metabolism disorders following significant TMAO elevation. Specifically, the lipolysis-related genes/proteins LPL and PPAR α were significantly downregulated at both transcriptional and translational levels, while the lipogenesis-related PPAR γ was significantly upregulated, suggesting TMAO may exacerbate lipid accumulation by regulating key transcription factors in lipid metabolism. Additionally, TMAO can inhibit bile acid synthesis and transport by downregulating key synthetic enzymes such as CYP7A1 and CYP27A1 [35]. Reduced CYP7A1 and CYP27A1 expression not only inhibits bile acid synthesis but also disrupts reverse cholesterol transport in the bloodstream, leading to intracellular cholesterol accumulation and bile acid metabolic disorders [36]. A case-control study also indicated that TMAO aggravates hepatic steatosis by suppressing BA-mediated hepatic FXR signaling [37]. Notably, this study found that under high-fat/high-choline diets, elevated TNF- α may inhibit CYP7A1 expression by activating the MAPK pathway [38], with this process synergistically regulated by gut-liver axis FXR and Tgr5 receptors, suggesting that TMAO may exacerbate metabolic abnormalities through an "inflammation-bile acid metabolism" axis. Furthermore, significant increases in body weight and abdominal white adipocyte diameter in the high-fat/high-choline group further support that TMA/TMAO may act as key effectors mediating high-fat diet-induced metabolic dysfunction.

From the perspective of interaction effects and TMA/TMAO biosynthetic pathways, the intestine is the primary site of TMA production, and high-fat diets are the core driver of gut microbiota structural remodeling. This study found that although high-fat and high-fat/choline diets did not significantly alter microbial relative abundance, they markedly affected overall community composition, with the gut microbiota structures shaped by these diets showing high similarity. Further analysis revealed that under high-fat diet induction, species expressing CutC/D enzymes were predominantly enriched in *Firmicutes* and *Proteobacteria*. Western high-fat dietary patterns have been shown to adversely alter gut microbiota and influence TMA/TMAO levels [24], particularly by increasing *Firmicutes* and *Proteobacteria* [39], as evidenced by the expansion of facultative anaerobes and opportunistic pathogens [40]. High-fat diets impair mitochondrial oxygen uptake in host intestinal cells and increase mucus nitrate levels, compromising healthy anaerobic intestinal function and enabling facultative anaerobes (e.g., pathogenic *Escherichia coli*) to dominate [21];

notably, *Escherichia coli* can convert choline to TMA. Our integrated metagenomics and machine learning identified high-fat/high-choline-induced *Serratia marcescens* as a facultative anaerobe belonging to *Enterobacteriaceae*. Sequence alignment analysis showed that the CutC/D nucleotide and protein sequences of *Serratia marcescens* shared high similarity with homologous sequences from known TMA-producing bacteria [20,41], with key amino acid residues being highly conserved, further confirming functional conservation in TMA production. Additionally, molecular simulation revealed its strong affinity for choline. Choline trimethylamine lyase belongs to the glycyl radical enzyme (GRE) family, which uniquely converts choline to TMA via C-N bond cleavage [42]. Experimental evidence highlights the critical role of CutC: TMA production was completely abolished when the *cutC* gene in *Desulfovibrio desulfuricans* was disrupted [43], directly demonstrating that CutC is indispensable for choline cleavage and TMA production. The CutC enzyme of *Desulfovibrio* and the homologous CutC protein of *Serratia marcescens* have high sequence homology, reflecting the functional conservation of this enzyme across species.

Collectively, this study is the first to demonstrate in a two-factor model that high-fat diets may promote TMA absorption and conversion by increasing the abundance of gut microbes expressing *CutC/D* genes and enhancing intestinal permeability, while high choline provides sufficient substrates for this metabolic pathway. These two factors exacerbate metabolic dysfunction through a “substrate–microbial function” synergism. This study clarified the independent and interactive effects of high fat and choline on metabolic disorders and microbiota regulation. Through integrated metagenomic and machine learning analyses, it precisely identified the functional TMA-producing genus *Serratia* under specific dietary conditions, with its conversion capacity validated by in vitro experiments.

Naturally, this study has limitations: In vitro metabolic assays using a single bacterial strain cannot fully recapitulate interaction effects in the complex intestinal microecosystem. Future studies could further combine germ-free mouse colonization experiments and clinical sample validation to clarify the pathological significance of *Serratia* in human metabolic diseases and explore intervention strategies targeting this genus or the TMA/TMAO metabolic axis.

5. Conclusions

In summary, this study first elucidated that the production of TMA/TMAO is co-regulated by high-fat diet and high choline via a 2×2 animal experiment. Compared with a high-fat diet alone, the content of TMA/TMAO significantly increases under the co-induction of a high-fat and high-choline diet. However, under a normal diet, the addition of choline does not increase TMA/TMAO levels. This indicates that high-fat diet is the primary factor for TMA/TMAO production, and the presence of choline enhances the conversion capacity of TMA/TMAO. A significant increase in TMA/TMAO further exacerbates metabolic disorders. Combining metagenomics with machine learning algorithms, we first identified the genus *Serratia* as a TMA-producing genus. In vitro experiments further confirmed the TMA-producing capacity and function of *Serratia marcescens*. Given the close association between high-fat diet, TMA/TMAO, and metabolic diseases, targeting *Serratia marcescens* may serve as a novel intervention strategy for obesity-related metabolic diseases induced by high-fat diets. This study also provides detailed experimental evidence for the potential regulatory mechanisms of the diet–microbiota–metabolite–metabolic disease axis.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/nu18040658/s1>. Table S1: Compositions of test diets; Table S2: Primer sequences for qRT-PCR; Table S3: qPCR cycling program; Table S4: Specific primer sequences for *CutC/D* genes of *Serratia marcescens*; Table S5: BLASTp comparison of metagenomic sequencing-identified microbial species expressing CutC/D enzymes with the NCBI database;

Table S6: Multiple comparison analysis of the relative abundance of CutC enzyme-expressing microorganisms; Table S7: Multiple comparison analysis of the relative abundance of CutD enzyme-expressing microorganisms; Table S8: The optimal parameters of the top 8 models among 60 prediction models for predicting TMA content based on the relative abundance of gut microbiota; Table S9: The optimal parameters of the top 7 models among 60 prediction models for predicting CutC enzyme activity based on the relative abundance of gut microbiota; Table S10: CutC amino acid sequence from CutC abundant strains; Figure S1 Changes in TMAO content in mouse feces under different dietary interventions and time periods. (A) Results of one - way ANOVA between groups in the same time period; (B) results of one - way ANOVA within the same group across different time periods. Figure S2: Electrophoresis image of qPCR products of *cutC* gene in colonic contents. (A) *16s* gene ($n = 6$); (B) *CutC* gene ($n = 6$); Figure S3: Average daily feed intake (over a 9-week period, $n = 12$); Figure S4: Correlation analysis of TMA and TMAO contents with phenotypic indices; Figure S5: Correlation analysis of TMA and TMAO contents in colonic content with microbial community abundance; Figure S6: Residual plots and prediction error plots based on the output of the random forest model. (A) Residual plot for predicting TMA content via relative abundance of gut microbiota; (B) prediction error plot for predicting TMA content via relative abundance of gut microbiota; (C) residual plot for predicting CutC enzyme activity via relative abundance of gut microbiota; (D) prediction error plot for predicting CutC enzyme activity via relative abundance of gut microbiota; Figure S7: Relative abundance of *Serratia fonticola* and *Serratia marcescens* ($n = 6$); Figure S8: Electrophoretogram of amplified products of *CutC/D* genes from *Serratia marcescens*. (A) Marker; (B) amplified band of *CutC* gene (925 bp); (C) amplified band of *CutD* gene (1122 bp).

Author Contributions: Z.W.: investigation, data curation, software, formal analysis, visualization, writing—original draft; J.W.: investigation, data curation, software, visualization; Z.H.: investigation, validation, data curation; X.L.: methodology, validation, data curation; S.L.: methodology; Z.F.: conceptualization, methodology; L.H.: methodology, resources; R.L.: methodology; L.T.: resources; C.L.: resources, supervision, funding acquisition; H.C.: resources, supervision, funding acquisition, project administration. All authors have read and agreed to the published version of the manuscript.

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