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Oral Microbiota Dysbiosis and Increased Inflammatory Cytokines with Different Stroke Subtypes

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Research Article

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ABSTRACT

Background: The possible correlation between oral microbiota dysbiosis and acute ischemic stroke, regarding different pathogenesis and stroke severity, remains unclear. Therefore, this study aimed to identify the specific microbiota for different subtypes of stroke to discover the underlying risk factors for ischemic stroke, which is of important clinical research value.

Methods: Oral microbiota communities from 162 stroke patients and 62 stroke-free controls were prospectively assessed by sequencing the V3–V4 region of the *16S DNA* gene. Demographic and clinical data were obtained for both groups. Triglycerides, total cholesterol, low density lipoprotein, homocysteine, high-sensitivity C-reactive protein, SLCO1B1, APOE, CYP2C19, IL6, IL8, IL1β, TNF-α, and sCD40L were measured, and their relationship with oral microbiota was analyzed. Cranial magnetic resonance and carotid artery ultrasound were performed for both groups within seven days of admission.

Results: IL6, IL8, IL1β, TNF-α, and sCD40L were significantly higher in stroke patients than in controls. Although the oral microbiota of the stroke and control groups were similar in diversity and structure, that of the severe stroke (national institutes of health stroke scale score>5) and cardio-embolic stroke subgroups differed from those of the control group. Linear discriminant analysis effect size analysis showed that *Megasphaera*, *Prevotella*, *Clostridia*, *Selenomonas*, *Prevotella*, and *Dialister* were mainly enriched in the severe stroke subgroup. *Prevotella*, *Staphylococcus*, and *Peptostreptococcus* were significantly enriched in the cardio-embolic stroke subgroup. Spearman correlation analysis revealed that IL6, IL8, IL1β, TNF-α, and sCD40L were significantly correlated with *Peptostreptococcus*, *Staphylococcus*, *Selenomonas*, *Megasphaera*, and other bacteria ($p<0.01$; $p<0.05$).

Conclusions: The oral microbiota in stroke patients was not significantly different from that in the stroke free controls. However, certain stroke subgroups, such as the severe or cardio-embolic stroke subgroups, exhibited significant oral microbiota dysbiosis, which was associated with elevated inflammatory cytokines.

Keywords: Ischemic stroke; Inflammatory cytokines; Oral microbiota dysbiosis; *Staphylococcus*; Spearman correlation

INTRODUCTION

According to a study conducted in 2010, approximately 50 million stroke survivors worldwide suffer certain functional disabilities, with 25%-74% requiring assistance or relying entirely on a caregiver for daily living activities. Therefore, investigating the risk factors for ischemic stroke is essential. In addition to traditional risk factors such as hypertension, hypercholesterolemia, diabetes mellitus, smoking, and obesity, bacterial inflammation may contribute directly or indirectly to atherosclerosis and atherothrombotic development [1]. Bacterial inflammation can also promote Atrial Fibrillation (AF) development, leading to cardio-embolic stroke. Oral bacterial inflammatory diseases, such as periodontal diseases, have been correlated with ischemic stroke. In the analysis of the Atherosclerosis Risk in Communities (ARIC) database, Sen, et al., revealed that periodontal diseases were not only associated with stroke but were also significantly associated with cardio-embolic and thrombotic stroke subtypes.

These findings suggest that oral microbiota dysbiosis may increase opportunistic pathogenic infection, consequently contributing to stroke development. However, the actual composition of oral bacterial communities in stroke patients remains unknown. Therefore, identifying specific stroke microbiota to discover underlying risk factors may significantly promote stroke prevention [2].

MATERIALS AND METHODS

Research participants and sample collection

In total, 224 patients (162 acute ischemic stroke patients and 62 controls with other neurological diseases or symptoms) from the department of neurology of the first affiliated hospital of Jinan university were prospectively included in this study. The inclusion criteria for stroke patients were those aged ≥ 18 years old; those diagnosed with acute ischemic stroke; those with stroke subtypes classified by Trial of Org 10172 in Acute Stroke Treatment (TOAST); and those within seven days of stroke onset. Patients in the control group were those ≥ 18 years; those diagnosed with benign paroxysmal positional vertigo, syncope, or cephalgia; and those without a history of stroke. The exclusion criteria for stroke patients and controls included the use of antibiotics or probiotics within one month prior to enrolment; those with upper respiratory tract infection, lung infection, or other infectious diseases; and those with dementia, mood disorders, schizophrenia, or other psychological or mental disorders. The first fasting blood and oral samples were obtained from all participants within 24 h after admission [3]. All participants underwent carotid artery ultrasonography and brain Magnetic Resonance Imaging (MRI).

In this study, the ischemic stroke group was divided into different subgroups. According to previous studies, stroke severity was classified into mild and severe based on the National Institutes of Health Stroke Scale score (NIHSS). The stroke severity group was labeled the SS group. The specific classification criteria included, mild stroke (SS1, n=96), NIHSS score of 0-5(7-9); severe stroke (SS2, n=66), NIHSS score of >5. According to TOAST classification, stroke patients were labeled ST group and divided into four subgroups, including the large artery atherosclerosis subgroup (ST1, n=79), small arterial occlusion subgroup (ST2, n=58), cardio-embolic stroke subgroup (ST3, n=20), and other subgroups (ST4, n=5). Owing to the small number of patients in the ST4 subgroup, it was excluded from statistical analysis [4].

Fasting Ethylenediamine Tetraacetic Acid (EDTA) plasma samples, buccal mucosa samples, and associated clinical data were obtained for all participants. Triglycerides (TG), Total Cholesterol (TC), Low Density Lipoprotein (LDL), Homocysteine (HCY), high sensitivity C-Reactive Protein (hs-CRP), SLCO1B1, APOE, and CYP2C19 were measured in the hospital. Plasma aliquots were centrifuged and frozen at -80°C for IL6, IL8, IL1β, TNF-α, and sCD40L measurements. Oral mucosa sample aliquots were frozen at -80°C immediately after collection. All participants (or their direct relatives) provided written informed consent, and the ethics committee of the first affiliated hospital of Jinan university approved all study protocols (KY-2020-030) [5].

DNA extraction, 16S sequencing and data analyses

Bacterial DNA was extracted from the buccal mucosa samples using a bacterial dna extraction mini kit (Mabio), and DNA purity and concentration were detected using Thermo NanoDrop One (Thermo Fisher Scientific, MA, USA). The V3-V4 regions of the *16S DNA* genes were amplified using a specific primer (338F) with a 12bp barcode. The process and parameters of the techniques such as amplicon generation, PCR product detection, pooling and purification, library preparation and sequencing and data analyses were used as described in our previous study and other researcher's study [6,7].

Statistical analyses

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 27.0. The t-

test was used to analyze continuous variables, the *Chi-square* test was used for categorical data, and the rank sum test was used for rank data. Statistical differences in bacterial species or OTUs between groups were compared using the Mann-Whitney U test (two groups) or the Kruskal-Wallis test (more than two groups). Spearman's correlation test was used to determine the correlation between blood test indices and different bacterial genera; p-values <0.05 were considered statistically significant [8-15].

RESULTS

Clinical characteristics of stroke and control groups

In the stroke group, the median age was 65 years (Interquartile Range (IQR), 55-73), and 105 (64.8%) were male. In the control group, the median age was 63 years (IQR. 55-70), and 25 (40.3%) were male. There were no significant differences in age (p=0.602), hypertension (p 0.092), diabetes (p=0.293), or coronary artery disease (p=0.856) between the two groups. Total cholesterol, triglyceride, low density lipoprotein, and homocysteine levels were not significantly different between the stroke and control groups; however, sCD40L, IL6, IL8, IL1β, and TNF-α levels in the stroke group were significantly different from those in the control group (p = 0.000) (Table 1) [16].

Table 1. Characteristics of the study participants. Baseline characteristics and inflammation markers of patients and controls.

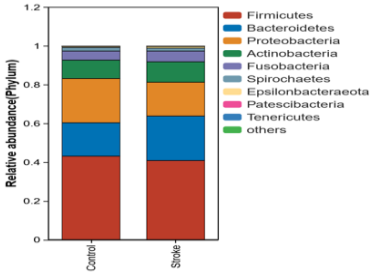
	AIS patients (n=162)	Controls (n=62)	p value
Male, n (%)	105 (64.8%)	25 (40.3%)	0.001
Age, (IQR)	65 (55-73)	63 (55-70)	0.602
History of hypertension, n (%)	106 (65.4%)	33 (53.2%)	0.092
History of diabetes, n (%)	48 (29.6%)	14 (22.6%)	0.293
History of CAD, n (%)	22 (13.6%)	9 (14.5%)	0.856
TC, mmol/L	4.783 ± 0.091	4.524 ± 0.136	0.578
TG, mmol/L	1.562 ± 0.808	1.175 ± 0.149	0.832
LDL, mmol/L	2.856 ± 0.065	2.493 ± 0.094	0.305
HCY	11.238 ± 0.715	9.083 ± 1.058	0.597
hs-CRP	9.001 ± 1.304	5.800 ± 2.031	0.502
sCD40L, ng/ml	17.118 ± 0.475	10.240 ± 0.466	0
IL8, pg/mL	104.752 ± 2.748	60.802 ± 4.244	0
IL1β, pg/mL	54.807 ± 1.918	32.830 ± 2.922	0
IL6, pg/mL	25.951 ± 0.989	11.186 ± 1.538	0
TNF-α, pg/mL	64.468 ± 1.634	36.903 ± 1.940	0

Note: IQR: Interquartile Range; CAD: Coronary Artery Disease; TC: Total Cholesterol; TG: Triglyceride; LDL: Low Density Lipoprotein; HCY: Homocysteine

Microbiota structure and diversity between different stroke subtypes and control groups

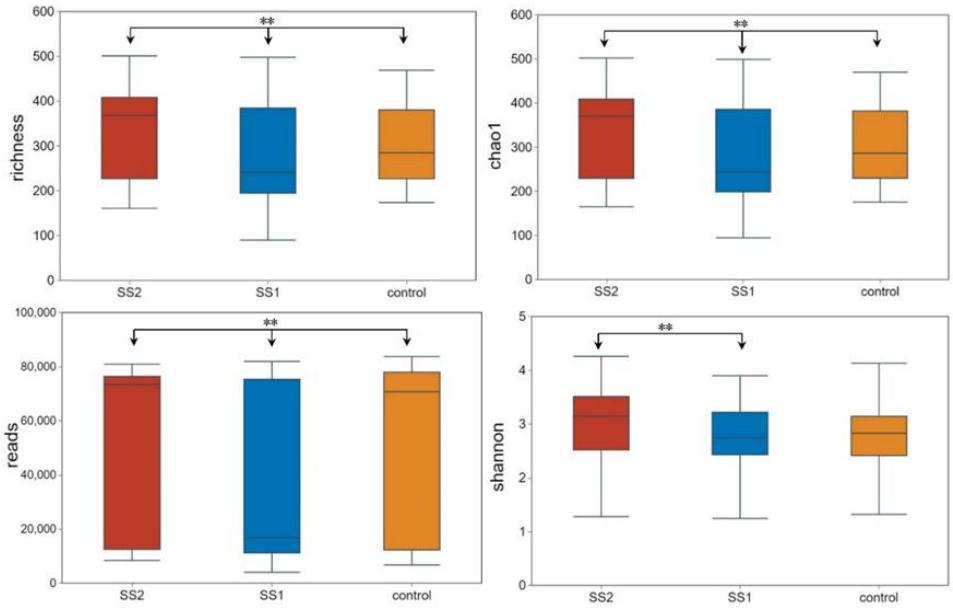
At the phylum level, the oral microbiota in the stroke group was primarily composed of *Firmicutes* (40.9%), *Bacteroidetes* (22.9%), *Proteobacteria* (17.6%), *Actinobacteria* (10.4%), and *Fusobacteria* (5.6%), and that in the control group was primarily composed of *Firmicutes* (43.2%), *Proteobacteria* (22.8%), *Bacteroidetes* (17.2%), *Actinobacteria* (9.6%), and *Fusobacteria* (4.5%) (Figure 1) [17].

Figure 1. Taxonomic summary of the oral microbiota of stroke patients and controls at phylum level.



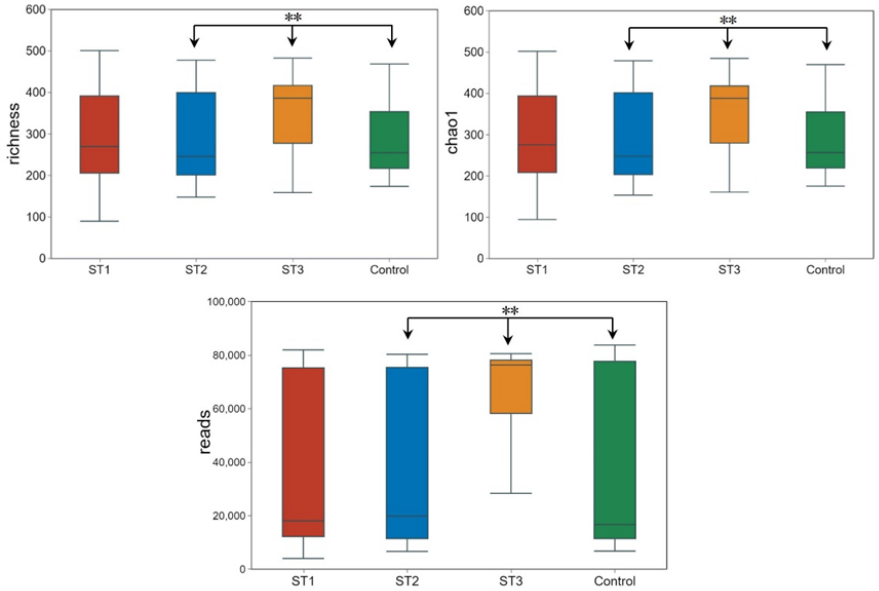
No significant differences in α-diversity were observed between the stroke and control groups. However, statistical significance was attained with Chao1, richness, and read indices between the SS2 subgroup and control ($p<0.05$). Regarding Chao1, richness, read index, and Shannon indices, the SS2 subgroup had greater diversity than the SS1 subgroup ($p<0.05$) and the ST3 subgroup had greater diversity with statistical significance than the control and ST2 subgroups (p 0.05) (Figures 2 and 3).

Figure 2. The α-diversity indices of the oral microbiota in the SS subgroup and control group. Four indices were used to represent the a-diversity which is richness, chao1, reads and Shannon index. Each box plot represents the median, interquartile range, minimum, and maximum values.



Note: SS1=mild stroke subgroup, SS2=severe stroke subgroup. **p<0.05.

Figure 3. The α-diversity indices of the oral microbiota in the ST subgroup and control group. Three indices were used to represent the a-diversity which is richness, chao1 and reads index. Each box plot represents the median, interquartile range, minimum, and maximum values.

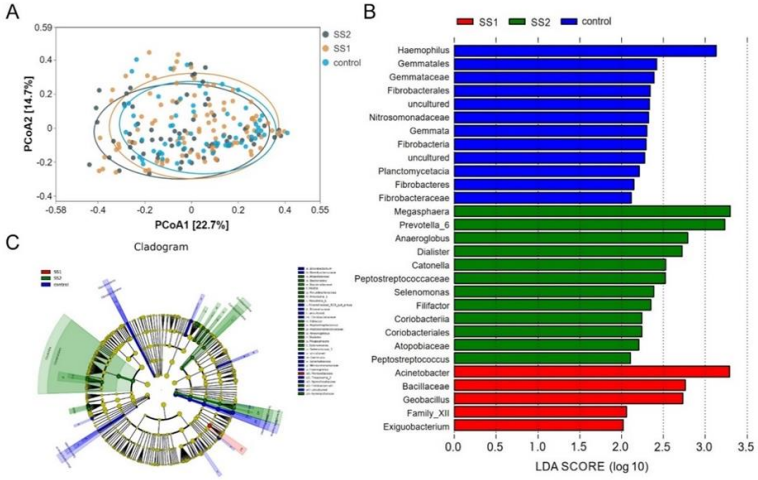


Note: ST1=Large artery atherosclerosis subgroup, ST2=Small arterial occlusion subgroup, ST3=Cardio-embolic stroke subgroup. **p<0.05.

In addition, the PCoA revealed no differences between the stroke and control groups. Further analysis showed that the SS2 subgroup differed from the SS1 subgroup (Adonis, R=0.0194; p=0.005) and the control group (Adonis, R=0.0264;

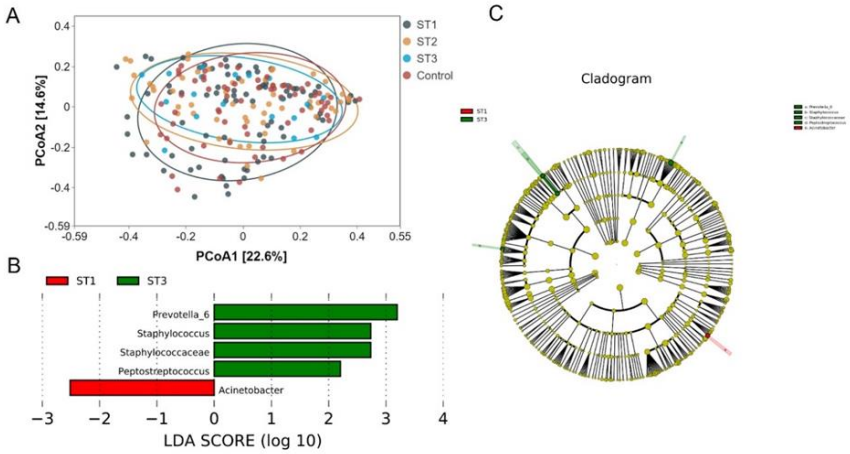
p=0.007) (Figure 4). The ST3 (Adonis, R=0.0315; p=0.016) subgroup also differed from the control group (Figure 5) [18].

Figure 4. A: Principal Coordinate Analysis (PCoA) plots of bacterial β-diversity based on the Bray-Curtis distances. Each circle represents a sample, and the corresponding group can be found in the legend. B: Linear Discriminant Analysis (LDA) effect size (LEfSe) analysis revealed significant bacterial differences in oral microbiota between the SS subgroup and control group. C: Cladogram using the LEfSe method indicating the phylogenetic distribution of oral microbiota associated SS subgroups and control group.



Note: SS1=mild stroke subgroup, SS2=severe stroke subgroup.

Figure 5. A) Principal Coordinate Analysis (PCoA) plots of bacterial β-diversity based on the Bray-Curtis distances. Each circle represents a sample, and the corresponding group can be found in the legend; B) Linear discriminant analysis (LDA) effect size (LEfSe) analysis revealed significant bacterial differences in oral microbiota between the ST subgroups and control group; C) Cladogram using the LEfSe method indicating the phylogenetic distribution of oral microbiota associated ST subgroups and control group.



Note: ST1=Large artery atherosclerosis subgroup, ST2=Small arterial occlusion subgroup, ST3=Cardio-embolic stroke subgroup. **p<0.05.

In this study, the sex distributions of the stroke and control groups were improperly balanced. To determine the possible effect of sex on oral microbiota, 38 age and sex matched pairs of samples were selected from the control and stroke groups and PCoA maps for the control and stroke groups were re-obtained (Table 2). The results showed no significant difference in the sample structure between the two groups after one-to-one matching of sex and age (Adonis, R=0.0215; p=0.095) (Figure 6). These results indicate that sex had no significant effect on the oral microbiota in this study [19].

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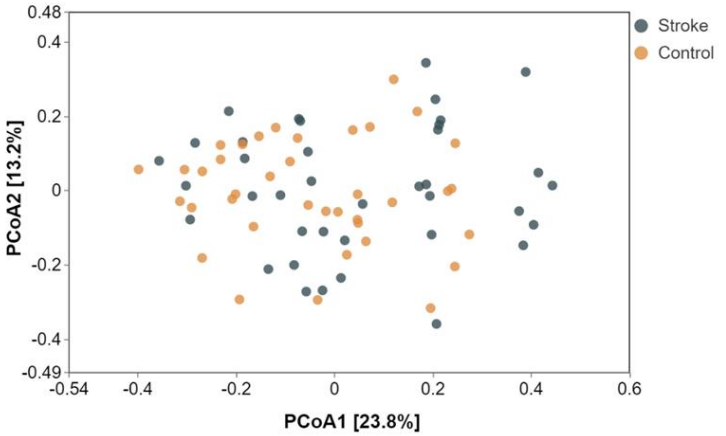
Table 2. Samples adjust for sex and age.

Sample ID	Group	Sex	Age	Sample ID	Group	Sex	Age
S172	Stroke	Male	42	C57	Control	Male	42
S168	Stroke	Male	43	C17	Control	Male	43
S171	Stroke	Male	50	C64	Control	Male	50
S178	Stroke	Male	52	C54	Control	Male	52
S8	Stroke	Female	53	C6	Control	Female	53
S146	Stroke	Male	55	C17	Control	Male	55
S149	Stroke	Female	55	C3	Control	Female	55
S101	Stroke	Male	55	C11	Control	Male	55
S43	Stroke	Male	57	C51	Control	Male	57
S90	Stroke	Male	58	C39	Control	Male	58
S46	Stroke	Female	61	C62	Control	Female	61
S95	Stroke	Male	62	C52	Control	Male	62
S177	Stroke	Female	62	C56	Control	Female	62
S67	Stroke	Male	63	C15	Control	Male	63
S51	Stroke	Male	63	C29	Control	Male	63
S4	Stroke	Female	63	C46	Control	Female	63
S50	Stroke	Female	63	C60	Control	Female	63
S174	Stroke	Male	63	C69	Control	Male	63
S126	Stroke	Male	64	C25	Control	Male	64
S173	Stroke	Male	65	C66	Control	Male	65
S133	Stroke	Female	65	C63	Control	Female	65
S24	Stroke	Female	66	C12	Control	Female	66
S91	Stroke	Female	66	C23	Control	Female	66
S129	Stroke	Female	66	C33	Control	Female	66
S141	Stroke	Female	67	C27	Control	Female	67
S176	Stroke	Female	69	C18	Control	Female	69
S76	Stroke	Female	70	C8	Control	Female	70
S136	Stroke	Female	70	C50	Control	Female	70
S140	Stroke	Male	70	C58	Control	Male	70
S112	Stroke	Male	71	C19	Control	Male	71
S162	Stroke	Female	72	C65	Control	Female	72
S153	Stroke	Male	72	C68	Control	Male	72
S118	Stroke	Male	76	C26	Control	Male	76
S175	Stroke	Male	81	C10	Control	Male	81
S157	Stroke	Female	82	C2	Control	Female	82
S130	Stroke	Male	84	C22	Control	Male	84
S109	Stroke	Female	84	C47	Control	Female	84
S87	Stroke	Male	88	C59	Control	Male	88

Figure 6. Principal coordinate analysis of 38 pairs of age and sex matched controls and stroke patients based on unweighted UniFrac.



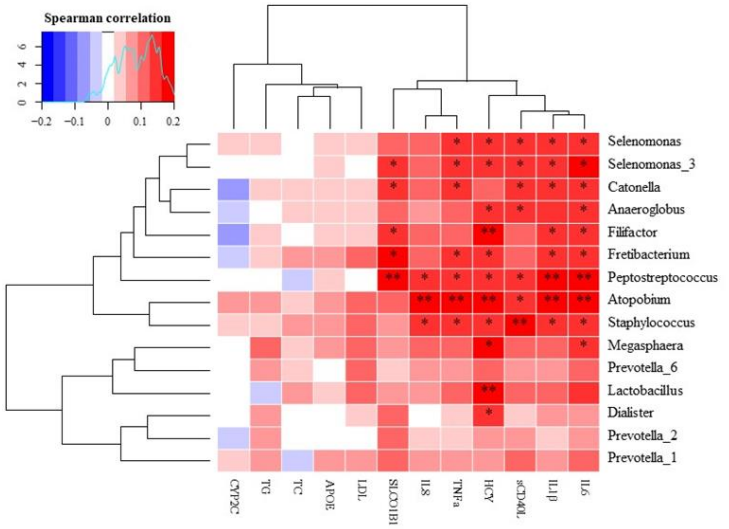
Oral microbiota alteration between different stroke subtypes and control groups

LEfSe analysis showed that *Megasphaera*, *Prevotella*, *Clostridia*, *Selenomonas*, *Prevotella*, *Dialister*, *Anaeroglobus*, *Catonella*, *Selenomonas*, *Filifactor*, *Coriobacteriales*, *Synergistetes*, *Synergistaceae*, *Atopobiaceae*, *Fretibacterium*, *Atopobium*, and *Peptostreptococcus* were significantly enriched in the severe stroke subgroup, and *Prevotella*, *Staphylococcus* and *Peptostreptococcus* were significantly enriched in the cardio-embolic stroke subgroup (Figures 4 and 5) [20].-

Dysregulated oral microbiota was associated with elevated inflammatory cytokines

The correlation heat map analysis revealed that IL6 was closely related to *Peptostreptococcus*, *Atopobium*, *Staphylococcus*, *Selenomonas*, and *Selenomonas*_3. A significant correlation existed among *Catanella*, *Filifactor*, *Fretibacterium*, *Anaeroglobus* and *Megasphaera* ($p<0.01$; $p<0.05$); IL1β was significantly correlated with *Peptostreptococcus*, *Atopobium*, *Staphylococcus*, *Selenomonas*, *Selenomonas*_3, *Catonella*, *Filifactor* and *Fretibacterium* ($p<0.01$; $p<0.05$); sCD40L was significantly correlated with *Selenomonas*, *Selenomonas*_3, *Catonella*, *Anaeroglobus*, *Peptostreptococcus*, *Atopobium*, and *Staphylococcus* ($p<0.01$; $p<0.05$); TNF-α was significant correlated *Peptostreptococcus*, *Atopobium*, *Staphylococcus*, *Selenomonas*, *Selenomonas*_3, *Catanella* and *Fretibacterium* ($p<0.01$; $p<0.05$); and IL8 was significantly correlated with *Peptostreptococcus*, *Atopobium* and *Staphylococcus* ($p<0.01$; $p<0.05$) (Figure 7) [21].

Figure 7. Spearman correlation analysis heat map between different bacteria and clinical indicators. The horizontal coordinate represents clinical test indicators such as IL6 and IL1β, and the vertical coordinate represents differential bacteria. Red color represents positive correlation, blue represents negative correlation, and the color darker, the correlation higher. *p<0.05, **p<0.01.



DISCUSSION

In this study, the V4 region of *16S rDNA* genes in the buccal mucosa and saliva samples from 162 stroke patients and 62 controls were sequenced. The results demonstrated that specific oral microbiota genera may be associated with severe or cardio-embolic stroke and clarified the correlation between oral microbiota composition changes and host inflammatory response [22].

The oral bacterial community composition in the stroke and control groups at the phylum level was mainly composed of *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, and *Fusobacteria*. The main oral microbiota in this study was consistent with those reported in previous studies [23-28]. Although no significant difference in oral microbiota diversity was observed between the stroke and control groups, the diversity analysis of each subgroup revealed that patients with severe or cardio-embolic stroke may have oral microbiota dysbiosis. The six major genera enriched in the severe stroke group were *Megasphaera*, *Prevotella*_1, *Clostridia*, *Selenomonas*_3, *Prevotella*_6, and *Dialister*. *Prevotella* is a common opportunistic pathogen associated with poor stroke outcomes [29]. Emerging human studies have correlated the increased abundance of *Prevotella* species at mucosal sites with localized and systemic diseases, including periodontitis, rheumatoid arthritis, metabolic disorders, and low grade systemic inflammation. *Prevotella* mediated mucosal inflammation induces systemic dissemination of inflammatory mediators, bacteria, and bacterial products, which may affect stroke outcomes. Moreover, in a study on intestinal microbiota, Yin and Wang et al., found that *Megasphaera* was abundant in patients with stroke. Chervinets, et al., demonstrated that *Clostridia* and *Peptostreptococcus* increased 2-3 times in patients with ischemic stroke. *Prevotella*, *Selenomonas*, and *Dialister* have been identified as periodontal disease pathogens and may be associated with stroke. However, the roles of these microbiotas in stroke mechanisms remain unclear and require further investigation [30].

AF is the main cause of cardio-embolic stroke [31]. AF is the most common persistent arrhythmia in clinical practice, and inflammation is speculated to play an important role in its occurrence and development. Systemic inflammatory markers, characterized by TNF-α, IL6, and CRP, are associated with a higher risk of AF. A previous study showed that *Staphylococcus* is a common pathogenic genus that can induce cross-infection and spread from the mouth to other body parts. Relevant studies found that bacteremia and a low degree of systemic inflammation caused by *Staphylococcus* may be associated with the development of infective endocarditis, which is one of the main causes of AF. Walls, et al., reported that endocarditis caused by *Staphylococcus aureus* and coagulase negative *Staphylococcus* was more likely to induce stroke than other causes [32]. Studies have also shown that *Streptococcus* is associated with cerebral ischemia and that *Peptostreptococcus* can cause bacterial endocarditis. In this study, *Staphylococcus* and *Peptostreptococcus* abundance were increased in cardio-embolic stroke patients, followed by a significant increase in blood inflammatory cytokines, such as IL6, IL8, IL1β and TNF-α. These results indicate that *Staphylococcus* and *Peptostreptococcus* may induce AF by promoting an inflammatory reaction, consequently inducing cardio-embolic stroke [33].

In this study, IL6, TNF-α, and other inflammatory cytokines were highly expressed in patients with ischemic stroke, indicating that these patients had chronic inflammation. Oral microbiota dysbiosis induced oral infectious diseases have been confirmed to induce an upregulation in inflammatory cytokines such as IL6 and TNF-α. Previous studies have also shown that *Prevotella* and *Selenomonas* are not only closely associated with the clinical symptoms of gingivitis but also with elevated levels of inflammatory cytokines such as IL1α, IL1β, IL-1Ra, and lactoferrin in gingival crevicular fluid. In this study, high expression of oral microbiota, such as *Staphylococcus*, *Peptostreptococcus*, *Selenomonas*_3, and *Megasphaera* in the severe and cardio-embolic stroke subgroups was accompanied by the expression of IL6 and/or IL1β, IL8, TNF-α, and other inflammatory cytokines. In addition, bacterial DNA was detected not only in fecal and oral samples but also in plasma and thrombus samples in our previous study. Therefore, it was speculated that the pathogenic link between oral dysbacteriosis and severe or cardio-embolic stroke might be based on two points. First, the upregulated oral microbiota causes low level bacteremia, through which oral bacteria enter the bloodstream of the oral inflammatory site, invade the heart and damage the vascular endothelium. Second, low grade systemic inflammation; the pathogenic bacteria produce pro-inflammatory cytokines, which not only spread into the mouth and saliva but also circulate and promote systemic inflammatory reactions, thus promoting ischemic stroke development. In addition, a previous study found that the microbiota interacts with the host immune system through immune cell polarization, stimulating Microbe Associated Molecular Pattern-Pattern Recognition Receptor (MAMP-PRR) and Aβ-NLPR3-ASC-SPECK signals and inducing anti or pro-inflammatory cytokines, which may play an important role in microbiota pathogenicity.

CONCLUSION

In conclusion, this study suggests that oral microbiota dysbiosis and an increase in blood inflammatory cytokine levels

occur in patients with severe or cardio-embolic stroke, expanding the understanding of the correlation between microbiota dysbiosis, inflammatory cytokines, and neurological diseases, although whether these changes occurred before or after stroke could not be determined. In addition, the causal relationship between dysbiosis and inflammation remains unclear; therefore, oral dysbacteriosis and its long term effect in stroke patients require further investigation.

DISCLOSURE STATEMENT

No potential conflict of interest was reported by the author(s).

AUTHOR CONTRIBUTIONS

Z.J. conceived the study and drafted the manuscript. L.H. sought funding support and guided the study. X.X. and J.G. collected oral mucosa samples and the first fasting blood of patients. J.Y., D.L. and J.L. collected all raw data. S.Z. analyzed data. X.L. and G.C. critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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