Methods

Animal rearing and sample collection

Healthy blunt snout bream specimens (n=300, weight=5.47±0.09g, age=four-month-old) were obtained from a fish-breeding base of the Huazhong Agricultural University, Wuhan. The fish were fed the control diet for 14 days to acclimate them to the laboratory conditions before the onset of the feeding trial in the Key Lab of Freshwater Animal Breeding of Huazhong Agricultural University. The fish were divided into 12 tanks, each containing 25 specimens: 4 diet groups × 3 replicates × 25 specimens = 300 (**Table S1**). The fish were fed twice daily at 2.0-3.0% of their body weight, regularly adjusted according to the amount of uneaten feed. The amount of feed consumed and fecal matter excreted by the fish in each tank were recorded twice daily. Water temperature, oxygen content, nitrites and total ammonia nitrogen (TAN = NH_3-N + NH_4^+-N) in the tanks were controlled: 24±2°C, >8 mg O_2/L, <0.1 mg NO_2^- /L, <0.1 mg TAN/L, respectively. Health status of fish was monitored daily, and apparently unhealthy fish were removed from the tanks and inspected (**Table S1**). Among the surviving fish, 15 apparently healthy specimens were selected randomly from each tank to obtain the blood, liver and gut microbiota samples. Blood was obtained from the caudal vein using sterile syringes with pre-added anticoagulant solution and then centrifuged (3000×g) at 4°C for 10 minutes to obtain the plasma, which was quickly frozen in liquid nitrogen and stored at -80°C for biochemical assays. After euthanising the fish by a trauma to the head, and subsequent dissection, livers and intestines were collected. Each liver was weighed and divided into two parts: one was placed in formalin for histological examination, and the remainder was quickly frozen in liquid nitrogen and stored at -80°C for quantitative real-time PCR (qPCR). Intestinal contents were collected into a tube filled with lumen wash solution, then gently agitated at 4°C for 10 minutes, followed by centrifugation (12000×g) for 5 minutes, and finally the supernatant was stored at -80°C for microbial DNA extraction.

Targeted mass spectrometry analysis of metabolites related to TMAO

This targeted MS analyses were performed by the Beijing Mass Spectrometry Medical Research Co., Ltd. (Beijing, China). The solvent was delivered to the column at a flow rate of 1 ml min^{-1}. Nitrogen gas was used as the collision gas in the multiple reaction monitoring mode. Calibration solution was used for calibration of the system and quantification of metabolites. The data were processed using Analyst software version 1.5.1

(Applied Biosystems).

T-RFLP (terminal restriction fragment length polymorphism) analysis

The 6-carboxyfluorescein (6-FAM) labeled 27f forward primer and non-labeled 1492r reverse primer were used for the amplification of *16S rRNA* fragments[1]. Thermal cycling was performed as follows: denaturation at 95°C for 7 min, followed by 35 cycles of 95°C for 30 s, annealing at 58°C for 30 s, and elongation at 72°C for 90s. PCR products were purified and their concentrations determined for the next step. Digestion reactions (25μl) were carried out with 500ng of purified PCR products and 25U of the *HaeIII* enzyme restriction endonuclease (NEB Biotechnology, Beijing, China) at 37°C for 3 h, followed by denaturation by heating at 80°C for 3 min, according to the manufacturer's instructions. Restriction endonuclease *MspI* enzyme digestion reactions were carried out in the same way. All procedures were performed in darkness. The resultant digestion products were mixed with size-standard solution (Liz 600bp) and analyzed the fluorescently-labeled terminal restriction fragments (T-RF) on the ABI 3730XL DNA analyzer (Applied Biosystems Instruments, Foster City, CA). T-RF data, consisting of T-RF lengths (bp) and T-RF peak heights, were analyzed using the ABI Peak Scanner v.1.0 software[2]. For each digestion product, each T-RF with the relative frequency of the peak height lesser than 1% of the total peak height, was excluded from subsequent analyses[3]. MiCA web tool[4] was used to determine the microbial community composition by assigning T-RFs from our samples to microbial taxa via T-RF length comparison with the *16S rRNA* sequence database[1]. On the basis of T-RF lengths and peak heights[2, 5], we evaluated changes in the microbial community composition using the following indices: Shannon-Wiener (H′), Simpson (D′) and Shannon evenness (E′). PCA analysis was adopted to infer the similarities and differences in the T-RF composition (relying on lengths and peak heights) and assess whether different dietary treatments could be discriminated by their T-RF composition[6]. Similarly, cluster analysis of T-RF composition was done to identify similar communities, and the numbers of clusters (types of communities) were independently assessed using statistical algorithms. T-RF was grouped into subclusters using Ward's method and Squared Euclidean Distances in dendrograms[2]. Hierarchically clustered heatmap analysis based on the T-RF profiles was conducted to corroborate results of these analyses[7].

qPCR analysis

We used genus-specific primers for *16S rRNA* of *Lactobacillus* sp. and *Clostridium* sp., and primers for *cutc/cnta/grdh/tora* to conduct a qPCR assay (SYBR Premix Ex Taq™). The qPCR assay was performed using SYBR Premix Ex Taq™ (TaKaRa, Dalian, China) on a Roche Light Cycler 480 machine (Roche, Sussex, UK). The qPCR conditions were as follows: denaturation at 95°C for 30 s, followed by 45 cycles of 95°C for 5 s, annealing at 58°C for 30 s, and elongation at 72°C for 15 s.

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