

# Overexpansion of *Proteobacteria* in the Thrombus of Stroke Patients Treated with Mechanical Thrombectomy and the Risk for Death

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## Research

**Keywords:** Bacteria, Acute ischemic stroke, Mechanical thrombectomy, 16S sequencing, FISH

**Posted Date:** May 21st, 2021

**DOI:** <https://doi.org/10.21203/rs.3.rs-498057/v1>

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# Abstract

**Background:** Extensive analysis has focused on the brain-gut axis interaction between stroke and the gut, but the most direct cause of stroke involves thrombi that block blood flow. The role of bacteria in thrombi on stroke onset has rarely been studied. Thus, it is of interest to fully characterize the microbial features of the thrombus and explore the underlying mechanism.

**Materials and methods:** Clot samples were collected from 104 acute ischemic stroke (AIS) patients who underwent clot retrieval with mechanical thrombectomy. After inclusion, oral, fecal, and isolated plasma samples of the same patients were synchronously gathered within 12 hours of admission. The microbial composition of all samples was compared using 16S rRNA gene amplicon next-generation sequencing. Fluorescent in situ hybridization (FISH) was selected to detect the bacteria in the thrombus with the bacterial probe EUB338. The markers of inflammatory cytokines and intestinal barrier integrity were measured using ELISAs. We performed a comprehensive analysis of the characteristics of the thrombus bacterial community in stroke patients and calculated the correlation between specific bacterial genera and clinical outcomes.

**Results:** Of the 104 AIS patients, the presence of bacterial DNA in the thrombus was confirmed by qPCR analysis of the 16S rRNA gene and FISH experiments. Operational taxonomic units (OTUs) from 14 different phyla were identified, with the majority of the OTUs belonging to *Proteobacteria* (73.3%); these were mainly predicted by BugBase to be potential opportunistic pathogens. Bray-Curtis distance analysis revealed that the microbiota in the thrombus were significantly different from the oral, fecal and plasma microbiota (all  $P < 0.001$ , Adonis test). A proportion of the clot microbiome was similar to that of plasma, and based on the FEAST algorithm, the bacteria in the thrombus were mainly derived from plasma (46.69%). Higher abundances of *Acinetobacter* and *Enterobacteriaceae* were associated with a risk of adverse events within 48 hours of admission, and a higher abundance of *Acinetobacter* was also associated with a risk of death by the 3rd month. Univariate and multivariate Cox regression analysis showed that, in addition to important clinical indicators (preoperative National Institutes of Health Stroke Scale (NIHSS) score, atrial fibrillation and history of stroke), the relative abundance of *Acinetobacter* in the thrombus was an important risk factor for 90-day mortality (HR 1.957, 95% CI 1.198-3.196,  $P = 0.007$ ; adjusted HR 2.664, 95% CI 1.384-5.129,  $P = 0.003$ ). Higher concentrations of IL 1 $\beta$  and IL 6 were associated with a higher risk of adverse events and death.

**Conclusion:** The study showed evidence for significant diversity of bacteria in the thrombus in ischemic stroke patients and visually confirmed their adherence in FISH. It is among the first to fully characterize the clot microbial feature as overexpansion of the phylum *Proteobacteria* and observe a close correlation between conditional pathogens and risk of death in patients receiving mechanical thrombectomy. It warrants further investigations on the role of bacteria in the thrombus in the pathogenesis and prognosis of stroke.

## Introduction

A considerable proportion of acute ischemic stroke (AIS) is a consequence of artery occlusion caused by an occlusive thrombus in the brain. Of these, the most devastating are secondary to large vessel occlusion (LVO)<sup>[1, 2]</sup>, tending to leave larger cerebral territories at ischemic risk and cause more severe brain injury if not treated urgently<sup>[3, 4]</sup>. In 2015, revascularization and tissue reperfusion through stent retriever thrombectomy and direct aspiration of cerebral arterial clots to reduce tissue damage, has been proved an effective treatment for AIS patients<sup>[5-10]</sup>. The emergence of mechanical thrombectomy has provided us with a unique opportunity to discover the microcosm of thrombi.

Association-based evidence has demonstrated that the commensal microbiota is closely related to arterial thrombosis<sup>[11, 12]</sup> and thrombin generation<sup>[13]</sup>. However, research into the role of the microbiota in the progression of thrombosis in stroke is in its infancy. Toll-like receptor 2 (TLR2) signaling induced by the microbiota stimulates Von Willebrand factor (VWF) synthesis in the hepatic endothelium of mice, promoting thrombus growth in the ligation-injured carotid artery<sup>[14]</sup>. Furthermore, gut microorganisms directly contribute to platelet overreactivity and enhance thrombosis potential by producing trimethylamine N-oxide (TMAO), a common bacterial metabolite<sup>[15]</sup>. Although extensive analysis has focused on the brain-gut axis interaction between stroke and the gut microbiome<sup>[16-20]</sup>, the key cause of stroke, thrombi that block blood flow, has rarely been studied, which may be attributed to the lack of ideal animal models and the difficulty in obtaining thrombus specimens from stroke patients.

Hyperpermeability of the intestinal barrier after stroke enhances translocation and transmission of selective strains from the host intestinal microbiota<sup>[21]</sup>. Recent studies have revealed that microbiota colonization in thrombi of patients with myocardial infarction might influence the progression of thrombosis<sup>[13, 22]</sup>. To date, limited studies have conducted microbial analysis of thrombi from stroke patients<sup>[23-25]</sup>, all, however, with a small sample sizes or simple research methods. QPCR amplification was performed with predesigned specific primers, and oral bacterial DNA in thrombus aspirates from stroke patients was affirmed<sup>[23]</sup>. These previous studies conflicted over the presence of bacteria in thrombus retrieved by mechanical thrombectomy<sup>[24, 25]</sup>. In addition, little information is available regarding the source of thrombus microbiota. Therefore, a comprehensive understanding of clot bacteria by means of advanced sequencing technology is imperative and conducive to the in-depth study of the pathogenesis of thrombosis.

In this study, we aimed to analyze the microbial features of thrombi collected by stent retriever from cerebral arteries of patients with AIS through 16S rRNA gene sequencing and fluorescent in situ hybridization (FISH) and to fully characterize the thrombus microbiome, by collecting oral, fecal and isolated plasma samples within 12 hours of admission for microbiome analysis.

## **Materials And Methods**

### **Study Subjects and Sample Collection**

This was an observational prospective cohort study. We recruited AIS patients admitted within 24 hours of symptoms onset to the Acute Stroke Unit of the First Affiliated Hospital of Jinan University, a university-affiliated academic hospital in Guangzhou, China, who were treated by intra-arterial thrombectomy between June 2019 and June 2020. Two experienced neurologists examined all patients when they arrived at the hospital and evaluated the possibility of revascularization using thrombectomy. The inclusion criteria included (a) adult patients (>18 years); (b) treatment with mechanical thrombectomy with retrieval of clot material; (c) availability of the clot for bacterial and histopathological analysis; (d) the cause of LVO was cardiogenic embolism (CE) or large-artery atherosclerosis (LAA), according to the Trial of ORG 10172 in Acute Stroke Treatment (TOAST)<sup>[26]</sup> classification; and (e) no infectious diseases or treatment with antibiotics or probiotics in the last 1 month and before sample collection. Other causes of LVO, such as tumor embolus, were excluded from the study. Upon inclusion, thrombus, fecal, oral, and plasma samples (Figure 1A) were collected within 12 hours after admission and transferred to a -80°C freezer immediately.

Medical history and other relevant information were collected. In this study, perioperative adverse events (within 48 hours of admission) were defined as hemorrhage conversion, intracerebral hemorrhage, progression requiring surgical treatment, or even cerebral herniation. 90-day survival status was documented for Cox analysis.

The collected thrombus was removed during surgery and placed directly into sterile tubes (CY-95000-P1, HCY Technology, China) for qPCR analysis under strict aseptic conditions. A histological sample was placed in another tube with 3.7% formaldehyde if the thrombus was large enough. All of the clot collection and preparation processes were carried out under sterile conditions, and the thrombus did not come in contact with any potential contaminants. After thrombectomy, another swab was applied to wipe the surgical instruments and the surgeon's gloves to obtain a blank control. AIS patients were generally under sedation after endovascular treatment, and no stool samples could be collected within 24 hours; therefore, rectal swabs were used as an alternative instead of stool samples in this study. A swab was inserted approximately 5-6 cm into the rectum, and the swab was turned 360 degrees. Once the collection was complete, the rectal swab was removed and examined for stool. Next, the physician placed the swab in a sterile tube containing 1 ml of phosphate-buffered saline (PBS). Oral samples were prospectively collected from participants by rubbing the insides of both cheeks with a swab and then placing the swab in a sterile tube containing 1 ml of PBS. Two fasting venous blood samples were collected from the subjects within 12 hours and then centrifuged at 4°C and 3000 rpm for 10 min. The isolated plasma was collected into two sterile tubes. One tube was used for 16S sequencing, and the other was prepared for ELISA.

## DNA Extraction and 16S Sequencing

Bacterial DNA was extracted from samples using a commercially available Bacterial DNA Extraction Mini Kit (MabioDNB361B, Mabio, China) according to the product instructions. Blank extractions (as reagent

controls) were also processed and used to identify potential contamination. The V3-V4 variable regions of 16S rRNA genes were amplified by polymerase chain reaction (PCR) using Illumina tagged primers 338F (5'-ACTCCTACGGGAGGCAGCA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). PCRs containing 25 µL 2x Premix Taq (Takara Biotechnology, Dalian Co., Ltd., China), 1 µL each primer (10 mM) and 50 ng DNA template in a volume of 50 µL, were amplified by thermocycling: pre-denaturation at 94°C for 5 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s, extension at 72°C for 30 s; and a final extension at 72°C for 10 min. Three replicates of the PCR products in the same sample were mixed, and the PCR instrument was a Bio-Rad S1000 (Bio-Rad Laboratories, CA). PCR products were evaluated by 1% agarose gel electrophoresis, and visible products 290–310 bp in size were used in further experiments. The PCR products were mixed in equimolar ratios, calculated by GeneTools Analysis Software (Version 4.03.05.0, SynGene), and then purified using an EZNA Gel Extraction Kit (Omega, USA). Sequencing libraries were set up using the NEBNext® Ultra™ DNA Library Prep Kit for Illumina® (New England Biolabs, USA) following the manufacturer's instructions, and the index codes were added. The Qubit® 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system were used to evaluate the library quality. Finally, the library was sequenced on an Illumina HiSeq 2500 platform, and 250-bp paired-end reads were obtained.

## Bioinformatics Analysis

The 16S rRNA gene sequences were processed using VSEARCH 2.15.1<sup>[27]</sup>, USEARCH 10.0.240<sup>[28]</sup>, and in-house scripts<sup>[29]</sup>. High-quality clean reads were obtained according to the Trimmomatic 0.33<sup>[30]</sup> quality control process, performing quality filtering on the paired-end raw reads under specific filtering conditions. Trimmed sequences were subsequently merged with VSEARCH (-fastq\_mergepairs). After dereplication (-derep\_fulllength), high-quality reads were denoised and clustered into operational taxonomic units (OTUs) using the UNOISE3 algorithm implemented in USEARCH. Chimeric sequences were filtered with UCHIME<sup>[31]</sup> de novo during OTU clustering. Based on the high-confidence 16S representative sequences and 97% sequence similarity, an OTU table was created according to VSEARCH (-usearch\_global). The taxonomy of the representative sequences was classified with the RDP classifier with VSEARCH (syntax\_cutoff 0.6). Sequences classified as eukaryotes, chloroplasts, or mitochondria were removed by in-house scripts with the aid of R software ver. 4.0.3.

OTUs with differential abundances were identified with a negative binomial generalized linear model in the edgeR package with a threshold of relative abundance of 0.1%. Venn diagrams and Manhattan and volcano plots were generated using the R packages "VennDiagram", "ggplot2", "dplyr" and "amplicon". Linear discriminant analysis effect size (LEfSe) was used to measure differentially abundant taxa across groups using the default parameters<sup>[32]</sup>. Based on high-quality sequences<sup>[33]</sup>, BugBase (<http://github.com/danknights/bugbase>) was used to calculate differences between different groups in terms of microbial phenotypes. Fast expectation-maximization microbial source tracking (FEAST) was performed to track the microbial source following protocols provided by the authors of the R package<sup>[34]</sup>. Gut microbiota alpha diversity indices were calculated by the "Vegan" package, and the Shannon index

was selected in our study. Then, ANOVA was used to test for significance, and a post hoc Tukey HSD test was applied to perform pairwise comparisons. The microbial beta diversity was compared using Bray-Curtis dissimilarity, and significance was determined by the Adonis test. Statistical analysis of the results was performed using R version 4.0.3 (<http://cran.r-project.org/>). Differences were considered statistically significant at  $P < 0.05$ .

## Fluorescent in Situ Hybridization

FISH of tissue sections included only 58 AIS patients due to an insufficient amount of tissue. The blood attached to the thrombus was rinsed with 0.9% normal saline, and the thrombus was fixed with 3.7% formaldehyde. Bacterial in situ hybridization experiments were carried out on 4  $\mu$ M paraffin tissue sections prepared in advance. Two slides of each specimen were chosen for the FISH experiment. The specimens were dewaxed with xylene three times, dehydrated in an ethanol series (98%, 85%, 70%), and then washed two times in PBS (each step for 5 min). The bacterial signals were labeled by the universal bacterial probe EUB338 (1:20, FB-0010B, HYCX, China), and the slides were processed with a FISH kit (D-0016, Exon Biotechnology, China) according to the manufacturer's instructions. Briefly, sections were immersed in blocking solution at 55°C for 2 hours and then hybridized overnight at 37°C with EUB338 diluted with hybridization buffer in a humid chamber. Unbound probe was subsequently removed by incubation in wash buffer. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) for 15 min. The slides were air-dried and analyzed by fluorescence microscopy (Zeiss, Scope A1+Iris, oil immersion lens, magnification,  $\times 1000$ ), and images were processed using ImageJ software.

## Enzyme-linked Immunosorbent Assay (ELISA)

Isolated plasma collected from AIS patients within 12 hours of admission was assayed for inflammatory cytokines and markers of intestinal barrier integrity using ELISA kits. Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ , CSB-E04740h), interleukin-1 $\beta$  (IL-1 $\beta$ , CSB-E08053h), interleukin-6 (IL-6, CSB-E04638h), lipopolysaccharide (LPS, CSB-E09945h), and lipopolysaccharide-binding protein (LBP, CSB-E09629h) were assayed by ELISA kits per the manufacturer's instructions (CUSABIO, China). D-lactate (CAK1177) was analyzed using a kit and protocol from Cohesion Bioscience, China.

## Statistical Analysis

Statistical analyses were performed using R4.0.3 and SPSS 27.0 (IBM, USA). A univariate Cox proportional hazards model of 90-day survival status was first performed, and the candidate variables with a P value of  $< 0.05$  were further included in the multivariate Cox regression model for adjustment. The candidate variables included demographic data (age and sex), comorbidities (diabetes, hypertension, atrial fibrillation, adverse events, and poststroke infection), plasma markers (white blood cell count, neutrophils, creatinine, C-reactive protein, total cholesterol, and low-density lipoprotein cholesterol),

indicators of critical illness (preoperative NIHSS score and length of hospital stay), intravascular treatment time (time to arrival and time to reperfusion), and z-scores of specific taxa abundances. Continuous variables were expressed as the median (interquartile range, IQR) and were analyzed using the Mann-Whitney U test and Kruskal-Wallis test. Subsequently, pairwise comparisons were adjusted by Bonferroni correction. Medians  $\pm$  quartiles are plotted in all box plots. The biodiversity difference was tested by ANOVA, and pairwise comparison was conducted by Tukey's HSD test.

## Result

### Patient Characteristics

We recruited 104 AIS patients who underwent thrombectomy, 57.7% (n=60) male and 42.3% (n=44) female. The mean age of the patients was 66.1 years. The median delay time between the onset of ischemic stroke and hospital arrival was 6 hours 58 min (range, 1-23 hours). The general clinical characteristics of LVO stroke patients are shown in Table 1.

### Presence of Bacterial DNA and Microbial Features in Thrombus Aspirates

Clot samples were collected from all participants. Notably, 96.2% (n=100) of aspirated thrombi were positive for bacterial DNA in qPCR, while 3.8% (n=4) were bacteria-negative thrombi. All blank control samples obtained from the interventional instruments and interventionalist's gloves were bacteria-negative in qPCR; thus, exogenous contamination (surgical procedures and sample disposal procedures) of bacteria can be ruled out.

To characterize the microbial features of aspirated thrombi, we collected rectal and oral swabs and isolated plasma from all stroke patients within 12 hours after admission (Figure 1A). Rectal swabs were selected to represent the fecal microbiota. As shown in Figure 1B, the average 16S rRNA concentration of clot (15.94 ng/ $\mu$ L) samples was lower than that of oral (186.24 ng/ $\mu$ L) and fecal (40.86 ng/ $\mu$ L) samples but higher than that of plasma (3.91 ng/ $\mu$ L) samples. Except for 9 samples that could not be collected (1 fecal, 6 plasma, 2 oral), 2.9% (n=3) of isolated plasma samples were negative for bacterial DNA in qPCR. Microbiota from 100 clot, 95 plasma, 103 fecal, and 102 oral samples were characterized by 16S rRNA amplicon sequencing.

The OTU data was rarefied to 9500 reads per sample. After rarefaction, there were 3800000 sequences, and these clustered into 7439 OTUs. The rarefaction curves show that the OTU numbers in the clot and plasma groups were similar, but both were lower than those in the fecal and oral groups (Figure 1C). In addition, in the Venn diagram, there were 70 highly abundant OTUs in the thrombus samples that were shared with bacteria from other sites. The number of unique sequences was smallest in the clot (33) group and largest in the oral (140) group (Figure 1D). In terms of alpha diversity, the Shannon index of the

clot group differed significantly from that of the fecal and oral groups (all  $P < 0.001$ ) but was similar to that of the plasma group ( $P = 0.48$ ) (Figure 1E). Additionally, unconstrained principal coordinate analysis (PCoA) of the Bray-Curtis distance revealed that the microbial composition of clot samples was obviously distinct from that of fecal, oral and plasma samples (Figure 1F, Adonis test, all  $P < 0.001$ ).

There were 14 phyla, 182 genera and 542 OTUs that had a relative abundance greater than 1% across clot samples. The top 15 relative abundances of genera in thrombi were *Acinetobacter* (12.8%), *Burkholderia* (12.5%), *Sphingomonas* (10.9%), *Pedobacter* (7.0%), *Serratia* (5.5%), *Stenotrophomonas* (5.3%), *Brevundimonas* (3.8%), *Bradyrhizobium* (3.1%), *Bacillus* (2.1%), *Elizabethkingia* (1.6%), *Prevotella* (1.6%), *Ochrobactrum* (1.5%), *Ralstonia* (1.4%), *Herbaspirillum* (1.3%) and *Chryseobacterium* (1.3%). At the phylum level (Figure 1G), most sequences of clot samples were assigned to the phyla *Proteobacteria* (relative abundance 73.3%), *Bacteroidetes* (12.9%) and *Firmicutes* (10.0%) and, to a lesser extent, *Actinobacteria* (2.0%). At the family level, the top 5 relative abundances of clot bacteria were *Moraxellaceae* (14.1%), *Burkholderiaceae* (14.0%), *Sphingomonadaceae* (11.5%), *Enterobacteriaceae* (8.0%), and *Sphingobacteriaceae* (7.0%). In general, the bacterial composition of the thrombus samples was similar to that of the plasma samples but distinct from that of the oral and fecal samples.

## Source Tracking of Clot Microbiota and Function Prediction by BugBase

A source tracking method, FEAST<sup>[34]</sup>, was used to track the origin of the thrombus microbiota based on the OTU data. Contributions from each source were calculated and represented as a percentage. The clot microbial community had approximately 46.69% of the community sourced from plasma, only 2.34% from oral samples, and 2.09% from fecal samples (Figure 1H). Compared with the LAA group, a larger proportion of the thrombosis bacteria in the CE group was derived from plasma (50.69% & 43.74%), but there was no significant difference between the two groups.

To better understand the possible function of the bacteria in the thrombus, BugBase<sup>[33]</sup> was used to infer and compare organism-level microbiome phenotypes among the different samples. The OTU contributions among the four groups are presented in Figure 2. We observed a significantly higher representation of aerobic bacteria, gram-negative bacteria, potentially pathogenic bacteria, bacteria related to biofilm formation and oxidative stress-tolerant bacteria in the clot and plasma groups (Figure 3).

## Signature of Microbiota in the Clot via FISH

To further confirm the presence and visualize the distribution of bacteria in thrombi, we selected the universal bacterial probe EUB338 to label the bacteria in the thrombus (Figure 4A) using FISH and then imaged them with a spectral fluorescence microscope. A total of 58 paraffin-embedded thrombus samples (50% from the CE group) were collected in the study due to an insufficient amount of tissue. As

described previously<sup>[35]</sup>, the main components of thrombi in stroke patients are red blood cells, white blood cells, fibrin, and aggregated platelets. The red blood cells, fibrin, and aggregated platelets were visualized in a purplish red color in FISH slices (Figure 4B), and the blue color represented nucleated cells such as neutrophils and macrophages. In FISH analysis, green dot fluorescence in clot samples was a positive signature of bacteria. Three distribution patterns of microbiota in thrombi were observed: free distribution, intracellular clustering (single green signal dot > 5) and extracellular clustering. Ten visual fields with obvious bacteria-positive signals in each clot sample were randomly selected, and microbial distribution patterns were recorded. The comparison among the three distribution patterns was significantly different between the CE and LAA groups (Table 2). The thrombi in the CE group (Figure 4C-4D) were characterized as a clustered microbial distribution pattern, with mainly intracellular clustering and extracellular clustering, while the LAA group had mainly free microbial distribution (Figure 4E-4F).

## Clot Microbial Peculiarity in Different Stroke Pathogeneses

The 16S rRNA concentration of clot samples in the CE group (average, 20.58 ng/ $\mu$ L) were remarkably higher than that in the LAA group (12.43 ng/ $\mu$ L,  $P = 0.007$ ). Although the pathogenesis of stroke was associated with the distribution pattern of bacteria within the thrombus, no significant difference in bacterial diversity among different pathogeneses was observed based on the number of OTUs (Figure 5A, 5C). The microbial composition of cerebral artery thrombosis with different pathogeneses is probably similar. The relative abundance of the dominant taxa in each clot sample from the CE and LAA groups at the phylum (Figure 5B) and family levels (Figure 5D) is illustrated in the stacked plot. Furthermore, for specific OTUs, 22 OTUs were obviously depleted and 25 were markedly enriched in the CE group compared with the LAA group (Figure 5E). To identify differentially abundant microbiota between the two groups, linear discriminant analysis (LDA) coupled with effect size measurement (LEfSe) was performed. With this approach, we observed that the dominant bacteria in thrombi were different among AIS patients with different pathogeneses (Figure 5F). The CE group featured the *Veillonellaceae* family in the phylum *Firmicutes*, while the *Chryseobacterium* and *Lactobacillaceae* families were more dominant in the LAA group.

## Features of the Clot Microbiome in Poor Clinical Outcomes

A total of 37.5% ( $n=39$ ) of patients had perioperative adverse events, represented as the With\_AE group. The remaining 65 patients were defined as the Without\_AE group. The relative abundance of the dominant taxa in the With\_AE and Without\_AE groups at the family level (Figure 6A) is illustrated in the stacked plot. To further investigate the differences in bacterial species of these two groups, we compared the relative abundances of all OTUs between the groups, as shown by the Manhattan plot in Figure 6B, and found that 47 OTUs were significantly different with a false discovery rate (FDR) < 0.05 (above the dotted line) in the With\_AE group. Further LEfSe analysis revealed that *Acinetobacter* (order *Pseudomonadales*, family *Moraxellaceae*) and *Enterobacteriaceae* were enriched in the thrombi of those

patients with adverse events (Figure 6C). During the follow-up period, 17 (16.3%) patients died within 90 days of admission. The stacked plot exhibits the microbial differences in the thrombus samples in the death and survival groups (Figure 6D), and 43 OTUs were markedly different in the death group compared with the survival group (Figure 6E). The LEfSe results suggested that higher abundances of *Pseudomonadales* and *Acinetobacter* were closely related to the death outcome (Figure 6F).

## Associations between Inflammatory Factors, Specific Microbiota and 90-day Mortality

LVO stroke patients who died within 90 days had higher white blood cell (WBC) levels at admission than those who survived at 90 days, suggesting a possible higher inflammatory response in the body ( $P = 0.025$ ). To further reveal the level of inflammation in stroke patients, plasma isolated within 12 hours after mechanical thrombectomy was used for the ELISA (Table 3). The results showed that the concentrations of IL 1 $\beta$  (mean, 72.1 pg/mL & 43.2 pg/mL,  $P=0.004$ ) and IL 6 (mean, 35.4 pg/mL & 26.6 pg/mL,  $P=0.002$ ) were obviously increased in the plasma of patients with adverse events. Furthermore, increased levels of IL 1 $\beta$  (mean, 147.6 pg/mL & 36.3 pg/mL,  $P=0.001$ ) and IL 6 (mean, 119.4 pg/mL & 13.1 pg/mL,  $P=0.001$ ) in stroke patients were also found to be closely associated with 90-day mortality.

A Cox proportional hazards regression model was constructed to examine the associations between the abundances of specific taxa and 90-day mortality after mechanical thrombectomy (Table 4). The results of the univariate model showed that three clinical parameters (WBC, neutrophils (NEU), creatinine) were associated with the 90-day mortality of stroke patients. In addition, the preoperative NIHSS score, adverse events within 48 hours, poststroke infection during hospitalization, and history of atrial fibrillation and stroke were important risk factors for death. Interestingly, we also found that *Pseudomonadales*, *Moraxellaceae* and *Acinetobacter* were associated with 90-day mortality. Moreover, *Acinetobacter* (HR 2.664, 95% CI 1.384-5.129,  $P=0.003$ ) remained significantly associated with 90-day mortality in the multivariate Cox regression analysis adjusted for other parameters listed in the table.

## Discussion

Our study demonstrates that bacterial DNA, 16S rRNA, is indeed expressed in thrombi samples retrieved from stroke patients with LVO and that clusters of bacterial signals can be seen in sections of bacterial in situ hybridization. Compared with the microbiome in other body regions, the thrombus microbiota was mainly characterized by excessive enrichment of *Proteobacteria*, and traceability analysis indicated that clot bacteria mainly originated from plasma. Furthermore, higher abundances of *Acinetobacter* and *Enterobacteriaceae* were associated with a risk of adverse events within 48 hours of admission, and an increased level of *Acinetobacter* was also associated with a risk of death within 3 months. In addition, the relative abundance of *Acinetobacter* in the thrombus was an important risk factor for 90-day mortality, as determined by univariate and multivariate Cox regression analysis. To the best of our knowledge, this is a prospective observational study with the largest cohort in which thrombus microbial features of AIS

patients have been fully characterized using 16S sequencing technology, and it explores the association between the abundances of specific bacteria and survival status during the 90-day follow-up.

In this prospective study, 104 thrombi were obtained via mechanical thrombectomy and collected from consecutive AIS patients with an onset of symptoms < 24 hours before collection; of these, 96.2% of thrombi were positive for bacterial DNA. A study with a sample size of 75 patients used qPCR to confirm the presence of *Streptococcus*, a representative oral microbiota, in the thrombus of stroke patients<sup>[23]</sup>. Their data also showed a higher rate of bacteria-negative samples than our study (16% & 3.8%), which may be due to the limitations of specific PCR primers. Our data showed that the relative abundance of *Streptococcus* in the thrombus samples was only 1.1%, as determined by next-generation sequencing. Another metagenomic analysis of thrombus samples involving only 4 patients<sup>[25]</sup> found that more than 27 bacteria existed in the clots of stroke patients and that the dominant bacteria were *Lactobacillus*, *Staphylococcus* and *Stenotrophomonas*, partially consistent with our study. In our clinical study, 182 genera and 542 OTUs had a relative abundance of more than 1% in thrombi. The bacteria with the highest average abundance were *Acinetobacter*, accounting for 12.8%, while *Stenotrophomonas* had an abundance of 5.3%, and *Lactobacillus* had an abundance of only 0.8%. It is possible that the majority of bacterial species in clots are commensal or low-grade pathogens around the human body, which possibly transgress into the bloodstream from the oral tract, gut, or skin without leading to overt infection in immunocompetent hosts.

Our study also found that the microbial characteristics of the thrombus were slightly different in strokes with different causes. In agreement with previous studies<sup>[25]</sup>, *Lactobacillus* and *Chryseobacterium*, a less-common non-lactose-fermenting bacteria that have emerged as important opportunistic pathogens<sup>[36]</sup>, significantly increased in the clots of stroke patients with LAA, while the relative abundance of the *Veillonellaceae* family increased in the CE group. Our results suggest that, bacteria may be involved regardless of the etiology of stroke, especially conditional pathogens, and that the contribution of microbiota in the thrombus to stroke may be underestimated. Moreover, in terms of the 16S bacterial DNA concentration in the thrombus, the concentration of DNA extracted from the CE group was obviously higher than that from the LAA group. Two hypotheses may explain the difference in DNA concentration of clot bacteria from different etiological sources. Previous studies have observed that cardiogenic thrombi contain a high level of fibrin and a large number of white blood cells<sup>[35]</sup>, and bacteria may be enriched by phagocytosis of white blood cells or by being wrapped in aggregated platelets and fibrin. The second reason may be that the cardiogenic clot is generally larger due to the anatomical location and hemodynamic reasons, whereas atherosclerotic stroke is possibly the consequence of artery-to-artery embolism arising from the rupture of a proximal in situ atherothrombosis.

Another innovation of this study is that oral, fecal and isolated plasma samples of AIS patients were collected synchronously within 12 hours of admission, aiming to illustrate the characteristics of the clot microbiome more comprehensively through multisite comparisons. The comparisons showed that the diversity of the thrombus microbiota was lower than that of the other samples and that the microbial

compositions were significantly different among the four types of samples. However, there were still 70 highly abundant OTUs in the clot overlapping with the other samples, suggesting that the source of the clot microbiome may be related to bacteria in these three regions. Furthermore, bacteria in the thrombus were similar to those in the plasma, and the majority of the bacteria that could enter the blood and be present in the clot were from the phylum *Proteobacteria*, accounting for 73.3% of the bacteria in the clot. Interestingly, we also found that, based on the FEAST algorithm, 46.69% of thrombus bacteria may originate from plasma samples, but the source of the circulating bacteria is unknown. Bacteria in systemic circulation may participate in the thrombosis process of stroke under the stimulation of certain triggers. An overabundance of *Proteobacteria* is generally considered a marker of gut dysbiosis<sup>[37]</sup> and a microbial signature of epithelial dysfunction<sup>[38]</sup>. The present study revealed for the first time that the thrombus of stroke patients was characterized by an overexpansion of *Proteobacteria*, predicted to be a potential pathogenic bacterium by BugBase. However, the role of enriched *Proteobacteria* in thrombi remains unclear and deserves further research.

By means of FISH with a universal bacterial probe, we confirmed the presence of bacteria in the thrombus and observed that the bacteria was localized in clusters or freely distributed. Interestingly, there were differences in the distribution patterns of bacteria in thrombi of different origins. A majority of the bacteria in CE stroke patients were aggregated in intracellular leukocytes and on the surface of fibrin, while bacteria in LAA stroke patients were clustered or found individually on the surfaces of red blood cells. The weakness of this study lies in the absence of specific markers of bacterial strains, so it was impossible to identify whether the clusters of bacteria were from a single strain or mixed community or to clarify whether the distribution of bacterial clusters is related to thrombosis. However, studies have demonstrated that, in the absence of atherosclerosis, the presence of bacteria is also conducive to thrombogenesis. Once bacteria gather in clusters, the specific spatial localization of *Bacillus* possibly affects the coagulation process and directly activates the coagulation of blood within minutes<sup>[39]</sup>. In addition, some bacterial species, such as *Staphylococcus aureus*, secrete procoagulant factors and promote plasma clotting to avoid deadly attack from the immune system<sup>[40]</sup>. Our study showed that both *Bacillus* and *Staphylococcus* were present in thrombus aspirates of stroke patients and that their relative abundances were 2.1% and 1.2%, respectively. Strikingly, based on the prediction of BugBase, the clot community was dominated by gram-negative bacteria, and the biofilm formation ability was significantly enhanced compared to that in other body regions. The bacteria in the clots of patients with AIS may play a pivotal role in promoting blood coagulation.

Early adverse events (hemorrhagic transformation, etc.) after mechanical thrombectomy are associated with poor prognosis in stroke patients<sup>[41-44]</sup>. In our study, the levels of opportunistic pathogens (*Acinetobacter* and *Enterobacteriaceae*) were significantly higher in patients with adverse events within 48 hours of admission than in those without. *Acinetobacter* species are omnipresent organisms that are widely distributed in nature and contribute to a range of nosocomial infections<sup>[45, 46]</sup>. Enrichment of *Enterobacteriaceae* in the gut is associated with a higher risk of stroke<sup>[47]</sup> and is an independent predictor of poor outcomes in stroke patients<sup>[18]</sup>. In addition, our study demonstrated that a higher abundance of

*Acinetobacter* was associated with higher 3-month mortality in stroke patients. Univariate and multivariate Cox regression analyses further showed that, in addition to three key clinical indicators, the abundance of *Acinetobacter* in the thrombus was an important risk factor for 90-day mortality. However, the underlying mechanism by which *Acinetobacter* contributes to poor clinical outcomes in stroke patients remains unclear. Accurate regulation by targeting the thrombus microbiome may be helpful for preventing thrombosis, reducing the risk of stroke and improving the clinical outcome of stroke patients.

## Conclusions

In conclusion, our study confirmed the existence of bacteria in the thrombus and shed light on the characteristics of the thrombus microbiome. The higher abundance of opportunistic pathogens in the thrombus was closely associated with increased mortality in stroke patients. This necessitates a larger study that involves a multicenter clinical cohort, bacterial culture, specific bacterial staining and receptor expression detection to verify the surrounding microbiota as additional risk factors for stroke.

## Abbreviations

AIS, acute ischemic stroke; FISH, Fluorescent in situ hybridization; OTU, Operational taxonomic unit; NIHSS, National Institutes of Health Stroke Scale; LVO, large vessel occlusion; TLR2, Toll-like receptor 2; VWF, Von Willebrand factor; TMAO, trimethylamine N-oxide; CE, cardiogenic embolism; LAA, large-artery atherosclerosis; TOAST, Trial of ORG 10172 in Acute Stroke Treatment; PBS, phosphate-buffered saline; LEfSe, Linear discriminant analysis effect size; FEAST, Fast expectation-maximization microbial source tracking; PCoA, principal coordinate analysis; AE, adverse event.

## Declarations

## Ethics Approval and Consent to Participate

Ethics approval was received from the Medical Ethics Committee of the First Affiliated Hospital of Jinan University (No. KY-2020-030), and all studies were conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all study participants or their legal representatives.

## Consent for Publication

Not applicable.

## Availability of Data and Materials

The data generated or analyzed during this study are included in this article and its supplemental information files. Other data and code that support the findings of this study are available from the

corresponding author, upon reasonable request.

## Competing Interests

No potential conflicts of interest were disclosed.

## Funding

This study was sponsored by the National Nature Science Foundation of China [81971120], Natural Science Foundation of Guangdong Province [2018A0303130264], Key Clinical Specialty Discipline Construction of Guangdong [711001] and National Science and Technology Action Plan for Prevention and Control of Major Diseases [ZX-01-C2016083].

## Author Contributions

YL and XMX performed the pathological experiments, and collected the samples included in this study. XLZ performed microbiota analyses, and wrote the manuscript. YL and LAH conceived the idea, provided funding support, and guided the experiments. XYL and WCW revised the manuscript. MG and HYQ perform the surgery of mechanical thrombectomy and provided the thrombus sample. SMH, DL and ZJ collected the clinical data and finished the follow-up process. All authors contributed to the article and approved the submitted version.

## Acknowledgements

Not applicable.

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## Tables

Table 1

Characteristics of patients with LVO (n=104).

Parameters	Value
Age, years	65 (58.25-74.75)
Sex (male)	60 (57.7%)
IVT	27 (26%)
Time to arrival, minutes	360 (230.5-592)
Time to reperfusion, minutes	454.5 (292.25-627.75)
Preoperative NIHSS	16.5 (13.25-20)
Discharged NIHSS	8 (3-12)
Hospital stays, days	12 (8-15)
TOAST	
CE	45 (43.3%)
LAA	59 (56.7%)
Patient history	
Smoke exposure	41 (39.4%)
Alcohol abuse	16 (15.4%)
History of stroke	21 (20.2%)
Comorbidities	
Diabetes mellitus	35 (33.7%)
Hypertension	71 (33.7%)
Atrial fibrillation	39 (37.5%)
Heart disease	35 (33.7%)
Adverse events	39 (37.5%)
Poststroke infection	56 (53.8%)
Affected vessel	
ICA	41 (39.4%)
MCA	40 (38.5%)
BA	21 (20.2%)
Clinical outcome	
90-day mRS	3 (1-4)

90-day survival	87 (83.7%)
LVO, large vessel occlusion. IVT, intravenous thrombolysis. ICA, internal carotid artery. MCA, middle cerebral artery. BA, basilar artery. TOAST, Trial of Org 10172 in Acute Stroke Treatment. CE, cardiogenic embolism. LAA, large-artery atherosclerosis. mRS, modified Rankin Scale. NIHSS, National Institute of Health Stroke Scale.	

Table 2

The distribution pattern of stroke classification.

	Intracellular clustering	Extracellular clustering	Free distribution
CE group (n=29)	6.5 (3)	3.5 (3)	11 (4)
LAA group (n=29)	2.5 (1)	3 (1)	29 (15)
P value	< 0.001	< 0.001	< 0.001
The P values were determined by the Mann-Whitney U test. CE, cardiogenic embolism. LAA, large-artery atherosclerosis.			

Table 3

Comparison of inflammatory factors and markers of intestinal barrier integrity.

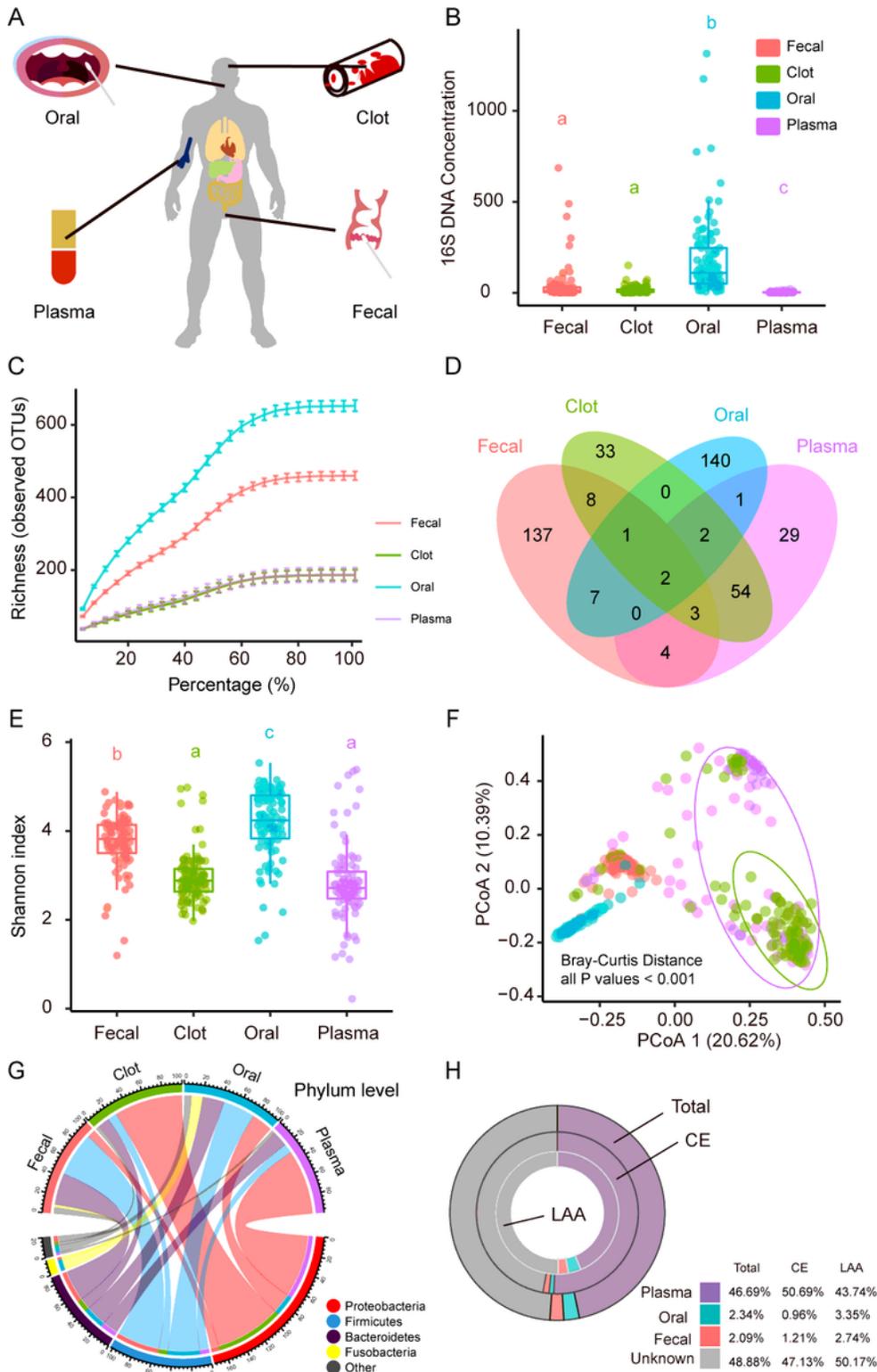
	With_AE group (n=35)	Without_AE group (n=60)	P value	Death group (n=15)	Survival group (n=80)	P value
WBC, 10 <sup>9</sup> /L	10.87 (4.06)	9.83 (4.42)	0.352	11.85 (5.63)	9.83 (4.17)	0.024
CRP, mg/L	9.67 (22.59)	7.85 (12.53)	0.547	13.66 (29.56)	8.36 (14.42)	0.368
IL 1 $\beta$ , pg/mL	41.17 (47.86)	29.56 (29.60)	0.004	56.48 (153.15)	29.56 (26.19)	0.001
IL 6, pg/mL	14.58 (32.12)	3.30 (12.25)	0.002	35.24 (65.10)	5.10 (16.84)	0.001
TNF $\alpha$ , pg/mL	48.77 (15.66)	46.55 (20.15)	0.371	41.70 (35.56)	49.04 (17.28)	0.345
LPS, pg/mL	49.25 (18.53)	48.04 (39.66)	0.963	43.21 (35.56)	49.04 (17.28)	0.571
LBP, $\mu$ g/mL	5.21 (1.76)	4.78 (2.55)	0.357	4.25 (3.43)	4.94 (2.23)	0.664
D-lactate, mmol/L	10.67 (6.03)	9.09 (4.41)	0.343	11.29 (4.61)	9.79 (5.15)	0.386
WBC, white blood cells. CRP, high-sensitivity C-reactive protein. Data are presented as the median (IQR). The P values were determined by the Mann-Whitney U-test. LPS, lipopolysaccharide. LBP, lipopolysaccharide-binding proteins. AE, adverse event.						

Table 4

Cox regression analysis of risk factors associated with 90-day mortality in LVO stroke patients (n=100).

Parameters	Univariate		Multivariate	
	HR (95% CI)	P value	aHR (95% CI)	P value
Candidate variables				
Creatinine, $\mu\text{mol/L}$	1.014 (1.001-1.027)	0.034		
WBC, $10^9/\text{L}$	1.192 (1.068-1.330)	0.002		
NEU, $10^9/\text{L}$	1.223 (1.094-1.367)	0.0004		
Hospital stay, days	0.840 (0.747-0.945)	0.004		
TC, $\text{mmol/L}$	0.586 (0.356-0.964)	0.036		
Preoperative NIHSS	1.088 (1.035-1.144)	0.001	1.140 (1.067-1.218)	0.0001
Adverse event	4.331 (1.504-12.474)	0.007		
Poststroke infection	3.885 (1.107-13.640)	0.034		
Atrial fibrillation	3.372 (1.188-9.011)	0.022	4.583 (1.258-16.695)	0.021
History of stroke	4.666 (1.748-12.457)	0.002		
The abundance of specific taxa in the thrombus (z-score)				
<i>Acinetobacter</i>	1.957 (1.198-3.196)	0.007	2.664 (1.384-5.129)	0.003
<i>Moraxellaceae</i>	1.554 (1.036-2.332)	0.033		
<i>Pseudomonadales</i>	1.502 (1.024-2.204)	0.038		
HR, Hazard ratio. aHR, adjusted hazard ratio. 95% CI, 95% confidence interval. LVO, large vessel occlusion. WBC, white blood cell. NEU, neutrophil. TC, total cholesterol. NIHSS, National Institute of Health Stroke Scale.				

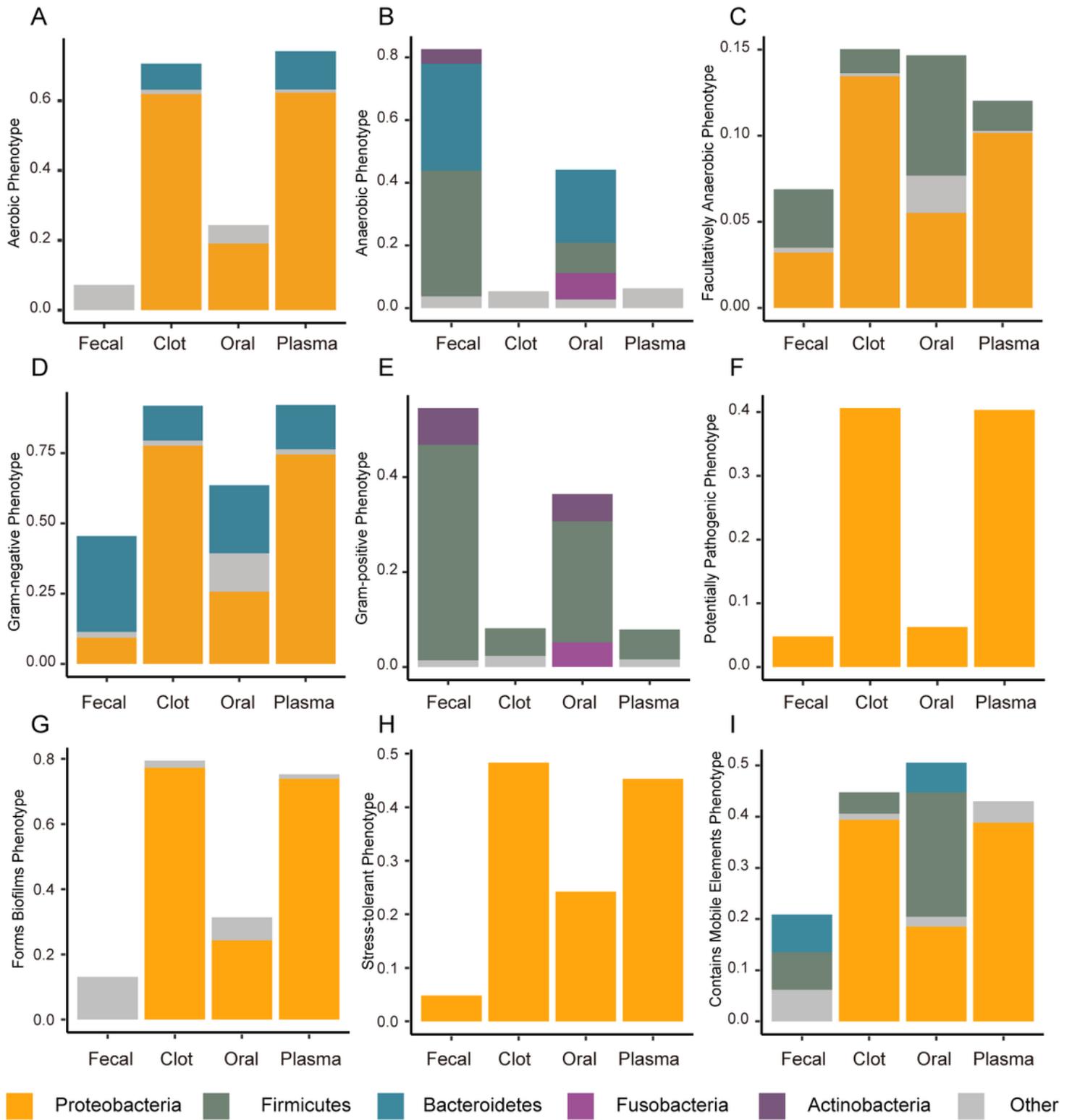
## Figures



**Figure 1**

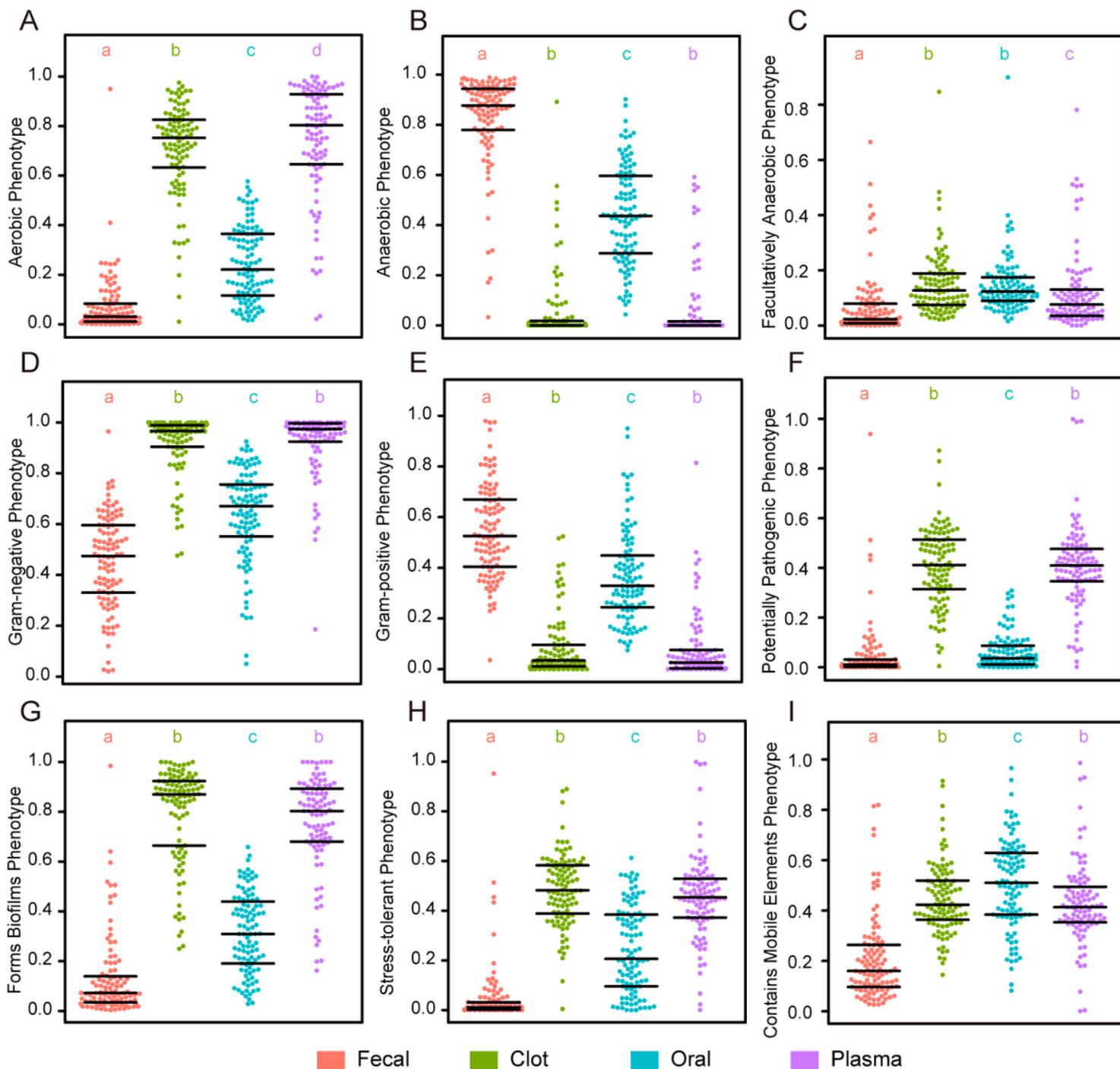
Bacterial composition of the different communities among the four body habitats. (A) Schematic diagram of the body sites of the bacterial communities in the present study. (B) The concentration of bacterial DNA obtained from the different samples. The significance among the four groups was determined by the Kruskal-Wallis test, and subsequent pairwise comparisons were adjusted by Bonferroni correction. (C) Rarefaction curves of detected bacterial OTUs among the four groups reaching the

saturation stage with increasing sequencing depth. Each vertical bar represents standard error. (D) A Venn diagram representing the overlapping OTUs in the different groups, where OTUs with a relative abundance greater than 0.1% were selected. (E) shows the Shannon index of each group. The biodiversity difference in the microbiota was tested by ANOVA, and the following pairwise comparison was performed by Tukey's HSD test. (F) The plot of the Bray-Curtis distance was scored on the relative abundance of OTUs (97% similarity level), and significance was determined by the Adonis test. The relative abundances of the dominant taxa among the four regions at the phylum level (G). (H) Source tracker of the microbiota in the thrombus. Based on the FEAST algorithm, the proportions of bacterial sources from plasma, fecal, and oral samples were calculated. In terms of the CE group, an increased proportion of bacteria were derived from plasma compared with the LAA group. The lowercase letters a, b, and c were used to represent the differences in compared groups. Different letters represent significant differences between the two groups; otherwise, the same letters represent no differences. Each dot denotes a sample. OTU, operational taxonomic unit. FEAST, fast expectation maximization for microbial source tracking. CE, cardiogenic embolism. LAA, large-artery atherosclerosis.



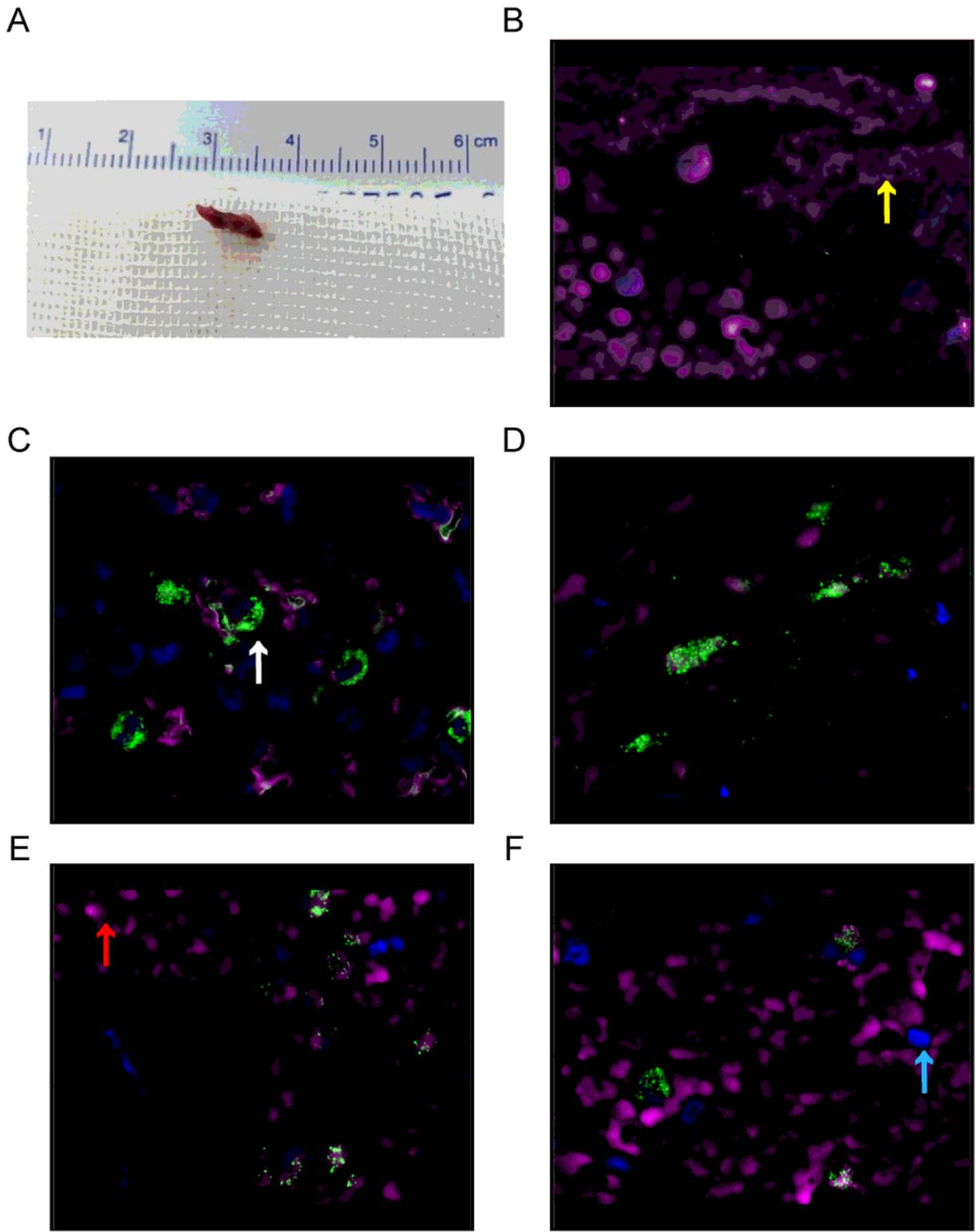
**Figure 2**

Bacterial composition of different body sites predicted by BugBase. Bacterial composition of each group, including Gram-positive, Gram-negative, Biofilm-forming, Pathogenic Potential, Mobile Element-containing, Oxygen Utilizing, and Oxidative Stress-tolerant.



**Figure 3**

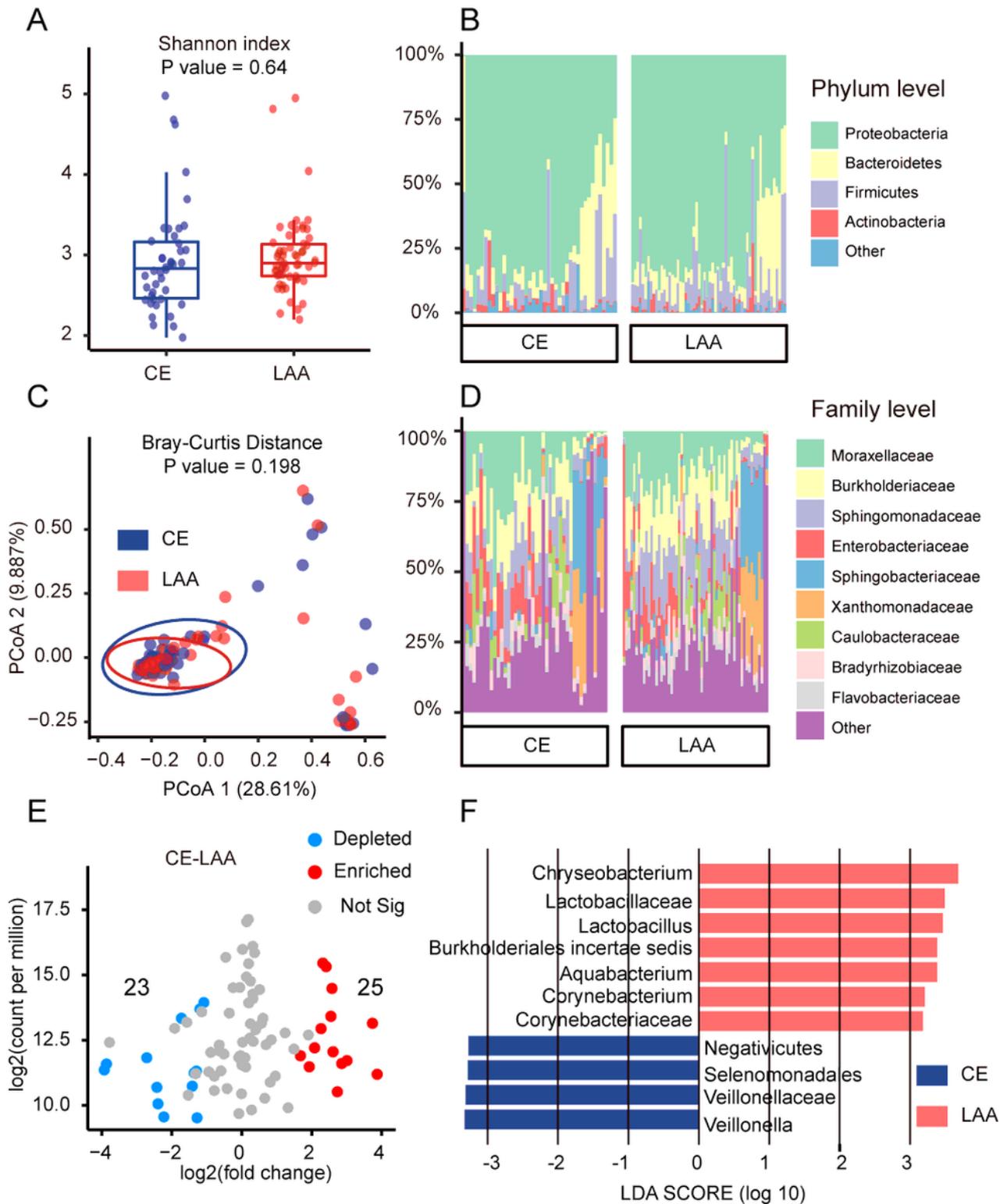
The bacterial compositions predicted by BugBase in different body sites. The significance among the four groups was determined by the Kruskal-Wallis test, and subsequent pairwise comparisons were adjusted by FDR correction. The lowercase letters a, b, c, and d were used to represent the differences in compared groups. Different letters represent significant differences between the two groups; otherwise, the same letters represent no differences. The data are summarized as the quartile and mean. FDR, false discovery rate.



**Figure 4**

Bacterial signature in the thrombus sample via fluorescence in situ hybridization (FISH). A. The classic appearance of a thrombus retrieved from a stroke patient by mechanical thrombectomy. B-F. The general composition of the thrombus in FISH was visualized by fluorescence microscopy (magnification,  $\times 1000$ ). The green fluorescence signal is the bacteria in the clot (white arrow), purple-red irregular round cells are red blood cells (red arrow), sheet of purple briquette is fibrin (yellow arrow), and the blue shapes are DAPI-

stained white blood cells or macrophages (blue arrow). Among them, C and D show typical bacterial distribution patterns in patients with cardiogenic embolism stroke, while E and F exhibit classic bacterial distribution patterns in patients with large-artery atherosclerosis stroke.



**Figure 5**

The microbial characteristics of stroke classification. No significant differences were found in the alpha diversity (A) and beta diversity (B) between the CE and LAA groups. The relative abundance of dominant

taxa among the four regions at the phylum level (B) and family level (D). (E) Numbers of differentially enriched OTUs with an average abundance higher than 0.1% between the CE and LAA groups with an FDR < 0.05 are presented as volcano plots. Each point represents an individual OTU, and the position along the y axis represents the log2 value of average abundance. (F) Significantly discriminative taxa between the CE and LAA groups were determined using linear discriminant analysis effect size (LEfSe) analysis. FDR, false discovery rate. CE, cardiogenic embolism. LAA, large-artery atherosclerosis.

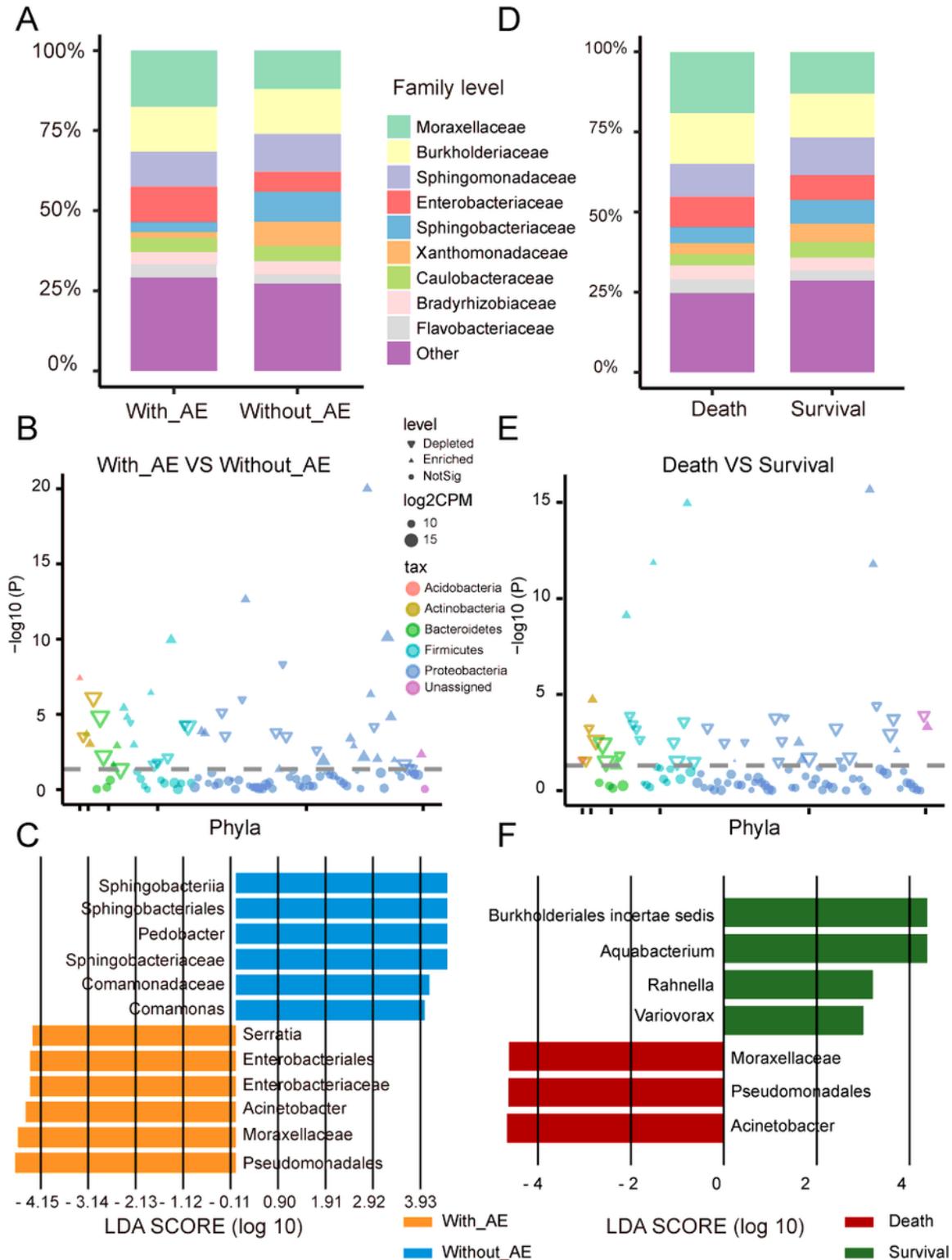


Figure 6

The microbial comparison between the different clinical outcomes of stroke patients. (A, B, C) The microbial characteristics between the With\_AE and Without\_AE groups. (D, E, F) The microbial features between the death and survival groups. (A, D) The relative abundance of dominant taxa at the family level. (B, E) Differential OTUs with an average abundance higher than 0.1% between the two groups with an FDR < 0.05 are shown by a Manhattan plot. (C, F) Significantly discriminative taxa were identified using linear discriminant analysis effect size (LEfSe) analysis. AE, adverse event.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supplementarytable1.Clinicaldata.xlsx](#)
- [Supplementarytable2.OTUtable.xlsx](#)
- [Supplementarytable3.Taxonomydata.xlsx](#)