Dysbiosis of Gut Microbiota with Increased Trimethylamine N-oxide Level in Patients with Large Artery Atherosclerotic and Cardioembolic Strokes

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Abstract

**Background** — With the establishment of the concept of the gut-brain axis, increasing evidence has shown that the gut microbiome plays an important role in the pathogenesis of cardiovascular diseases. Gut bacteria can transform dietary choline, L-carnitine, and trimethylamine N-oxide (TMAO) into trimethylamine, which can be oxidized into TMAO again in the liver and participate in atherogenesis. However, only few studies have described alterations in the gut microbiota composition and function in cardioembolic (CE) and large artery atherosclerotic (LAA) strokes.

**Methods and Results** — A case-control study was performed on patients with LAA and CE strokes. TMAO was determined via liquid chromatography tandem mass spectrometry. Gut microbiome was profiled through Illumina sequencing of the 16S ribosomal RNA gene (V4-V5 regions). The TMAO levels in the plasma of patients with LAA and CE strokes were significantly increased (TMAO: LAA stroke, 2931±456.4 ng/mL vs. CE stroke, 4220±577.6 ng/mL vs. control, 1663±117.8 ng/mL; \( P < 0.05 \)). The TMAO level in patients with LAA stroke was positively correlated with the carotid plaque area (\( \rho = 0.333 \), 95% confidence interval = 0.08 to 0.55, and \( P = 0.0093 \)). The composition and function of gut microbiomes in the LAA and CE stroke groups were significantly different from those of the asymptomatic control. In addition to the significantly increased \( \alpha \) and \( \beta \) diversities, the gut microbiome composition and function showed that the LAA group had more microorganisms than the asymptomatic control group; such microorganisms convert dietary source choline, TMAO to TMA. *Parabacteroides* and *Streptococcus* exhibited the strongest association with LAA and CE strokes.

**Conclusions** — This study established the compositional and functional alterations of gut microbiomes in patients with LAA and CE strokes and the relationship between plasma TMAO and gut microbiota. The findings suggest the potential of using gut microbiota as a biomarker for patients with LAA and CE strokes.

**Introduction**

The interest in and evidence on the role of gut microbiota as an environmental factor affecting human health and disease are growing [1]. Dysbiotic gut microbiome has been reported in multiple diseases, such as obesity [2, 3], type 2 diabetes mellitus (T2DM) [4, 5], atherosclerosis [6, 7], and
cardiovascular disease (CVD) [8, 9]. At present, atherosclerosis or adverse CVD is the center of research on the relationship between intestinal microorganisms and cardio- and cerebrovascular diseases. Stroke is associated with high morbidity, mortality, disability, and recurrence; it incurs considerable healthcare costs and imposes a burden on society [10]. In addition, stroke rarely occurs as an entirely separate entity. Trimethylamine N-oxide (TMAO), a gut microbial-dependent metabolite, has emerged as a biomarker for CVD risk and atherothrombotic diseases [11, 12]. Phosphatidylcholine, choline, L-carnitine, TMAO, and other trimethylamine (TMA)-containing nutrients can be transformed to TMA by the following distinct gut microbial enzyme complexes: choline TMA-lyase (CutC) and choline TMA-lyase activating enzyme (CutD) [13], the carnitine Rieske-type oxygenase/reductase system (CntA/B and YeaW/X) [14], and trimethylamine N-oxide reductase (TorA/C/D) [15]. TMA then enters the portal circulation and is further oxidized by host enzymes called flavin-containing monooxygenases (FMO, predominantly FMO3) to produce TMAO. Although several studies have found a correlation among the plasma levels of choline, TMAO, and CVD, not all of these studies showed a consistent association between dietary intake of TMAO and CVD risk. A recent study revealed that dietary L-carnitine supplementation can increase the circulating TMAO level but decrease the aortic atherosclerotic lesion area in Apoe-/- mice transgenically expressing human cholesteryl ester transfer protein [16]. Another recent clinical study showed that TMAO levels are significantly decreased in patients with large-artery ischemic stroke (LAA) and transient ischemic attack (TIA) compared with healthy control [17]. Therefore, the present work focused on the relationship between and the alterations of intestinal microorganisms in TMAO and stroke.

Methods
1.1 Study Design And Sample Collection
A total of 203 Chinese subjects were recruited from the Neurology Department of Dongyang Affiliated Hospital of Wenzhou Medical University from March 2018 to February 2019. The subjects comprised 144 consecutively recruited patients who experienced acute cerebral ischemia within 7 days after symptom manifestation and 59 subjects without atherogenesis in the carotid arteries as asymptomatic control. Figure 1 shows that patients initially diagnosed with acute ischemic stroke
and considered to have LAA (stenosis > 50%, 85) or CE stroke (atrial fibrillation, 23) were consecutively recruited on the basis of the Trial of Org 10172 in Acute Stroke Treatment classification. Only blood samples were obtained from the 36 remaining patients with acute cerebral ischemia (without atrial fibrillation and stenosis < 50%) to explore the difference in TMAO levels in patients with acute stroke with varying degrees of artery stenosis. The exclusion criteria were ongoing infectious diseases, cancer, congestive cardiac failure, respiratory failure, renal failure, severe liver dysfunction, or consumption of probiotics or antibiotics within two months before admission. The 59 asymptomatic control patients did not use any type of antibiotics or probiotics within two months before recruitment.

Sixty-one out of 85 patients who met the LAA inclusion criteria successfully provided fasting EDTA-plasma samples and fresh fecal samples, 20 of the 23 patients with CE stroke provided blood and fecal specimens, and 51 of the 59 asymptomatic controls submitted fecal and blood specimens. Plasma aliquots were centrifuged and immediately frozen at – 80 °C. Fecal sample aliquots were frozen at – 80 °C immediately after collection. All participants (or their immediate relatives) provided a written informed consent form. The ethics committee of Dongyang Affiliated Hospital of Wenzhou Medical University approved all study protocols.

1.2 Quantification of inflammation indicators, plasma TMAO, and serum zonulin
Blood samples for clinical chemistry analyses were collected after overnight fasting for at least 8 h. γ-GT, creatine kinase (CK), total bilirubin and direct bilirubin, hsCRP, and lipid profile (including triglycerides or TG, TC, high-density lipoprotein cholesterol, and LDL cholesterol) were measured with an auto-analyzer (HITACHI-7600-120). The quantitative sandwich enzyme immunoassay technique was used to quantitatively determine human zonulin concentrations in serum (CUSABIO, catalogue number CSB-EQ027649HU). The TMAO levels in plasma were determined through stable isotope dilution liquid chromatography tandem mass spectrometry. Plasma (50 µL) was aliquoted in a 1.5 mL tube and mixed with 200 µL of a 10 µmol/L internal standard composed of trimethylamine-d9 N-oxide or d9-TMAO (catalogue number: T795792, lot number: 3-LXM-155-1) in methanol. The samples were vortexed for 1 min, and the supernatant was recovered following centrifugation at 12,000 g at 4 °C.
for 25 min. The supernatant was injected directly into a silica column (2.1 mm × 50 mm, 1.7 µm, ACQUITY UPLC BEH C18, Part No.188002350) at a flow rate of 0.4 mL/min with 90% A (0.1% formic acid in water) and 10% B (acetonitrile). TMAO and d9-TMAO were monitored in the positive multiple reaction monitoring mass spectrometry mode by using characteristic precursor–production transitions, namely, m/z 76/58 and m/z 85/66, respectively. For TMAO concentration calculation, 5 µL of trimethylamine N-oxide standard (USA, Lot # SHBK2341) with various concentrations (7.8125 ng/mL–2000 ng/mL) and 200 µL of a 10 µmol/L internal standard composed of d9-TMAO were added to 45 µL of control plasma. The ratio of the TMAO standard peak area to the d9-TMAO peak area was taken as the abscissa, and the concentration of the TMAO standard was adopted as the ordinate to construct the calibration curves.

1.3 Quantification of infarct volumes and carotid atherosclerotic plaque burden
The area of carotid artery plaque was measured with a Philips IE33 instrument by calculating the sum of areas of unstable carotid artery plaque (hypoechoic) and stable carotid artery plaque (hyperechoic). Patients underwent imaging before receiving any reperfusion therapy via a 1.5 T scanner (Magnetom Aera1.5T) within 24 hours of hospitalization. Infarct volumes indicated by diffusion-weighted imaging (DWI) were measured with the software called Medical Image Processing, Analysis, and Visualization (version 8.0.2). Acute diffusion lesions were identified on a slice-by-slice basis by using a semiautomatic segmentation method and consulting apparent diffusion coefficients to distinguish acute from non-acute diffusion signals [18]. The images were analyzed by experienced stroke neurologists.

Infarct volumes = total area of lesions × (slice thickness + spacing between slices)

1.4 Analysis Of Intestinal Microbiota Composition And Function
The 16S rRNA (V4–V5 regions) of fecal samples from a cohort of 132 individuals was sequenced to determine if a correlation exists between the alteration of gut microbiota and stroke. The cohort comprised 51 non-stroke controls, 20 subjects with CE, and 61 patients with LAA. The microbial composition of the three groups was compared in terms of ecological composition and microbial function (PIRUST). The relationship between laboratory (TMAO, zonulin, etc.) or clinical values (infarct
volumes and Activity of Daily Living score) and gut microbiota in the patients was analyzed. A prediction model was constructed to distinguish between stroke and control. This model was established based on the data on the differences in species composition.

1.4.1 DNA Extraction

Samples were stored at – 80 °C until DNA extraction. DNA was extracted from 200 mg samples by using the QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany) in accordance with the manufacturer's instructions.

1.4.2 PCR amplification of 16S rRNA genes and Miseq sequencing

16S rRNA genes were amplified via polymerase chain reaction (PCR) by using general bacterial primers (515F 5’-GTGCCAGCMGCCGCGGTAA-3’ and 926R 5’-CCGTCAATTCMTTTGAGTTT-3’). The primers also contained Illumina 5’ overhang adapter sequences for two-step amplicon library construction in accordance with Illumina instructions. The initial PCR reactions were carried out in 25 µL reaction volumes with 1-2 µL DNA template, 250 mM dNTPs, 0.25 mM of each primer, 1X reaction buffer, and 0.5 U Phusion DNA polymerase (New England Biolabs, USA). The PCR conditions were as follows: initial denaturation at 94 °C for 2 min, 25 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 30 s, with a final extension of 72 °C for 5 min. Second-step PCR with dual 8-base barcodes was used for multiplexing. Eight cycle PCR reactions were adopted to incorporate two unique barcodes into either end of the 16S amplicons. The cycling conditions were as follows: one cycle at 94 °C for 3 min, eight cycles at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s, followed by a final extension cycle of 72 °C for 5 min. Prior to library pooling, the barcoded PCR products were purified using a DNA gel extraction kit (Axygen, China) and quantified using FTC-3000 TM real-time PCR. The libraries were sequenced via 2 x 300 bp paired-end sequencing on the MiSeq platform by using a MiSeq v3 Reagent Kit (Illumina, Tiny Gene Bio-Tech Co., Ltd., Shanghai).

1.4.3 Bioinformatics Analysis

The raw FASTQ files were demultiplexed based on the barcode. The PE reads for all samples were run through Trimmomatic (version 0.35) to remove low-quality base pairs by using the parameters SLIDINGWINDOW: 50:20, MINLEN: 50. The trimmed reads were further merged using the Flash
program (version 1.2.11) with default parameters. The low-quality contigs were removed via the screen.seqs command by using the following filtering parameters: maxambig = 0, min-length = 200, max-length = 580, and maxhomop = 8. The 16S sequences were analyzed by a combination of the software mothur (version 1.33.3), UPARSE (USEARCH version v8.1.1756, http://drive5.com/uparse/), and R (version 3.2.3). The demultiplexed reads were clustered at 97% sequence identity into operational taxonomic units (OTUs) by using the UPARSE pipeline (http://drive5.com/usearch/manual/uparse.cmds.html). The OTU representative sequences were assigned for taxonomic analysis against the Silva 128 database with a confidence score ≥ 0.6 via the classify.seqs command in mothur. OTU taxonomies (from phylum to species) were determined based on NCBI.

1.5 Statistical analysis
Statistical tests were conducted using SPSS Statistics 21 or R (version 3.2.3). The tested indices obeying the normal distribution were expressed as the mean ± SEM, and the significance of the differences was analyzed through an independent t-test. The indicator of disobedience was expressed as the median (interquartile range) and analyzed via the Kruskal-Wallis test. The chi-square test was used for categorical variables. For α-diversity analysis, Shannon, Simpson, Chao1, and abundance-based coverage estimator indices and rarefaction curves were calculated using mothur and plotted in R. For β-diversity metrics, weighted and unweighted UniFrac distance matrices were calculated using mothur and visualized with principal coordinate analysis (PCoA) and tree by R. The Bray-Curtis metrics were calculated and visualized by R. Enterotype analysis was implemented using BiotypeR, and receiver operating characteristic (ROC) analysis was performed with pROC (version 1.15.3.). The PICRUSt (version 1.1.0) bioinformatics tool was used to predict the metagenomic content from the 16S rRNA sequencing data of the samples. The predicted KEGG orthologues were summarized at hierarchy level 3, and the differential abundances by group were determined and displayed using R.

Results
2.1 Baseline characteristics of the study cohort
The demographic and clinical characteristics of patients are shown in Table 1. Patients with LAA and
CE strokes showed a higher inflammatory state than the patients in the control group. hsCRP (LAA stroke vs. CE stroke vs. control: 4.57 ± 0.7 mg/L vs. 3.14 ± 0.92 mg/mL vs. 2.20 ± 0.42 mg/L) and leukocyte count (LAA vs. CE vs. control: 7.00 ± 0.26 × 10^9/L vs. 6.31 ± 0.32 × 10^9/L vs. 5.63 ± 0.23 × 10^9/L) significantly increased. In addition, D-dimer (LAA vs. CE vs. control: 0.99 ± 0.14 mg/L vs. 1.24 ± 0.25 mg/L vs. 0.47 ± 0.05 mg/L) showed a significant increase in stroke patients compared with asymptomatic controls (Fig. 2A).

2.2 Increased plasma TMAO and serum zonulin in patients with stroke

A total of 126 plasma samples (including stroke and control groups that met the inclusion criteria but did not provide fecal samples) were analyzed to study the plasma TMAO level difference between stroke patients and asymptomatic control. The results showed that patients with LAA and CE had significantly increased TMAO compared with asymptomatic controls (Fig. 2B) (TMAO: LAA stroke, 2931 ± 456.4 ng/mL vs. CE stroke, 4220 ± 577.6 ng/mL vs. control, 1663 ± 117.8 ng/mL). An additional 36 non-LAA and CE (stenosis < 50%) acute cerebral ischemia blood samples (Fig. 1) were included to study the difference between TMAO and the degree of vascular stenosis. The TMAO difference between patients with stroke with mild stenosis (stenosis < 50%) and moderate–severe stenosis (LAA, stenosis > 50%) was not significant. The TMAO of the mild stenosis (stenosis < 50%) acute cerebral ischemia group and the control group had no significant difference (Fig. 2C) (TMAO: LAA, 2292 ± 481.1 ng/mL vs. mild stenosis, 1892 ± 214.3 ng/mL vs. control, 1663 ± 117.8 ng/mL).

Furthermore, the relationship between plasma TMAO and infarct volume or carotid plaque area in the stroke patients was studied. Although TMAO and infarct volume were not directly correlated (Supplementary Fig. S1), the plasma TMAO and carotid plaque area among patients with LAA (stenosis > 50%) were positively correlated (Fig. 2E; Spearman $r = 0.333, P = 0.0093$). However, in patients with acute ischemic stroke (< 50%), this correlation disappeared (Fig. 2F, $P = 0.7252$). The difference in serum zonulin levels between patients with stroke and the asymptomatic controls was analyzed using 161 serum samples (including stroke group or control group that met the inclusion criteria but did not have stool samples). As expected, patients with LAA and CE stroke showed significantly increased zonulin in serum compared with asymptomatic controls (Fig. 2D) (zonulin: LAA,
2.71 ± 1.17 ng/mL vs. CE, 2.17 ± 1.00 ng/mL vs. control, 0.43 ± 0.07 ng/mL).

Table 1
Demographic and clinical characteristics of the study participants

|                          | LAA               | CE               | Controls          | P value |
|--------------------------|-------------------|------------------|-------------------|---------|
| Blood samples            | 85 (61 ± 24*)     | 23 (20 ± 3*)     | 59 (51 ± 8*)      | < 0.05  |
| Fecal samples            | 61                | 20               | 51                |         |
| Demographic              | 66                | 72               | 58                | < 0.05  |
| Age, years (mean)        | 40/21             | 11/9             | 19/32             |         |
| Gender (male/female)     |                   |                  |                   |         |
| Drinking (yes/no)        | 19/42             | 19/42            | 19/42             |         |
| Smoking (yes/no)         | 22/39             | 22/39            | 22/39             |         |
| Hypertension (yes/no)    | 42/19             | 42/19            | 42/19             |         |
| T2DM (yes/no)            | 24/37             | 3/17             | 3/17              |         |
| Laboratory values        |                   |                  |                   |         |
| Neutrophilic granulocyte, 10^9/L | 4.80 ± 0.22 | 4.48 ± 0.34     | 3.41 ± 0.20       | < 0.05^ab |
| ApoA I, g/L              | 1.06 ± 0.27       | 1.16 ± 0.54      | 1.21 ± 0.04       | < 0.05^a |
| ApoB, g/L                | 0.97 ± 0.03       | 0.72 ± 0.053     | 0.87 ± 0.37       | < 0.05^a |
| ApoAI/ApoB               | 1.17 ± 0.06       | 1.66 ± 0.15      | 1.51 ± 0.09       | < 0.05^a |
| HDL-C, mmol/L            | 1.05 ± 0.032      | 1.12 ± 0.06      | 1.19 ± 0.04       | < 0.05^a |
| LDL-C, mmol/L            | 2.89 ± 0.12       | 2.21 ± 0.238     | 2.67 ± 0.78       | < 0.05^b |
| Folic acid               | 13.8 ± 1.27       | 16.83 ± 3.01     | 20.4 ± 2.2        | < 0.05^a |
| GPDA, U/L                | 83.18 ± 2.33      | 83.8 ± 3.33      | 98.6 ± 3.11       | < 0.05^ab|
| α-HBDH, U/L              | 155.68 ± 6.87     | 175.4 ± 16.73    | 140.24 ± 3.7      | < 0.05^ab|
| Total cholesterol, mmol/L| 4.64 ± 0.13       | 3.84 ± 0.23      | 4.52 ± 0.12       | > 0.05  |
| Total bilirubin, µmol/L  | 11.45(7.55)       | 15.9(9.025)      | 10.45(5.75)       | < 0.05^b |
| Direct bilirubin, µmol/L | 4(2.33)           | 5.85(2.93)       | 3.15(1.7)         | < 0.05^ab|
| γ-GT, U/L                | 25(23.5)          | 31(33.25)        | 19.5(14.5)        | < 0.05^a |
| α-L-fucosidase, U/L      | 19(4)             | 21.5(10.25)      | 20(6)             | < 0.05^b |
| β-HB, mmol/L             | 0.08(0.19)        | 0.07(0.07)       | 0.05(0.03)        | < 0.05^a |

* Indicates blood specimens, including specimens that meet the inclusion criteria but do not have the fecal specimens needed for TMAO and zonulin analyses.

The tested indices with a normal distribution indicates mean ± SEM via one-way ANOVA. The indicator of disobedience is expressed as the median (interquartile range) and analyzed through a Kruskal-Wallis test. LAA indicates large artery atherosclerosis, CE denotes cardioembolism, HDL-C is high-density lipoprotein cholesterol, and LDL-C is low-density lipoprotein cholesterol. P > 0.05 means no significant statistical difference among the three groups. ^aP suggests a significant statistical difference between LAA and controls. ^bP implies a significant statistical difference between CE and controls. ^abP indicates a significant statistical difference among LAA, CE, and controls.

2.3 Alterations of gut microbiome composition in patients with stroke
2.3.1 Increased α Diversity In LAA And CE

A total of 132 fecal samples from patients with LAA and CE and asymptomatic controls were analyzed.
The species accumulation curve was used to determine whether the detected sample size was sufficient. The curve was smooth, and the sampling was sufficient; thus, data analysis can be performed (Fig. 3A). Rarefaction Curves and Chao indices were examined to characterize species richness. The results showed that the curves were nearly saturated in each group (Fig. 3B), and there was an increased trend in LAA compared with asymptomatic controls (Fig. 3C, $P = 0.06318$ for Chao richness). Species diversity and phylogeny were compared with other parameters, including Shannon and Simpson indices and phylogenetic diversity (PD) whole tree. The three ecological parameters showed that gut microbial diversity in the LAA group was much higher than that in the asymptomatic controls (Figs. 3D–3F). The species diversity between CE and the control increased according to the result of the Shannon indices and PD whole tree (Figs. 3G and 3H). This trend was also observed in the Simpson index (Fig. 3I, $P = 0.063$) but without statistical significance.

2.3.2 Increased β Diversity In Laa And Ce

The overall diversity in microbial composition was assessed via principal co-ordinates analysis (PCoA) and non-metric dimensional scaling (NMDS) analysis. Figures 4A–4B show that patients in the LAA group were more dispersed and had higher heterogeneity than those in the control group. On the basis of weighted UniFrac distance, significant differences were observed via PCoA and NMDS analysis between LAA and the control (Figs. 4A–4B, qualitative analysis by multi-response permutation procedure (MRPP), $P = 0.002$). The microbial communities between LAA and the control were significantly different for the unweighted UniFrac distance (MRPP, $P = 0.01$), Bray–Curtis distance, and Jaccard distance at the OUT level (Table 2). Although this trend was also observed when CE was compared with the control based on weighted UniFrac distance (Table 2, MRPP, $P = 0.077$), only unweighted UniFrac distance reached statistical significance (Figs. 4C–4D). Thus, LAA and CE may harbor a distinct gut microbial community composition compared with the control.

| Group         | A                  | Observed Delta | Expected Delta | P     |
|---------------|--------------------|----------------|----------------|-------|
| LAA-control   | Weighted_unifrac   | 0.03535        | 0.25339        | 0.26268 | 0.002 |
| LAA-control   | unWeighted_unifrac | 0.0078         | 0.50602        | 0.51   | 0.01  |
| LAA-control   | Bray_curtis_OTU    | 0.01071        | 0.71603        | 0.72378 | 0.001 |
| LAA-control   | Jaccard_OTU        | 0.00263        | 0.62787        | 0.62952 | 0.037 |
| CE-control    | Weighted_unifrac   | 0.01009        | 0.24088        | 0.24334 | 0.077 |
| CE-control    | unWeighted_unifrac | 0.00847        | 0.51435        | 0.51875 | 0.026 |
Table 2: $\beta$ diversity among LAA, CE, and control groups. A value $> 0$ indicates that the difference between groups is greater than the difference within the group. A value $< 0$ indicates the opposite. The smaller the value of ObserveDelta, the smaller the difference within the group; the smaller the ExpectDelta value, the smaller the difference between groups.

2.3.3 Altered Community Types In Laa And Ce
Microbial enterotype features were studied to investigate the shift in the gut microbiota community structure in the stroke patients and asymptomatic controls. Microbial enterotype features were calculated using the Jensen–Shannon distance of genera abundance and clustered samples with partitioning around medoids. A total of 132 samples were divided into three clusters (Fig. 5A). Enterotypes 1, 2, and 3 were dominated by Bacteroides, Prevotella, and Ruminococcus, respectively (Figs. 5B, $P = 2e-16$, $P = 6.1e-16$, and $P = 0.0041$, respectively; Kruskal–Wallis test). The association between enterotype distribution and stroke status was determined, and the results showed that patients with LAA were underrepresented in enterotype 2 (Prevotella) but overrepresented in enterotype 3 (Ruminococcus, Fig. 5C, Fisher’s exact test, $P = 0.001999$). CE stroke patients and the asymptomatic control exhibited a similar enterotype distribution (Figs. 5D–5F, $P = 0.7536$). Enterotype was investigated to determine the relationship between TMAO and gut microbiota. Previous research has revealed that the TMAO-producer phenotype should be identified [19], so we attempted to identify it based on enterotype stratification. The results showed that no significant difference existed among the three enterotypes (Supplementary Fig. 2). We failed to distinguish the TMAO producer phenotype via enterotype stratification.

2.3.4 Changed taxonomic composition profile in patients with LAA and CE
First, the taxonomic profile of gut microbiota in LAA was compared with that of the asymptomatic control individuals from the taxonomic composition plots. At the phylum level, Bacteroidetes, Firmicutes, Proteobacteria, Fusobacteria, and Actinobacteria represented more than 90% of the total bacterial community in the gut (Fig. 6A). Bacteroidetes and Firmicutes were the most dominant phyla in LAA and asymptomatic controls, and the changes observed between the two groups were significant at the phylum level. Bacteroidetes were significantly decreased in LAA, whereas
Firmicutes, Proteobacteria, and Actinobacteria were significantly increased in LAA (Fig. 6A). When CE was compared with the asymptomatic control, significant changes were observed only in Bacteroidetes and Fusobacteria (Fig. 6B). A subsequent analysis of the microbiota relative abundances at other taxonomic levels (class, order, family, and genera) was performed. The 21 genera levels were significantly different between CE and the control. Among the genera, the relative abundances of *Fusobacterium, Streptococcus, Dorea, Enterococcus*, and *Desulfovibrio* were significantly increased in CE (top 20, Fig. 6C). Four genera, including *Streptococcus*, were significantly different at various taxonomic levels (Figs. 7A–7B; class, order, family, and genera). Twenty-seven genera were significantly different between LAA and the control (top 20, Fig. 6D). *Parabacteroides, Klebsiella*, and *Lactobacillus* relative abundances were significantly increased in LAA, and seven genera had obvious differences (Fig. 8; class, order, family, and genera).

### 2.3.5 Different Biomarkers Of Laa And Ce

Linear discriminant analysis effect size (LDA effect size) was performed on the fecal microbiota to identify the biomarkers of LAA, CE, and control. The differences in the taxa at different levels with a logarithmic LDA score > 2.0 and a P-value < 0.05 were considered. At the phylum level, Bacteroidetes was significantly enriched in the asymptomatic control, whereas Firmicutes and Proteobacteria were enriched in LAA. Significant differences were also observed at the genera level. The following eight bacterial taxa showed distinct relative abundances between the two groups in fecal microbiota: *Collinsella, Parabacteroides, Alloprevotella, Lactobacillus, Anaerotruncus, Fastidiosipila, Mogibacterium*, and *Klebsiella* (Figs. 9A–9B). Consistent with the results of the taxonomic composition profile (Fig. 6D), all of them were significant increased in LAA. A general linear model (GLM) [20] was designed to control the possible influences of age, gender, drinking, smoking, hypertension, and diabetes and further validate the results. The abundances of *Parabacteroides* and *Fastidiosipila* differed between the two groups (*P* = 1.33E-06 and *P* = 8.42E-06, Table 3).

Several species between CE and the control exhibited significant differences in abundance. At the phylum level, Bacteroidetes was the most enriched in the asymptomatic control, but Fusobacteria was significantly increased in CE. The following bacterial genera showed a distinct increase in relative...
abundances between CE and the control: *Lactobacillus, Streptococcus, Peptostreptococcus, Fastidiosipila, Mogibacterium, and Fusobacterium* (Figs. 10A–10B). The taxonomic composition profile produced the same result (Fig. 6C). Notably, after the possible confounding factors were adjusted (age, gender, drinking, smoking, hypertension, and diabetes), the *Streptococcus* abundance in the two groups was still statistically significant (*P* = 1.0286e-06, Table 3).

**Table 3**

| Group       | Names                | B value | P value  |
|-------------|----------------------|---------|----------|
| LAA_Control | g__Parabacteroides   | 18.32   | 1.33E-06 |
| LAA_Control | g__Fastidiosipila    | 7.42    | 8.42E-06 |
| CE_Control  | g__Streptococcus     | 2.25    | 1.0286e-06 |

2.3.5.1 Gut microbiome-based signature-discriminated stroke and control

The potential of using gut microbiota as biomarkers for discriminating LAA and CE from control was assessed. First, *Fastidiosipila* and *Parabacteroides* were used separately to generate area under receiving operating characteristics (AUC) curves of 0.54 and 0.62, respectively (Fig. 11A). Second, multivariable stepwise logistic regression analysis [21] was applied to the list of LAA-associated genera identified by LEFSe to generate AUC. We found that the combination of eight genera, namely, *Collinsella, Parabacteroides, Alloprevotella, Lactobacillus, Anaerotruncus, Fastidiosipila, Mogibacterium, and Klebsiella*, could discriminate LAA from non-stroke controls effectively with an AUC of 0.843 (Fig. 11B). All diversity levels of microbiome identified by LEFSe (Fig. 9B) could significantly improve predictive performance (Fig. 11C; AUC, 0.931). The potential value between CE and the control was assessed, and *Streptococcus* generated an AUC of 0.7 (Fig. 11D). The combination of six bacterial taxa at the genus level could also effectively discriminate CE from non-stroke controls with an AUC of 0.762 (Fig. 11E). However, using all microbiomes identified by LEFSe (Fig. 10B) did not improve predictive performance (*Supplementary Fig.* 3; AUC, 0.751).

2.4 Role of baseline characteristic factors in the change in intestinal microbes

Studies have reported that microbiota composition changes with aging [22], and the present study is limited by the non-balanced age distribution of the included patients and controls (*P* > 0.05, Table 1). Therefore, whether age factors account for the main factors in the alterations of intestinal microbes in patients with stroke was explored. A subgroup analysis was performed on 75 samples with a balanced
age distribution for further analysis (Supplementary Table 1). Consistent with the aforementioned analysis results, a significant difference was observed between the two groups (group 1: stroke and group 2: control) in the α and β diversity analyses (Figs. 12A-12D). The gut microbiota of the control group can be separated from that of the patient group based on the weighted UniFrac matrix ($P = 0.001$, Figs. 12A-12B).

Additionally, although HTN and T2DM are widely known to be connected with gut microbiome dysfunction, we did not exclude stroke patients or control with comorbidities (HTN and T2DM) to reflect the real signature of clinical practice. Nineteen non-diabetic, hypertensive stroke patients (including LAA and CE) and 32 controls without diabetes and hypertension were screened to evaluate the disrupted patterns of gut microbiome resulting mainly from stroke (Supplementary Table 2; BS, Stroke; Con, control). The age distributions were well balanced between the two groups ($P = 0.257$, independent $t$-test). The α and β diversity indices showed significant differences between BS and Con ($P = 0.018$, Figs. 13A-13F). The gut microbiota of patients (non-diabetic, HTN, and control) could be separated from that of others (non-diabetic, HTN, and stroke group) via PCoA and NMDS (Figs. 13A-13B). The subgroup analyses replicated the previous results obtained after adjusting for possible confounding factors (Table 3). This finding indicates that Parabacteroides can still serve as a marker of stroke (Table 4). Consequently, the difference in gut microbiome between the stroke and control groups was mainly due to stroke.

Existing literature shows that age has a considerable influence on TMAO. The plasma TMAO between the two groups was analyzed after age equalization. The TMAO levels increased significantly in patients with stroke (Fig. 14; LAA, $2763 \pm 408.7$ ng/mL vs. control, $1642 \pm 144.1$ ng/mL). The relationship between intestinal microbes and stroke was also analyzed. Fecal samples of patients with stroke were analyzed according to the location of vascular stenosis (internal carotid and vertebrobasilar arteries), and the location of cerebral infarction (brain stem, cerebellar, and other parts of infarction). The difference between α and β diversity indices was not significant (results are not shown), indicating that the changes in intestinal microbes are not associated with the location of the infarct nor the location of vascular stenosis.
| Biomarker                                                                 | LDA | P       |
|--------------------------------------------------------------------------|-----|---------|
| p_Bacteroidetes.c_Bacteroidia.o_Bacteroidales.f_Porphyromonadaceae       | 3.1 | 0.009   |
| p_Bacteroidetes.c_Bacteroidia.o_Bacteroidales.f_Porphyromonadaceae.g_Para bacteroides | 2.85 | 0.024 |

Table 4. Biomarker between stroke patients and the control after adjusting for confounding factors

HTN and T2DM. P, phylum; c, class; o, order; f, family; g, genera; and s, species.

2.5 Relationship Between Bacteria And Laboratory Or Clinical Indices

The relationship between bacteria in the genera detected in all samples and clinical indices was explored. Several genera played catalytic roles in the pathogenesis of stroke. We found that certain microorganisms had high correlation with multiple clinical indicators (Fig. 15A). Parabacteroides, a discriminative bacteria detected by LEFSe, could be regarded as a biomarker in LAA. Parabacteroides was positively correlated with infarct volume (measured using DWI) and carotid artery plaque area (measured using carotid duplex ultrasound), negatively correlated with ADL, and positively correlated with serum neutrophilic granulocyte, creatine kinase, and total cholesterol. It was also positively correlated with zonulin, which exhibited a significant increase in patients with LAA and CE. Further exploration revealed that the association of Parabacteroides can be attributed to P. distasonis (Fig. 15B) and Parabacteroides_sp._HGS0025 (Fig. 15C). The abundance of Alistipes also showed a positive correlation with infract volumes, carotid artery plaque area, and creatine kinase and a negative correlation with ADL. The abundance of Alistipes and other genera (Anaerotruncus, Odoribacter, and Ruminococcus) exhibited a significant correlation with TMAO. Streptococcus, as a biomarker in CE, showed a positive correlation with fasting blood glucose but a negative correlation with total cholesterol. Notably, several genera levels may have played an opposite role in the course of stroke. Haemophilus was negatively correlated with infract volume and positively correlated with the ADL score. Haemophilus was significantly elevated in the control group compared with the LAA group (Fig. 15D), indicating that these genera may play roles in preventing stroke.

2.6 Alterations in gut microbiome function in patients with stroke

The functional and metabolic changes in the microbial communities in LAA, CE, and the controls were studied by inferring the metagenomes from the 16sRNA data and predicting the potential function of
the gut microbiota using PICRUSt. The predicted functional categories were based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologue (KO). In the level 2 KEGG pathway, amino acids, glycan, secondary metabolites, terpenoids, polyketides, cofactors, and vitamins were significantly low in the LAA microbiome. Membrane transport, signal transduction, and xenobiotic biodegradation and metabolism were high in LAA (Fig. 16B). In the level 3 KEGG pathway (Fig. 16A), unsaturated fatty acids, ketone body biosynthesis, fatty acid, and glycerolipid metabolism were significantly increased in the LAA microbiome. Excessive accumulation of extracellular glutamate, a principal excitatory neurotransmitter, is a major factor that contributes to the death of ischemic penumbra [23]. Several genes associated with D-glutamine and D-glutamate metabolism had low representation in the microbiome of LAA compared with the control. In addition, the functional modules related to bacterial invasion of epithelial cells were significantly increased in the LAA microbiome, and combined with the elevated serum zonulin, these may indicate an increased intestinal permeability in patients with LAA. Several genes related to antigen processing and presentation and several immunology pathways on innate immunity, such as the peroxisome proliferator-activated and nucleotide-binding oligomerization domain (NOD)-like receptor signaling pathways, were significantly decreased in the LAA microbiome (Fig. 16A). Furthermore, secondary metabolites associated with antibiotics, such as biosynthesis of vancomycin group antibiotics and streptomycin, were significantly decreased in the LAA microbiome. Such metabolites may promote the growth of pathogenic microorganisms and change the gut microbiome composition in patients with stroke.

The functional difference between CE and the control was compared. In the level 2 KEGG pathway (Fig. 17A), the microbial gene functions related to membrane transport were significantly higher in CE than in the control. Inversely, cofactors, vitamins, glycan biosynthesis, and metabolism were significantly decreased in CE. The top 20 differences in the level 3 KEGG pathway are shown in Fig. 17C. Consistent with the functional changes in LAA, glycerolipid metabolism was significantly high, and amino sugar, nucleotide sugar metabolism, and protein digestion and absorption were significantly low in CE. In the function of the LAA microbiome, the NOD-like receptor signaling
pathway and several secondary metabolites associated with antibiotics, such as streptomycin and vancomycin, also decreased in CE (Fig. 17B).

The levels of circulating TMAO were significantly increased in the stroke patients. The relationship between the microbiome function and TMAO in the study cohort was studied. In the microbiota of LAA patients, the genes involved in the synthesis, transport, and metabolism of methylamine-containing source nutrients [24] were significantly increased (Table 5). Examples of the genes include lipopolysaccharide cholinephosphotransferase (K07271), choline (K00108, K02168), betaine (K00130, K03762, K05020), phosphatidylcholine (K01004), carnitine (K08279), L-carnitine (K05245), and TMA (K14084, K14083). Gut microbial enzyme complexes are involved in the formation of primary metabolite TMA. Carnitine can be converted by microbiota into TMA via carnitine TMA lyases, such as CntA/CntB. Choline can be converted into TMA via choline TMA lyases, such as CutC/CutD, and TMAO can be converted to TMA via TMAO reductase. From the results of the PICRUSt analysis, we could not detect the KOs linked to the first four enzymes, namely, K20038 for choline trimethylamine-lyase CutC, K20037 for choline trimethylamine-lyase activating enzyme CutD, K22443 for carnitine monooxygenase subunit CntA, and K22444 for carnitine monooxygenase subunit CntB. Several KOs could be defined to TMAO reductase (table.6). Among such KOs, K07811, K07821, and K03532 were significantly higher in the LAA microbiome compared with the control (Fig. 18A). Searching the NCBI nucleotide database by using the phrase “TMAO reductase” showed that Enterobacteriaceae (family), Helicobacter (genus), and many other human intestinal origin bacteria can reduce TMAO to TMA [25]. The results of the present work indicate that the relative abundance of Enterobacteriaceae and Helicobacter increased more significantly in the LAA microbiome than in the control (Fig. 18B). LEFSe analysis indicated that Enterobacteriaceae was significantly increased in patients with stroke and may be considered a biomarker (Table 7). Thus, the microorganisms in the LAA microbiome can convert more dietary sources of TMAO to TMA than those in the control. This phenomenon may partly contribute to the increased plasma TMAO levels in LAA. We noticed that such KOs could be projected onto the same metabolic pathway, that is, K000680 methane metabolism (Fig. 19, part of methanogenesis, TMAO as a substrate). Methane metabolism was significantly higher in LAA than in
the control (Fig. 12B), but other information about the pathway that we could obtain from PICRUSt analysis was limited. Metagenomics sequencing technology was necessary for further research.

Table 5

| Entry  | Name     | Definition                                                      | P    |
|--------|----------|-----------------------------------------------------------------|------|
| K00108 | betA, CHDH | choline dehydrogenase                                           | 0.003|
| K00130 | betB, gbsA | betaine-aldehyde dehydrogenase                                  | 0.014|
| K01004 | pcs      | phosphatidylcholine synthase                                    | 0.037|
| K02168 | betT, betS | choline/glycine/proline betaine transport protein               | 0.041|
| K03762 | proP      | proline/betaine transporter                                     | 0.024|
| K05020 | opuD, betL | glycine betaine transporter                                     | 0.008|
| K05245 | caIF     | L-carnitine/gamma-butyrobetaine antipporter                    | 0.034|
| K07271 | licD      | lipopolysaccharide cholinephosphotransferase [EC:2.7.8.-]      | 1.27E-05|
| K08279 | caiE      | carnitine operon protein CaiE                                   | 0.044|
| K14083 | mttB      | trimethylamine—corrinoid protein Co- methyltransferase [EC:2.1.1.250] | 9.83E-04|
| K14084 | mttC      | trimethylamine corrinoid protein                                | 0.043|

Table 6

| Entry  | Name     | Definition                                                      |
|--------|----------|-----------------------------------------------------------------|
| K07811 | torA     | trimethylamine-N-oxide reductase (cytochrome c) [EC:1.7.2.3]   |
| K07821 | torY     | trimethylamine-N-oxide reductase (cytochrome c), cytochrome c-type subunit TorY |
| K07812 | torZ     | trimethylamine-N-oxide reductase (cytochrome c) [EC:1.7.2.3]   |
| K03532 | torC     | trimethylamine-N-oxide reductase (cytochrome c), cytochrome c-type subunit TorC |
| K03533 | torD     | TorA specific chaperone                                         |

Table 7: Biomarkers in patients with stroke. P, phylum; c, class; o, order; f, family; g, genera.

11413148: K18277(ttm); 1.5.8.2: K00317(dmd-tmd) ;1.5.8.1: K00317 (dmd-tmd)

17.2.3: K07811 (torA), K03532 (torC), K03533 (torD), K07812 (torZ), K07821 (torY)

MtbA: K14082(mtbA) MttB: K14083(mttB) MttC: K14084(mttC)

MtbA: K14082(mtbA) MtbB: K16176(mtbB) MttB: K16179(mttB)

MtbA: K14082(mtbA) MtbB: K16176(mtbB) MttC: K16177(mttC)
Discussion
This study found evidence delineating the features of stroke-related gut dysbiosis through the integration of 16sRNA gene sequencing and microbiome function prediction PICRUSt. New information was obtained about TMA O level and gut microbiome in patients with LAA and CE strokes. The most significant findings are as follows. ① Compared with the asymptomatic control group, the LAA and CE stroke groups showed significant changes in the composition and function of the gut microbiome. ② *Parabacteroides* and *Streptococcus* could be regarded as biomarkers for patients with LAA and CE, respectively. ③ TMAO plays a more important role in the pathophysiological mechanism of LAA and CE strokes than in other types of strokes (non-LAA, CE, and acute cerebral ischemia).

To the best of our knowledge, high bacterial diversity has been recurrently documented in multiple diseases, such as atrial fibrillation [26] and myocardial infarction [27]. The fecal microbiomes of patients with LAA and CE strokes showed increased species richness and diversity compared with that of the asymptomatic control. Consistent with the findings of a previous study, the patients with stroke and transient ischemic attacks showed increased diversity of the gut microbiota [17]. According to the present study, the evaluated richness and diversity of the gut microbiota can reflect the imbalanced gut microenvironment and the dysbiosis of gut microbiota characterized by the overgrowth of varieties of harmful bacteria (e.g., *Desulfovibrio* and *Enterococcus*), opportunistic pathogens (e.g., *Eubacterium*), and a few commensal or beneficial genera (e.g., *Bifidobacterium* and *Lactobacillus*).

The gut microbiota dysbiosis in LAA could also be determined according to enterotype. The gut microbiome in LAA shifted from enterotype 2 represented by *Prevotella* to enterotype 3 represented by *Ruminococcus*, which is known to possess pro-inflammatory properties and exerts a contributory effect on the development of inflammatory bowel disease [28, 29]. Previous laboratory studies have shown that *Ruminococcus* transplanted in germ-free mice can enhance the level of IL-17 and other inflammatory cytokines [30]. IL-17 and IL-17+γδ T cells have also been implicated in stroke [31]. Additionally, γδ-T cell infiltration and IL-17 secretion have been documented in ischemic human brain tissue [32, 33]. These may indicate that the type of gut dysbiosis (shift from enterotype 2 to enterotype 3) plays an important role in LAA pathophysiology.
The dysbiosis of intestinal milieu in LAA could be further characterized by increased relative abundances in *Collinsella, Parabacteroides, Alloprevotella, Lactobacillus, Anaerotruncus, Fastidiosipila, Mogibacterium,* and *Klebsiella.* Among those bacteria, *Parabacteroides* showed the strongest association with LAA. Consistent with previous research, all of our results showed that *Parabacteroides,* a discriminative bacteria filtered by LEFSe, can be regarded as a biomarker in LAA [17]. It is correlated not only with the severity of the stroke reflected by infarct volume and ADL score, but also with high inflammatory status reflected by serum neutrophilic granulocyte count.

*Parabacteroides* showed high correlation with risk factors for cardiovascular and cerebrovascular diseases. This correlation is reflected by the total cholesterol and carotid artery plaque area. Further research revealed that *P. distasonis* and *Parabacteroides* sp. HGS0025 significantly increased in LAA, although previous animal experiments have shown that *P. distasonis* plays a beneficial role in alleviating obesity and metabolic dysfunctions [34]. Several studies have also revealed that *P. distasonis* is a potential pathogen for coronary artery disease [35]. Such results may be due to the differences between animal and clinical experimentation or the result of strain-level variations. Existing literature has shown that in a clinical context, species- and strain-level variations can contribute to functional differences between individuals but are always ignored by researchers [36, 37]. Therefore, the role of these microorganisms in LAA should be further studied via metagenomics to determine the possible role in the etiology of stroke. We also found that *Streptococcus* played an important role in CE, and *Haemophilus* played a beneficial role in the asymptomatic control.

*Haemophilus,* which includes commensal organisms and several pathogenic species, was significantly higher in the control than in patients with stroke. The abundance of *Haemophilus* was negatively correlated with stroke severity (infarct volume and ADL) and may play a beneficial role in the pathogenesis of stroke. As mentioned above, *Streptococcus* detected by LEFSe could be regarded as a biomarker of CE stroke. Consistent with a previous study, the present study showed that patients with atrial fibrillation [26] and atherosclerotic cardiovascular disease [9] have increased *Streptococcus;* the accrual of *Streptococcus* is always concomitant with increased *Enterobacteriaceae,* which is enriched in cardiovascular disease, and decreased commensal or
beneficial genera, such as *Faecalibacterium* [9, 26]. The same results can be obtained from LEFSe in our research cohort. *Enterobacteriaceae* was high in patients with CE stroke. Different subgroup analyses showed that alterations in the gut microbiome composition and function in LAA and CE strokes were mainly due to stroke itself rather than age, diabetes, and hypertension. Microbial differences in patients with stroke may not be related to the site of arterial stenosis and the location of cerebral infarction.

A growing body of literature suggests that increased permeability of oral and intestinal epithelial barriers enables a small number of bacteria to enter into the systemic circulation and ultimately into host tissues to promote diseases [38]. Zonulin, a protein that modulates the intercellular tight junctions of endothelial cells, can reversibly modulate intestinal permeability. In the current study, patients with LAA and CE strokes showed significantly increased serum zonulin, which means increased intestinal permeability. In line with the current study, a previous work showed that serum zonulin is also elevated in patients with coronary artery disease [39]. The zonulin/zonula occludens toxin (Zot) receptor plays an important role in the pathophysiological regulation of the blood–brain barrier (BBB) [5], so elevated serum zonulin may mean increased BBB permeability in patients with stroke. Cynomolgus monkey experiments have revealed that an increase in serum zonulin occurs after left middle cerebral artery occlusion [40]. Hence, we speculate that zonulin-regulated intestinal permeability or BBB permeability may also occur after stroke. Furthermore, functional predictions indicated that the functional modules of the microbiome of patients with LAA have high expression in the bacterial invasion of epithelial cells. These results indicate that the increased intestinal permeability in stroke patients may be related to bacterial translocation and post-stroke infection.

Previous studies have obtained different findings on the relationship between TMAO and cardio- or cerebrovascular diseases [11, 12, 17, 41, 42]. When the relationship between TMAO and cerebrovascular diseases (stroke) is explored, relevant changes in TMAO elevation can be determined regardless of serology or the compositional and functional prediction of the gut microbiome. The serological results indicated that plasma TMAO levels in patients with LAA and CE were significantly higher than those of patients in the control group. After adjusting for age factors, this
difference still existed. The plasma TMAO level was significantly high in patients with LAA and CE stroke but did not increase in patients with acute ischemic stroke with mild arterial stenosis (< 50%) compared with the asymptomatic control. The difference in plasma TMAO between patients with acute ischemic stroke with mild arterial stenosis (< 50%) and patients with moderate-to-severe arterial stenosis (> 50%) was not significant. Plasma TMAO showed a significant positive correlation with carotid plaque area only in patients with stroke with moderate-to-severe arterial stenosis (> 50%). These results indicate that increased TMAO occurs mainly in patients with LAA or CE stroke, suggesting that TMAO may play an important role in the pathophysiological mechanisms of patients with stroke with aortic atherosclerosis or atrial fibrillation. The increased TMAO in CE may be correlated with the ability of TMAO to promote platelet hyper-reactivity and increase thrombosis risk [41].

The taxonomic profile of gut microbiota indicated that patients with LAA stroke harbored abundant TMA-producing bacterial species. According to previous research, Klebsiella and Erysipelotrichaceae both encode CutC to potentially produce TMA from choline [9]. The majority of CutC homologs (88–61% amino acid identity) are found in Proteobacteria (Gammaproteobacteria and Deltaproteobacteria) and Firmicutes (Clostridia and Bacilli) [43]; in the current study, they were significantly increased in patients with LAA compared with the asymptomatic control (Figs. 6–8).

The functional profile prediction of gut microbiota suggested that the genes involved in the synthesis, transport, and metabolism of methylamine-containing source nutrient were significantly increased in patients with LAA. Although we did not find CutC/D- and CntA/B-related KOs from the results of microbiome functional prediction, KO definition to TMAO reductase (TMAO-TMA: K07811, K07821, K03532) showed obviously increased microbial gene expression among patients with LAA compared with the asymptomatic control. Moreover, consistent with the functional prediction, the results of the gut microbiota composition profile showed that Helicobacter and Enterobacteriaceae with TMAO reductase [25] were significantly increased in patients with LAA. Enterobacteriaceae can be regarded as a biomarker of LAA stroke. Hence, the LAA group had more microorganisms that convert dietary source TMAO to TMA (enrichment in fish at concentrations of 20–120 mg/100 g of fish fillet [44]).
compared with the control. In addition, microbial methane metabolism is significantly positively correlated with TMAO levels [45], and methanogen archaea may be used to prevent cardiovascular diseases [46]. However, PICRUSt analysis showed that methane metabolism increased significantly in LAA; other detailed information about the pathway that we could derive from PICRUSt was limited. Such a result may be due to the limitations of 16sRNA, which restricts data interpretation in terms of species level and functional tests. Thus, metagenomics sequencing technology are necessary to study the role of *P. distasonis* in patients with stroke and analysis of microbial composition and function related to ko00680 methane metabolism in stroke patients.

Other limitations of the current study should be noted. Information about the patients’ diet and lifestyle was not collected, which may have affected the composition of the gut microbiome in patients with LAA and CE strokes.

In addition, metagenomics sequencing and microbiota transplantation are needed in future studies to assess if and how disease-associated bacteria, especially *Parabacteroides* and *Streptococcus*, play a role in LAA and CE stroke pathogenesis.

**Conclusions**

We find that significant alterations in the gut microbiome composition and function and an increase in the plasma TMAO level occur in LAA and CE strokes. And the abundance of specific gut microbiota correlates with clinical phenotypes and disease severity in stroke patients. The findings suggest the potential of using gut microbiota as a biomarker for patients with LAA and CE strokes.

**Abbreviations**

| Abbreviation | Description |
|--------------|-------------|
| TMAO         | Trimethylamine N-oxide |
| 16S rRNA     | 16S ribosomal RNA |
| CE           | cardioembolic |
| LAA          | large artery atherosclerotic |
| T2DM         | type 2 diabetes mellitus |
| CVD          | cardiovascular disease |
| CutC         | choline TMA-lyase |
| CutD         | choline TMA-lyase activating enzyme |
| TorA/C/D     | trimethylamine N-oxide reductase |
| FMO          | flavin-containing monooxygenases |
| TIA          | transient ischemic attack |
| MIPAV        | Medical Image Processing, Analysis, and Visualization |
| PCoA         | principal coordinate analysis |
| NMDS         | non-metric dimensional scaling |
| LEFSe        | linear discriminant analysis effect size |
| GLM          | general linear model |
| AUC          | area under receiving operating characteristics |
| KEGG         | Kyoto Encyclopedia of Genes and Genomes (KEGG) |
| KO           | Kyoto Encyclopedia of Genes and Genomes ortholog |
| ADL          | Activity of Daily Living score |

**Declarations**
Acknowledgments

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Authors’ contributions

KKJ, DJX, and LC were responsible for the study concept and design. KCW, HFL, YYX, and LYW collected the data. KCW, LBY, and QQL analyzed and interpreted the data, and KCW and LBY drafted the manuscript. KKJ critically revised the manuscript for important intellectual content and supervised the study. All authors read and approved the final manuscript.

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Availability of data and materials

All data are available upon request. The datasets used and analyzed in the current study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

All participants provided informed consent, and the study was approved by the Institutional Ethics Board 2017-KY-036, Medical Ethics Committee of Dongyang People’s Hospital.

Consent for publication

The paper has not been published previously and is not under consideration for publication elsewhere. All co-authors have read and approved the submission.

Competing interests

All authors declare that they have no competing interests.

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Supplementary Tables
| Group1 | ID   | Group2 | ID   |
|-------|------|--------|------|
| Group1 | D07  | Group2 | 12   |
| Group1 | D64  | Group2 | 24   |
| Group1 | D47  | Group2 | 26   |
| Group1 | D11  | Group2 | 15   |
| Group1 | D02  | Group2 | 116  |
| Group1 | D74  | Group2 | 32   |
| Group1 | D24  | Group2 | 31   |
| Group1 | D72  | Group2 | 114  |
| Group1 | D73  | Group2 | 108  |
| Group1 | D60  | Group2 | 11   |
| Group1 | D63  | Group2 | 108  |
| Group1 | D61  | Group2 | 23   |
| Group1 | D75  | Group2 | 112  |
| Group1 | D101 | Group2 | 10   |
| Group1 | D54  | Group2 | 107  |
| Group1 | D57  | Group2 | 110  |
| Group1 | D106 | Group2 | 02   |
| Group1 | D51  | Group2 | 07   |
| Group1 | D79  | Group2 | 33   |
| Group1 | D37  | Group2 | 25   |
| Group1 | D116 | Group2 | 27   |
| Group1 | D09  | Group2 | 42   |
| Group1 | D113 | Group2 | 22   |
| Group1 | D115 | Group2 | 106  |
| Group1 | D18  | Group2 | 18   |
| Group1 | D35  | Group2 | 117  |
| Group1 | D108 | Group2 | 01   |
| Group1 | D34  | Group2 | 20   |
| Group1 | D03  | Group2 | 19   |
| Group1 | D114 | Group2 | 104  |
| Group1 | D117 | Group2 | 16   |
| Group1 | D66  | Group2 | 02   |
| Group1 | D104 | Group2 | 40   |
| Group1 | D33  | Group2 | 36   |
| Group1 | D43  | Group2 | 105  |
| Group1 | D70  | Group2 | 113  |

**Supplementary Table 1. Subgroup analysis of 75 samples with a balanced age distribution (mean age: Group1, 58 years vs. Group2, 56 years; P > 0.05).** Group 1 patients are selected from stroke cases, and group 2 patients are selected from the control.
Supplementary Table 2. Biomarker between patients with stroke and patients in the control group after adjusting for confounding factors HTN and T2DM.

Supplementary Figure Legends

Supplementary Fig. S1: Relationship between plasma TMAO and infarct volume in patients with stroke. P > 0.05 means no significant statistical difference.

Supplementary Fig. 2: Identification of TMAO-producer phenotype based on entrotype stratification. ns means no statistical significance.

Supplementary Fig. 3 Using the relative abundance of CE, all diversity taxonomic levels generated an area under the receiving operating characteristic curve.

Figures
Flow diagram of stroke patient selection and sample collection. * indicates that patients with acute cerebral ischemia (CTA/MRA, stenosis < 50%) only need to provide blood samples to explore the difference in TMAO levels in patients with acute stroke with varying degrees of artery stenosis.
Baseline characteristics of the study cohort. (a) The inflammation index of patients with stroke was significantly higher than that of the control group. Leukocyte with a normal distribution as indicated by ANOVA. hsCRP and D-dimer did not have a normal distribution as per the Kruskal–Wallis test (b–c). Difference in plasma TMAO between patients with
stroke and the control group. Stenosis < 50% indicates non-LAA and CE acute cerebral ischemia as calculated by the Kruskal-Wallis test. (d) Difference between patients with stroke and the control group in serum zonulin (e-f). Relationship between plasma TMAO and the area of carotid plaque calculated using Spearman analysis. $r$ is rho, and the error bar indicates the standard error of mean (SEM). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$. 
Figure 3

Alpha diversity indices in patients with LAA and CE and controls. (a–b) Accumulation and rarefaction curves between patients with LAA and CE, and controls. (c–f) Box plots depicting differences in the fecal α diversity indices between LAA and control according to the Chao, PD whole tree, and Shannon and Simpson indices based on the OUT counts. (g–i) Box plots depict differences in the fecal α diversity indices between CE and the control according to the PD whole tree, Shannon, and Simpson indices. Boxes represent the interquartile ranges, lines inside the boxes denote medians, and circles are outliers. P < 0.05 indicates statistical significance, Wilcoxon rank sum test *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
β diversity differences among LAA, CE, and control. (a–b) PCoA and NMDS based on weighted UniFrac matrix showed that the overall fecal microbiota composition was different between LAA and controls. (c–d) NMDS and PCoA based on unweighted UniFrac matrix showed that the overall fecal microbiota composition was different between CE and controls. PCoA means principal coordinate analysis. NMDS means non-metric dimensional scaling.
Figure 5

Altered community types in patients with LAA and CE, and the control. (a) A total of 112 samples of LAA and control are clustered into enterotype 1 (green), enterotype 2 (blue), and enterotype 3 (cyan) by using principal component analysis (PCA) of Jensen–Shannon divergence values at the genera level. (b) Relative abundances of the top genera in each enterotype: Bacteroides in enterotype 1, Prevotella in enterotype 2, and Ruminococcus in enterotype 3. Boxes represent the interquartile ranges, lines inside the boxes denote medians, and circles are outliers. P<0.05 indicates a statistical difference as per Kruskal-Wallis test. (c) Percentage of control and LAA samples distributed in enterotypes 1, 2, and 3. Enterotype distribution between LAA and control (P = 0.001999, Fisher’s exact test). (d) Seventy-one samples of CE and control are clustered into enterotype 1 (green), enterotype
2 (blue), and enterotype 3 (cyan) by PCA of Jensen–Shannon divergence values at the
genera level. (e) Relative abundances of the top genera in each enterotype: Bacteroides in
enterotype 1, Prevotella in enterotype 2, and Ruminococcus in enterotype 3 with \( P=3.0 \times 10^{-9}, \)
\( P=5.4 \times 10^{-9}, \) and \( P=0.016, \) respectively, as per Kruskal–Wallis test. (f) Percentage of CE and
control samples distributed in enterotypes 1, 2, and enterotype 3 \( (P=0.7536, \) Fisher’s exact
test).
Figure 6

Taxonomic composition profile at the phylum and genus levels. (a–b) Taxonomic profile of gut microbiota at the phylum level between LAA-control and CE-control. (c–d) Taxonomic profile of gut microbiota at the genus level among LAA, CE, and control. P < 0.05 indicates a statistical difference as per the Wilcoxon rank sum test. The error bar indicates the standard error of mean (SEM), *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
Figure 7

Taxonomic composition profile between CE and the control. (a–b) The taxonomic profile of gut microbiota is statistically different at the main taxonomic levels between CE and the control: class, order, family, and genera. P < 0.05 indicates a statistical difference as per the Wilcoxon rank sum test. The error bar indicates the standard error of mean (SEM), *P < 0.05, **P < 0.01, ***P < 0.001
Taxonomic composition profile between LAA and the control. The taxonomic profile of gut microbiota between LAA and the control is statistically different at the main taxonomic levels: class, order, family, and genus. $P < 0.05$ shows a statistical difference as per the Wilcoxon rank sum test. The error bar indicates the standard error of mean (SEM), *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. 
Figure 9
Biomarkers between LAA and the control. (a) Cluster tree between LAA and the control. The circle diagram radiating from inside to outside is a clustering tree, which represents the classification level of the phylum, class, order, family, genus, or species. Each small circle on a different circle layer represents a classification at that level, and the diameter of the small circle is proportional to the relative abundance of the classification. The red and green areas in the image represent different groupings: yellow indicates species that are not significantly different between the two groups, red nodes indicate microbial species that play an important role in the red group, and green nodes indicate that they are in the green group. The red and green nodes in the figure are identified by letters, and the microbial species represented by the nodes are summarized and annotated on the right side of the cluster tree. (b) Linear discriminant analysis (LDA) effect size (LEfSe) reveals significant bacterial differences in fecal microbiota between the patients with LAA (negative score) and the controls (positive score). P, phylum; c, class; o, order; f, family; g, genera; and s, species.
Figure 10

Biomarker between CE and the control. (a) Cluster tree between CE and the control. (b) Linear discriminant analysis (LDA) effect size (LEfSe) reveals significant bacterial differences in fecal microbiota between the patients with CE (negative score) and the controls (positive score). P, phylum; c, class; o, order; f, family; g, genera; and s, species.
Figure 11
Disease classification based on gut microbiome signature. The classification performance of the multivariable logistic regression model using the relative abundance of stroke-associated genera was assessed by AUC. (a) Using *Fastidiosipila* or *Parabacteroides* as a predictor generated an AUC. (b–c) Using the relative abundance of LAA-associated genera or all diversity taxonomic levels generated an AUC. (d) Using only *Streptococcus* as a predictor generated an AUC. (e) Using the relative abundance of CE-associated genera generated an AUC.

Figure 12

α and β diversity differences between stroke and control patients after adjusting for the
confounding factors age. (a–b) PCoA and NMDS based on weighted UniFrac matrix showed that the overall fecal microbiota composition was different between stroke and control patients. (c–d) Box plots depict differences in fecal α diversity index between patients with stroke and the control according to Shannon and Simpson indices based on the OUT counts. Boxes represent the interquartile ranges, lines inside the boxes denote medians, and circles are outliers. P<0.05 indicates statistical significance as per the Wilcoxon rank sum test. PCoA means principal coordinate analysis. NMDS means non-metric dimensional scaling. Group 1 pertains to patients with stroke after adjusting for age. Group 2 pertains to the control group after adjusting for age. *P < 0.05, **P < 0.01, ***P < 0.001.
\( \alpha \) and \( \beta \) diversity differences between stroke and control patients after adjusting for
confounding factors HTN and T2DM. (a–b) PCoA and NMDS based on weighted UniFrac matrix showed that the overall fecal microbiota composition was different between stroke and control patients. (c–f) Box plots depict differences in the fecal α diversity index between patients with stroke and the control according to Sobs, PD whole tree, and Shannon and Simpson indices based on OUT counts. Boxes represent interquartile ranges, lines inside the boxes denote medians, and circles are outliers. P<0.05 indicates statistical significance as per the Wilcoxon rank sum test. PCoA means principal coordinate analysis. NMDS means non-metric dimensional scaling. BS pertains to patients with stroke without HTN and T2DM. Con refers to control groups without HTN and T2DM. *P < 0.05, **P < 0.01, ***P < 0.001.
Differences in plasma TMAO between patients with stroke (LAA) and the control after adjusting for the confounding factor age. The error bar indicates the standard error of mean (SEM). **P < 0.01.
Figure 15

Relationship between bacteria and laboratory or clinical indices. (a) Heat maps showing Spearman correlation coefficients between microbiota genera and clinical and laboratory characteristics of patients with stroke. (b–d) Parabacteroides was significantly over-represented in patients with LAA. (d) Control group with a high abundance of Haemophilus.

The error bar indicates the standard error of mean (SEM). *P < 0.05, **P < 0.01.
Figure 16

Microbial functional change in LAA. Functional categories between LAA and the control in
the level 2 KEGG pathway (b) and level 3 KEGG pathway (a). Wilcoxon rank sum test. The error bar indicates the standard error of mean (SEM). * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.
Microbial functional change in CE. Functional categories between CE and the control in level 2 KEGG pathway (a) and level 3 KEGG pathway (b, c). Wilcoxon rank sum test. The error bar indicates the standard error of mean (SEM). * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.
Figure 18

Microbial functional change associated with TMAO reductase in LAA. (a) Difference between LAA and the control in KO definition to TMAO reductase. (b) High abundance of Enterobacteriaceae and Helicobacter in LAA compared with the control. Wilcoxon rank sum test. The error bar indicates the standard error of mean (SEM). * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.
Metabolic pathway: K000680, methane metabolism. Part of methanogenesis with TMA as a substrate. Searched from KEGG (https://www.kegg.jp/). 11413148: K18277(ttm); 1.5.8.2: K00317(dmd-tmd); 1.5.8.1: K00317(dmd-tmd) 17.2.3: K07811(torA), K03532(torC), K03533(torD), K07812(torZ), K07821(torY) MtbA: K14082(mtbA) MttB: K14083(mttB) MttC: K14084(mttC) MtbA: K14082(mtbA) MttB: K16178(mtbB) MttC: K16179(mtbC) MtbA: K14082(mtbA) MtmB: K16176(mtmB) MtmC: K16177(mtmC)

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