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Dysbiosis of Gut Microbiota in Patients with Large-Artery Atherosclerotic Stroke: A Pilot Study

(Gut Dysbiosis in LAA Stroke)

Chatpol Samuthpongtorn[1], Abhinbhen W. Saraya[2,3], Yutthana Joyjinda[3,4], Apaporn Rodpan[3,5], Nijasri C. Suwanwela[6*]

[1]Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

[2]Division of Neurology, Department of Medicine, King Chulalongkorn Memorial Hospital, Bangkok, Thailand

[3]Thai Red Cross Emerging Infectious Diseases-Health Science Centre and WHO Collaborating Centre for Research and Training on Viral Zoonoses, King Chulalongkorn Memorial Hospital, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

[4]WHO-CC for Research and Training on Viral Zoonoses, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

[5]Program in Biotechnology, Faculty of Science, Chulalongkorn University, Bangkok, Thailand

[6]Division of Neurology, Department of Medicine, Chulalongkorn Comprehensive Stroke Center, King Chulalongkorn Memorial Hospital, Bangkok, Thailand

Research Article

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***For Correspondence:** Nijasri C. Suwanwela, Division of Neurology, Department of Medicine, Chulalongkorn Comprehensive Stroke Center, King Chulalongkorn Memorial Hospital, Bangkok, Thailand

E-mail: Nijasris@yahoo.com

Phone number: (+66)89-799-8993

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ABSTRACT

Introduction: Increasing data demonstrate an association between gut microbiome in brain diseases *via* the gut-brain axis. However, few studies have evaluated the association between gut microbiome and Large-Artery Atherosclerotic (LAA) ischemic stroke patients.

Methods: A cross sectional pilot study was conducted among 14 patients with LAA stroke and 15 asymptomatic persons. LAA were diagnosed using TOAST classification. The control group was selected based on age-match and sex-match with the patient group. Participants provided a stool sample profiled by 16S rRNA sequencing. Mann-Whitney U test was used to compare the differences in gut microbiota profile between groups. Alpha-diversity and beta-diversity evaluated gut microbial diversity. Gut microbial genus and stroke were correlated using generalized linear mixed effects models which were adjusted for age, BMI, underlying disease (diabetes, hypertension and dyslipidemia), and alcohol use.

Results: The average age of stroke patients was 61.1 ± 7.1 and 59.2 ± 8.2 in the control group. Beta-diversity (Bray-Curtis dissimilarity) of the gut microbiome was statistically significant in order, family and genus level (P-value=0.017, 0.011 and 0.003, respectively) between stroke and control groups; however, there was no statistically significant difference in alpha-diversity (Shannon diversity index; P-value=0.852). Using generalized linear mix effect model, we found 6 genera were significantly associated with stroke after multivariate adjustment. *Ruminococcus* spp. (P-value=0.017), *Streptococcus* spp. (P-value=0.019), *Actinomyces* spp. (P-value=0.02) and *Dorea* spp. (P-value=0.021) showed positive association while *Bifidobacterium* spp. (P-value=0.04) and *Faecalibacterium* spp. (P-value=0.041) showed negative association with stroke.

Conclusion: Patients with LAA stroke had a decreased microbiome beta-diversity and certain gut microbiota genera may be related to LAA stroke.

Keywords: Stroke; Large-Artery Atherosclerotic; Gut microbiota; Dysbiosis; Sample; Diseases; Patients; Sequencing

INTRODUCTION

Stroke is a major public health concern worldwide, resulting in substantial morbidity, disability, Disability-Adjusted Life Years (DALYs) lost, and mortality for both sexes. In 2014, the prevalence of stroke among adults aged 45 and older was estimated to be 1.88%. The average age of onset for a stroke is 65, and it is responsible for nearly 50,000 deaths annually [1]. There are two most common types of strokes; ischemic stroke, which is caused by a blockage in a blood vessel supplying the brain, accounts for about 80% of all stroke cases, while hemorrhagic stroke, which is caused by bleeding within the brain, accounts for the remaining 20%. These two types of stroke necessitate distinct treatment approaches and management strategies, highlighting the significance of an accurate diagnosis [2]. In 2010, the global burden of disease due to hemorrhagic stroke and ischemic stroke was a staggering 62.8 million DALYs and 39.4 million DALYs, respectively [3].

Among the different types of ischemic stroke, Large-Artery Atherosclerotic (LAA) stroke is the most prevalent, especially in the asian population, where it accounts for approximately 33% of cases. In addition, LAA stroke has the highest annual growth rate, estimated at 5.7% [4]. Several traditional risk factors are associated with the occurrence of LAA stroke, such as advanced age, male sex, hypertension, diabetes mellitus, dyslipidemia, a family history of cardiovascular disease, current smoking, binge alcohol consumption, and obesity [5,6]. Emerging evidence also suggests that the gut microbiome and microbiota-metabolites may contribute to the development of LAA stroke [5,7]. It is believed that the immune response and microbial metabolites play a crucial role in the pathogenesis of LAA stroke, and that they may represent potential therapeutic intervention targets [7,8]. Thus, further investigation of the mechanisms underlying the association between the gut microbiome and LAA stroke is warranted, as it may pave the way for the development of novel preventive and therapeutic strategies for this debilitating condition.

Gut microbiota, which consists of microorganisms such as bacteria, fungi, and viruses, inhabits a variety of regions of the human body, including the gastrointestinal tract. The gut microbiota consists of approximately 10^{13}-10^{14} microbial cells, with *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria* constituting the predominant bacterial phyla [9-11]. The potential association between gut microbiota and stroke has been hypothesized under the model of the gut microbiota-brain axis [12-14]. A previous cohort study examined how ischemic stroke affects the abundance of gut microbiota, finding that *Lactobacillus ruminis* increases with markers of inflammation in stroke patients, while SCFAs (Short-Chain Fatty Acids) decrease [8]. In addition, animal models have demonstrated that occlusion of the MCA for 60 minutes increases intestinal permeability and results in gut dysbiosis [12,13]. However, the etiopathogenesis of the relationship between gut microbiota and stroke remains unknown, and there are few clinical studies examining this association. In order to investigate the potential of gut microbiota as a therapeutic target for ischemic stroke, our study aims to establish an association between gut microbiota and ischemic stroke patients, with a focus on LAA subtypes due to their high prevalence.

MATERIALS AND METHODS

A cross-sectional pilot study was conducted among a total of 29 participants, consisting of 14 acute ischemic stroke patients with Large Artery Atherosclerosis (LAA) and 15 age and sex-matched asymptomatic volunteers, between the years 2019 and 2020. The participants were recruited from the King Chulalongkorn Memorial Hospital (KCMH) stroke unit and an outpatient clinic within the same hospital. Participants were required to provide information on multiple lifestyle variables and their medical history upon enrollment. Using the TOAST classification system, a neurologist diagnosed a stroke caused by LAA within 48 hours after the onset of symptoms. Exclusion criteria included patients who had taken antibiotics within one month prior to stool collection, as well as those with

underlying diseases such as metastatic cancer, autoimmune diseases, immunodeficiency, renal failure, chronic heart failure, and parkinson's disease. The study was approved by the institutional review board of the faculty of medicine, Chulalongkorn University (IRB 029/62). All findings were reported in accordance with the STROBE guidelines (Strengthening the Reporting of Observational Studies in Epidemiology) for cross-sectional studies [15].

Baseline assessment

All participants underwent a comprehensive assessment that measured the followings:

1. Demographic characteristics including age, sex, BMI.
2. Potential risk factors for ischemic stroke, such as hypertension, dyslipidemia, diabetes mellitus, atrial fibrillation, ischemic heart disease, a smoking habit, a history of stroke, a family history of ischemic heart disease/ischemic stroke and alcohol consumption.
3. NIHSS score to assess severity of ischemic stroke.
4. Onset of acute ischemic stroke.
5. Laboratory variables, including lipid profile, HbA1C, creatinine, uric acid.
6. Brain imaging and vascular imaging including Computed Tomography (CT) scan and Computed Tomography Angiography (CTA).
7. Assessment of current medications (antibiotic, anti-hypertensive drugs, statins, proton pump inhibitors/H2 blockers, anti-thrombotic drugs).

Assessment of acute ischemic stroke

We categorized patients with acute ischemic stroke depending on TOAST classification which include Large-Artery Atherosclerosis (LAA), cardioembolism, small vessel occlusion, stroke of other determined etiology and stroke of undetermined etiology [16]. We only included LAA patients with clinical and brain imaging findings of either substantial (>50%) stenosis of a major cerebral artery, presumedly related to atherosclerosis [16].

Biochemical assays

Blood samples were obtained from patients with stroke at the time of admission and from control subjects during the outpatient clinic visit. Serum levels of glycated haemoglobin (HbA1c), High-Density Lipoprotein (HDL) cholesterol, Low-Density Lipoprotein (LDL) cholesterol, Triglyceride (TG), glucose, blood urea nitrogen, creatinine and uric acid were measured.

Gut microbiome

Fecal samples of all stoke participants were collected within 1 day after admission and stored in -80°C within 60 minutes at the Thai red cross emerging infectious disease health science centre laboratory by research assistants. Also, fecal samples from healthy individuals were collected at outpatient clinics and were sent by the same process. DNA extraction from stool samples were carried out, amplified and sequenced.

Bacterial DNA extraction

Approximately 200 mg of stool samples were resuspended in 1 ml of Inhibitex buffer, incubated at 70°C for 5 minutes, and centrifuged at 20,000 × g for 1 minute. The QIAamp fast DNA stool mini kit (QIAGEN) was used to extract bacterial DNA from 200 µl of aqueous phase per the manufacturer's instructions. All of the extracted DNA was quantified using the Qubit™ dsDNA HS test kit on a Qubit 4 Fluorometer (Life Technologies).

16S rRNA (V3-V4) amplification and sequencing

The PCR products were separated on agarose gel electrophoresis, followed by gel extraction and purification using nucleospin gel and a PCR clean up kit (Macherey-Nagel). The indices of Illumina were applied to both ends of PCR products so that samples could be multiplexed. The indexed PCR products were then purified using Agencourt AMPure XP beads (Beckman Coulter, Inc.) and concentration was measured with a Qubit™ dsDNA HS test kit (Life Technologies). Prior to library pooling and sequencing, the accurate size of indexed libraries was determined through QIAxcel capillary electrophoresis (QIAGEN) utilizing paired-end (2 × 301 bp) sequencing on Illumina MiSeq with Illumina V3 reagent kit.

Diversity calculations

The sample-taxa frequency table was re-summarized at the phylum and genus levels by summing read counts belonging to the same phylum or genus together. Operational Taxonomic Units (OTUs) with unassigned phylum or genus were discarded from further analyses at the respective levels. Shannon alpha-diversity indices was calculated for each sample based on their mathematical definitions using vegan R package [17]. We used taxonomic data at the genus, family and order level to conduct a principle coordinates analysis based on Bray-Curtis dissimilarity using the Phyloseq R package. PERMANOVA (Adonis) was used to test for differences in Bray-Curtis beta diversity by stroke diagnosis. To evaluate beta-diversity structure, a phylogenetic tree containing all identified taxa was reconstructed using the phylogeny module in QIIME2.

Statistical analysis

Descriptive data analysis was performed using SPSS Statistics version 21 (SPSS Inc., Chicago, IL, USA). Categorical data were expressed as numbers and percentages. Normally distributed continuous data were presented as means with SD, while non-normally distributed continuous data were reported as medians with inter-quartiles range. AP value of less than 0.05 was considered as statistically significant.

We examined the association between each gut microbial genus and acute ischemic stroke using generalized linear mix effect model with multivariate adjustment for underlying disease including diabetes mellitus, hypertension and dyslipidemia, lifestyle variables including age, BMI, and alcohol intake. Analyses were performed using R version 4.0.1:

Glm (stroke~microbial genus+age+BMI+diabetes+hypertension+dyslipidemia+alcohol intake, family=binomial)

The circular phylogenetic tree was generated using GraPhlAn in Python version 3.6.4 to illustrate the relationship between each microbial species and stroke diagnosis.

RESULTS

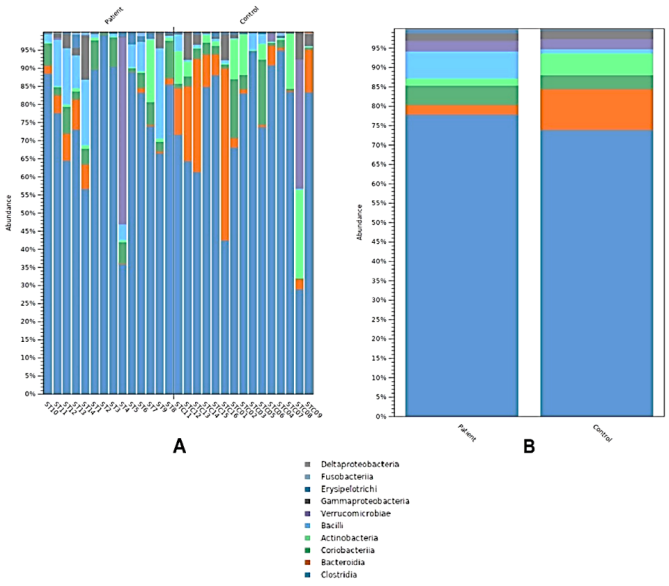
There were 29 participants in the study, 14 in the stroke group and 15 in the control group. The average age of the stroke group was 61.1 ± 7.1 years, while that of the control group was 59.2 ± 8.2 years. Hypertension and dyslipidemia were the most prevalent traditional risk factors for ischemic stroke in both groups, comprising 62% of participants. Baseline characteristics of the patients were shown in Table 1.

Table 1. Baseline characteristics of included participants.

Characteristics	Stroke patients N=14	Control N=15	P-value
Male (%)	85.7	80	1.000
Age (mean ± SD)	61.1 ± 7.1	59.2 ± 8.2	0.720
Risk factors (%)			
History of diabetes mellitus	7.1	13.3	0.543
History of hypertension	35.7	26.7	0.690
History of dyslipidemia	28.6	33.3	0.690
Smoking	14.3	0	0.143
Previous stroke	7.1	0	0.390
Lab investigation (mean ± SD)			
Total cholesterol	181.9 ± 42.9	179.3 ± 28.2	0.842
HDL	38.1 ± 10.4	46.1 ± 17.2	0.145
LDL	115.9 ± 40.1	109.4 ± 25.5	0.948
TG	139.9 ± 38.6	119.0 ± 33.2	0.941
HbA1c	5.7 ± 0.3	5.9 ± 0.7	0.416
NIHSS on admission (mean ± SD)	1.28 ± 1.09	-	-

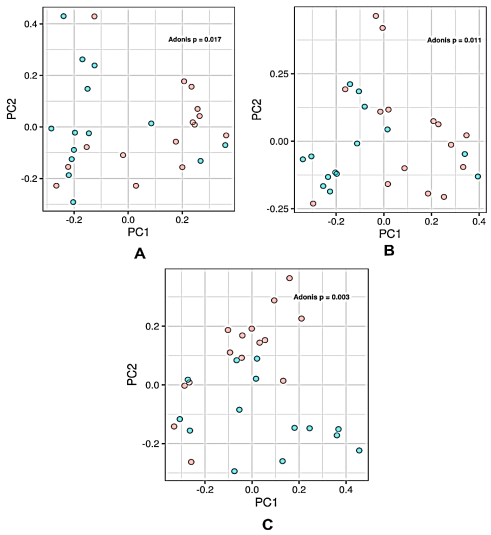
The majority of intestinal bacteria discovered in both patient and control groups were *Clostridia* class, which belong to the phylum *Firmicutes*, according to taxonomic classification at the class level (Figure 1). A significant difference in *Bacteroidota* was found in approximately 7% of the stroke group but only 2% of the control group (Mann Whitney U test; P-value 0.014). (Supplementary Figure 1).

Figure 1. Class-level taxonomic overview of the gut microbiota. (A) Summary of gut microbiota in each specimen; (B) Summary of gut microbiota between stroke and control group. **Note**: *Deltaproteobacteria*; *Fusobacteria*; *Erysipelotrichi*; *Gammaproteobacteria*; *Verrucomicrobiae*; *Bacillus*; *Actinobacteria*; *Coriobacteriia*; *Bacteroidia*; *Clostridia*.



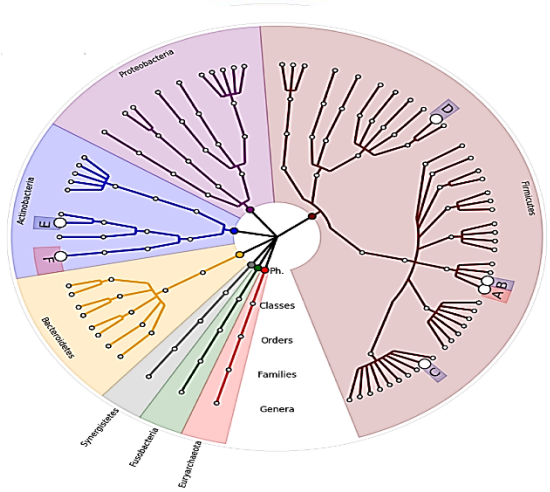
Stool samples were underwent 16S rRNA sequencing and analyses. We found no significant differences between stroke and control groups in alpha-diversity index (Shannon) (P-value 0.852) (Supplementary Figure 2). Beta-diversity evaluation using PCoA showed significant differences at the order family and genus levels between strokes and controls (P-value 0.017, P-value 0.011 and P-value 0.003, respectively) (Figure 2).

Figure 2. Beta-diversity of bacterial (A) order, (B) family, and (C) genus in stroke and control group. PCoA based on Bray–Curtis dissimilarity was performed with the significant differences in gut microbial community structure at the order (P-value 0.017), family (P-value 0.011) and genus levels (P-value 0.003) between stroke and control group. **Note**: ○ No; ○ Yes.



Using generalized linear mix effect models, we found that 6 genera were significantly associated with stroke after multivariate adjustment (P-value <0.05) (4 positive association and 2 negative association). *Ruminococcus* spp. (Beta 18.70, P-value 0.017), *Streptococcus* spp. (Beta 9.25, P-value 0.019) and *Actinomyces* spp. (Beta 63.37, P-value 0.02) and *Dorea* spp. (Beta 46.10, P-value 0.021) showed positive association while *Bifidobacterium* spp. (Beta -4.4, P-value 0.04) and *Faecalibacterium* spp. (Beta -7.16, P-value 0.041) showed negatively association with stroke (Figure 3).

Figure 3. GraPhlAn visualization of the annotated phylogenies and taxonomies compared between stroke and control group for (A) *Faecalibacterium*, (B) *Ruminococcus*, (C) *Dorea*, (D) *Streptococcus*, (E) *Actinomyces*; (F) *Bifidobacterium*. **Note**: ● Negative association; ● Positive association.



DISCUSSION

In this study, a correlation was found between Large-Artery Atherosclerotic stroke and gut microbiome diversity and as well as specific microbial genus. Bray-Curtis dissimilarity was decreased in gut microbial community structures at the order, family and genus in stroke group. Although the underlying mechanism remains unclear, it was hypothesized that communication from gut-microbiota to acute ischemic stroke or from acute ischemic stroke to gut-microbiota or both may be involved [12,13]. Regarding gut-microbiota to stroke communication, low-diversity dysbiosis may modify the metabolic flow of bacteria and their direct interactions with the host immune system [17]. Due to low microbial diversity, many anaerobes in a healthy gut convert complex carbohydrates to short chain fatty acids (SCFAs) [17]. Owing to the anti-inflammatory and cholesterol-blocking effects of SCFAs [18], patients with reduced microbial diversity are more susceptible to LAA stroke than the control group. Regarding the latter hypothesis that stroke leads to gut microbial dysbiosis, a recent study on mice revealed that ischemic stroke affects the gut microbiome, reduces microbial diversity, and boosts the immune system [19]. Also, in an another mice study, reduced microbiome diversity was also found as a characteristic of post-stroke dysbiosis, which was related with decreased intestinal motility [8]. This finding was supported by some hypotheses, the first of which is that the autonomic nerve system mediates the effect of stroke on dysbiosis. Houlden et al. determined that stroke affected the composition of caecal microbiota which these microbiome changes were mediated by the release of noradrenaline from the autonomic nervous system, affecting the synthesis of caecal mucoproteins and the quantity of goblet cells [20]. In addition, it was hypothesized that the post-stroke stress response increases intestinal permeability *via* the production of corticotropin-releasing and glucocorticoid hormones, resulting in enhanced bacterial translocation in the gut [21,22].

We found a negative association between *Bifidobacterium* spp. and stroke. *Bifidobacterium* spp. has been commonly referred to as beneficial bacteria that perform necessary functions in the human colon [23] and was developed as a widely used probiotics [24]. Nowadays, *Bifidobacterium* spp. probiotics are now commonly used to treat irritable bowel syndrome and ulcerative colitis by altering the composition of the microbiota in the gut [25]. Decreased numbers of these species in the colon have been linked to numerous diseases, such as antibiotic-associated diarrhea, irritable bowel syndrome, inflammatory bowel disease, obesity, allergies, and regressive autism. *Bifidobacteria* spp. has served multiple purposes, including the development of the immune system in early life, maintenance of the intestinal barrier, and protection against pathogens [26].

Regarding *Bifidobacterium* spp. and stroke, a number of research have demonstrated that *Bifidobacterium* treatment can effectively enhance the long-term rehabilitation of mice with cerebral ischemia [27]. It was hypothesized, while the mechanism remained unknown, that *Bifidobacterium* spp. generated metabolites of Short-Chain Fatty Acid (SCFA), which can decrease inflammation and improve stroke recovery [28,29]. Also, *Bifidobacterium*-treated mice exhibited an upsurge in a range of metabolites, including prostaglandin B1, which may promote stroke recovery [30,31].

Faecalibacterium spp. abundance were lower in the stroke group than in the control group, according to our findings. *Faecalibacterium prausnitzii* is one of the most essential gut microbiota components in the human colon, which has been considered a bioindicator of human health [32]. Changes in the abundance of *Faecalibacterium prausnitzii* have been linked to dysbiosis in a variety of human disorders [33-35]. As a butyrate-producing bacteria, it was found to be reduced in cardiovascular disease and metabolic syndrome. Butyrate is an essential fatty acid with

a short chain that promotes intestinal health [36]. In addition, this microbe possesses a variety of anti-inflammatory and metabolic properties, which give it an important role in human health [37]. Butyrate protects the intestinal lining, thereby preventing infections from entering the body *via* the gastrointestinal tract. It stimulates the growth of villi and the production of mucin, a protective gel that coats the digestive tract lining [38]. Concerning stroke, studies on mice have demonstrated a correlation. In a previous mice study from China, for instance, *Faecalibacterium* was used for transplantation [39]. These bacteria decreased post-stroke neurological deficits and inflammation and increased SCFA concentrations in the stomach, brain, and plasma of aged mice with stroke [39]. The majority of studies have shown an association between *Faecalibacterium prausnitzii* and stroke; however, our study only analyzed genus data but not species data due to 16S NGS analysis. Therefore, future studies should conduct 16S metagenomic analysis in order to determine the link between *Faecalibacterium prausnitzii* and stroke.

Our findings indicated that four microbial genera (*Ruminococcus* spp., *Streptococcus* spp., *Actinomyces* spp., and *Dorea* spp.) were positively correlated with stroke group. The relationship between these microbial genera and stroke has been demonstrated in a small number of studies. *Ruminococcus* spp. and *Streptococcus* spp. have been associated with metabolic syndrome and cardiovascular disease in certain research [40]. First, Kurilshikov et al. discovered the association between the *Ruminococcus* species and the onset of cardiovascular diseases [41]. The production of L-methionine by *Ruminococcus* species is associated with cardiovascular characteristics in obese people [41]. Second, *Streptococcus* spp., a morbid oral bacterium species, has also been shown to be raised in hypertension [42], and atherosclerotic cardiovascular disease [43,44]. Finally, *Actinomyces* spp. was discovered to be prevalent in obese adolescents [45,46], and a positive association was also identified between *Dorea* spp. and BMI and blood lipids [40]. Consequently, these four genera may have an impact on LAA stroke *via* the cardiovascular disease pathway, thereby contributing to the development of stroke. Even while no studies showed the potential mechanism of these microbial genera, future research should investigate the relationship between these microbial genera and large-artery atherosclerotic stroke. Our study had some limitations. First, our study had small sample size which may affect our results. Second, we have no metabolomic data in our databases and the causality between this associations have not been conducted due to the cross-sectional design of the study. For future research to clarify the relationship between gut microbiota, its metabolites, and stroke, cohort studies with a metabolomic profile should be implemented. Finally, our research is based on the results of 16S rRNA sequencing within the limitations of resolution. A subsequent study is planned to compare these findings, which should broaden our understanding through functional microbiome analysis. The long-term objective of this research is to develop metatranscriptomic and metatranscriptomic analysis between gut microbiome and large-artery atherosclerotic stroke.

CONCLUSION

Our findings suggests that patients with large-artery atherosclerotic stroke had a decreased beta-microbiome diversity, and certain gut microbiota genera may be related to large-artery atherosclerotic stroke. Future implications of this study could include the development of targeted interventions to modulate gut microbiota in order to improve outcomes for patients with large-artery atherosclerotic stroke.

ABBREVIATIONS

BMI: Body Mass Index; CT: Computed Tomography; CTA: Computed Tomography Angiography; LAA: Large-Artery Atherosclerosis; NIHSS: National Institute of Health Stroke Scale; PCR: Polymerase Chain Reaction; SD: Standard Deviation; TOAST: Trial of Org 10,172 in Acute Stroke Treatment.

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

All participants provided written informed consent at the time of data collection. The study was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University (IRB 029/62) and adheres to the tenets of the Declaration of Helsinki. All participants data were fully anonymized.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

AUTHORS CONTRIBUTIONS

Chatpol Samuthpongtorn: Data gathering, interpretation of data, draft writing, editing, and revision of manuscript. Abhinbhen W. Saraya: Study conception, supervision, editing, and revision of manuscript. Yutthana Joyjinda: Interpretation of data. Apaporn Rodpan: Interpretation of data. Nijasri C. Suwanwela: Study conception, interpretation of data, supervision, editing, and revision of manuscript.

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DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this article. Further enquiries can be directed to the corresponding author.

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