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Altered salivary microbiota profile in patients with abdominal aortic aneurysm

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ABSTRACT

Evidence suggests that the DNA of oral pathogens is detectable in the dilated aortic tissue of abdominal aortic aneurysm (AAA), one of the most fatal cardiovascular diseases. However, the association between oral microbial homeostasis and aneurysm formation remains largely unknown. In this study, a cohort of individuals, including 53 AAA patients and 30 control participants (CTL), was recruited for salivary microbiota investigation by 16S rRNA gene sequencing and bioinformatics analysis. Salivary microbial diversity was decreased in AAA compared with CTL, and the microbial structures were significantly separated between the two groups. Additionally, significant taxonomic and functional changes in the salivary microbiota of AAA participants were observed. The genera Streptococcus and Gemella were remarkably enriched, while Selenomonas, Leptotrichia, Lautropia and Corynebacterium were significantly depleted in AAA. Co-occurrence network analysis showed decreased potential interactions among the differentially abundant microbial genera in AAA. A machine-learning model predicted AAA using the combination of 5 genera and 14 differentially enriched functional pathways, which could distinguish AAA from CTL with an area under the receiver-operating curve of 90.3 %. Finally, 16 genera were found to be significantly positively correlated with the morphological parameters of AAA. Our study is the first to show that AAA patients exhibit oral microbial dysbiosis, which has high predictive power for AAA, and the over-representation of specific salivary bacteria may be associated with AAA disease progression. Further studies are needed to better understand the function of putative oral bacteria in the etiopathogenesis of AAA.

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Importance: Host microbial dysbiosis has recently been linked to AAA as a possible etiology. To our knowledge, studies of the oral microbiota and aneurysms remain scarce, although previous studies have indicated that the DNA of some oral pathogens is detectable in aneurysms by PCR method. We take this field one step further by investigating the oral microbiota composition of AAA patients against control participants via high-throughput sequencing technologies and unveiling the potential microbial biomarker associated with AAA formation. Our study will provide new insights into AAA etiology, treatment and prevention from a microecological perspective and highlight the effects of oral microbiota on vascular health.

1. Introduction

Abdominal aortic aneurysm (AAA) is a permanent dilation of the abdominal aorta that exceeds the normal aortic diameter by 50 % [1]. Most AAAs are asymptomatic and, if left untreated, may lead to fatal aortic rupture. Major treatment options include open surgical and endovascular repair of AAA [2]. Surgical intervention is not recommended unless the aorta reaches a size where the risk of rupture surpasses the risk of repair [3]. There are still no effective pharmacological therapies to halt aneurysm progression and reduce the risk of later rupture [4]. Therefore, a better understanding of the pathological factors implicated in AAA may help to unveil biomarkers and therapeutic strategies.

Accruing evidence indicates a significant correlation between poor oral health status and the incidence of cardiovascular diseases [5,6]. The oral cavity serves as a reservoir for opportunistic pathogens that are capable of driving inflammatory diseases both locally and systemically. Oral pathogens can disseminate to the bloodstream or translocate to the intestine and take part in extra-oral diseases [7]. It has been proposed that oral dysbiosis potentially influences the occurrence and disease severity of atherosclerosis, hypertension and myocardial infarction [8–10]. Conversely, intensive periodontal treatment or improved oral hygiene attenuates systemic inflammation and alleviates the symptoms of cardiovascular diseases [5,11].

The effect of oral microbiota in aortic aneurysms has been growingly appreciated. AAA patients tend to have worse periodontal status, and DNA of periodontal pathogens can be discovered in the vascular samples of AAA patients [12–14]. The presence of the keystone pathogen *Porphyromonas gingivalis* (*P.g.*) in oral samples is correlated with AAA diameters and volumes [15]. Moreover, *P.g.* can accelerate the progression of experimental AAA in animal models [16,17]. These studies support the hypothesis that the oral microbiota plays important roles in AAA.

In this study, we characterized the salivary microbiota of AAA patients and aimed to further investigate the associations between salivary microbiota and the clinical features of AAA. These findings suggest that the salivary microbiota is a potential driver affecting the disease progression of AAA and that microbiota-oriented interventions are promising strategies for the prevention and treatment of AAA.

2. Methods

2.1. Study cohort

A total of 53 untreated AAA patients (AAA group) and 30 controls (CTL group) were recruited from the Department of Vascular Surgery at Shanghai Ninth People's Hospital. Participants with an infrarenal aorta that is greater than 3 cm in diameter are diagnosed with AAA by computed tomography angiography (CTA). Patients with ruptured AAA, coexisting malignant disease, autoimmune disease, gastrointestinal diseases, heart failure, renal failure, and the use of antibiotics or probiotics within the last two months were excluded. Control participants free of aneurysmal symptoms were screened by abdominal ultrasound following the same exclusion criteria used for AAA patients. Multiplanar and three-dimensional images were reconstructed using EndoSize software version 3.1 (Therenva, Rennes, Bretagne, France) to calculate the morphological features of aneurysms in an anonymized fashion. A participant was diagnosed with periodontitis if at least 2 interproximal sites had clinical attachment loss $\geq$3 mm and probing depth $\geq$4 mm (not on the same tooth) [18]. All participants were Han Chinese living in Shanghai for years. Socio-demographic and clinical information of participants was collected from face-to-face interviews and medical records.

2.2. Sample collection and DNA extraction

Salivary specimens were collected and processed as previously described [19]. Briefly, participants were advised to rinse their mouth and avoid eating or drinking at least an hour before saliva collection in the morning. Two milliliters of unstimulated saliva was collected in a sterile tube within 10 min. Equal volumes of Saliva DNA Preservation Solution (Huayueyang Biotech, Beijing, China) were immediately added to the saliva. Salivary specimens were transported to the laboratory in cool boxes with ice packs. Total genomic DNA was extracted using OMEGA Soil DNA Kits (Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer's protocol. The concentration of DNA was evaluated by a NanoDrop NC2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and the purity of DNA was assessed by agarose gel electrophoresis. DNA was stored at -20 °C until use.

2.3. 16S rRNA gene sequencing and processing

The sequencing procedures were performed as previously reported [19] with minor modifications. Briefly, PCR amplification of the bacterial 16S rRNA gene V3–V4 region was carried out using the forward primer 338F (5′-ACT CCT ACG GGA GGC AGC A-3′) and reverse primer 806R (5′-GGA CTA CHV GGG TWT CTA AT-3′). Sample-unique barcodes were integrated into the forward primers to identify sequences from different samples. The PCR system contained 0.25 μL of DNA Polymerase (ABclonal, Wuhan, China), 5 μL of 5 × reaction buffer, 5 μL of 5 × high GC buffer, 2 μL of 2.5 mM dNTP, 1 μL of 10 μM forward primer, 1 μL of 10 μM reverse primer, 2 μL of DNA template and 8.75 μL of ddH_2O. The PCR program began with an initial denaturation for 5 min at 98 °C, followed by 25 cycles of denaturation (30 s at 98 °C), annealing (30 s at 52 °C) and extension (45 s at 72 °C) with a final extension for 5 min at 72 °C. PCR products were purified with DNA clean beads (Vazyme, Nanjing, China) and quantified using a fluorescence assay kit (Invitrogen, Carlsbad, CA, USA). Next, the library was constructed using a DNA library preparation kit (Illumina, San Diego, CA, USA) and quantified using a fluorescence assay kit. Finally, sequencing data were produced using a NovaSeq reagent kit (Illumina) by 2 × 250 bp paired-end sequencing on the NovaSeq platform at Personal Biotechnology (Shanghai) Co., Ltd.

2.4. Bioinformatics and statistical analysis

The sequencing data were mainly analyzed and visualized via the QIIME2 and R software packages. Raw FASTQ files were demultiplexed, quality-filtered, denoised, merged and chimera-removed according to the official tutorials [20]. An average of 69,541 non-singleton reads were produced from saliva samples. Non-singleton amplicon sequence variants (ASVs) were treated with MAFFT to construct a phylogeny [21,22]. The taxonomy was assigned to ASVs against the Human Oral Microbiome Database (HOMD, 16S rRNA RefSeq V15.2). Alpha diversity indexes were calculated based on the ASV table. Beta diversity analysis was carried out to explore the structural variation in bacterial communities using Jaccard distances and UniFrac distances. PCoA was used to illustrate beta diversity, and the significance of differences in microbial structure was evaluated by permutational multivariate analysis of variance (PERMANOVA). The significance of the intergroup separation trend in PCoA was tested by analysis of similarities (ANOSIM). Associations of community composition with microbiota-external factors were quantified using the 'adonis2' implementation of PERMANOVA. Linear discriminant analysis effect size (LEfSe) was performed to identify taxa that were differentially abundant [23]. Random forest analysis was performed to select genera that best contributed to the discrimination of the two groups via a machine learning technique in QIIME2 [24]. The 20 most important predictors of AAA vs CTL were ranked by the Gini index calculated from the random forest algorithm. Receiver operating characteristic (ROC) curves were used to represent the classification accuracy of the machine-learning models based on different variables. A greater area under the curve (AUC) of the ROC curve indicates better

Table 1
Demographic and clinical characteristics of participants.

Characteristic	CTL (n = 30)	AAA (n = 53)	P value
Demographics			
Age (years)	69.67 ± 4.30	71.60 ± 7.72	0.21
Gender (male/female)	26/4	47/6	1.00
Height (cm)	169.30 ± 7.32	170.85 ± 7.98	0.38
Weight (kg)	68.58 ± 9.57	70.60 ± 11.39	0.41
BMI	23.87 ± 2.52	24.10 ± 3.04	0.73
BSA	1.79 ± 0.15	1.82 ± 0.17	0.38
Periodontitis, n (%)	26 (86.67 %)	48 (90.57 %)	0.72
SBP (mmHg)	127.22 ± 12.78	132.53 ± 11.70	0.06
DBP (mmHg)	78.18 ± 6.78	78.74 ± 9.63	0.76
Smoking history, n (%)	18 (60.00 %)	41 (77.36 %)	0.09
Antihypertensive treatment, n (%)	23 (76.67 %)	37 (69.81 %)	0.50
Lipid-lowering treatment, n (%)	6 (20.00 %)	15 (28.30 %)	0.40
Blood test			
TG (mmol/L)	1.29 ± 0.56	1.51 ± 1.00	0.27
TC (mmol/L)	3.92 ± 0.81	4.45 ± 1.08	0.02
LDL (mmol/L)	2.59 ± 0.77	3.01 ± 0.91	0.04
HDL (mmol/L)	1.29 ± 0.32	1.12 ± 0.27	0.01
FBG (mmol/L)	5.67 ± 0.88	5.34 ± 0.91	0.11
UA (μmol/L)	368.37 ± 72.49	380.92 ± 76.67	0.47
ALT (U/L)	19.27 ± 5.20	17.64 ± 8.42	0.34
Parameters of AAA			
Aortic maximal diameter (mm)	NA	47.73 ± 12.12	NA
Infrarenal aortic length (mm)	NA	116.55 ± 31.00	NA
Infrarenal aortic volume (mL)	NA	93.62 ± 77.68	NA

Data are shown as mean ± SD or n (%). Fisher's exact test was used for statistical analysis of gender and periodontitis. Chi-square test was used for statistical analyses of smoking history, antihypertensive treatment and lipid-lowering treatment. Student's *t*-test was used for statistical analyses of age, height, weight, BMI, BSA, SBP, DBP and blood test. AAA, abdominal aortic aneurysm; BMI, body mass index; BSA, body surface area; SBP, systolic blood pressure; DBP, diastolic blood pressure; TG, triglyceride; TC, total cholesterol; LDL, low-density lipoprotein; HDL, high-density lipoprotein; FBG, fasting blood glucose; UA, uric acid; ALT, alanine aminotransferase; NA, not applicable.

performance. Co-occurrence networks were identified using the Spearman correlation coefficient $|r| > 0.4$ and statistical significance ($P < 0.05$). The correlation networks represented potential interactions among co-occurring microbial genera, which were depicted with Cytoscape software. Microbial functional characteristics were predicted by PICRUSt2 (Phylogenetic investigation of communities by reconstruction of unobserved states) against KEGG databases [25]. Heatmaps were used to display the microbe–microbe and microbe-clinical index associations on the basis of the Spearman correlation coefficients.

3. Results

3.1. Baseline characteristics of the study subjects

A total of 53 patients with AAA and 30 age- and sex-matched normal controls participated in this study. The baseline characteristics of the 83 participants are displayed in Table 1 and Supplemental Table 1. There was no significant difference in demographics between the two groups, except for a slightly higher systolic blood pressure in subjects with AAA. The proportions of participants receiving antihypertensive treatment were 76.67 % (23/30) and 69.81 % (37/53) in the CTL group and AAA group, respectively. In addition, 9 participants with AAA might have potentially undiagnosed hypertension, whereas 1 participant might have undiagnosed hypertension according to the blood pressure record (Supplemental Table 1) and hypertension practice guidelines [26], which might explain why the AAA group had a higher systolic blood pressure. Participants with AAA showed significantly higher blood total cholesterol and low-density lipoprotein levels and significantly lower high-density lipoprotein levels than those without AAA, which indicates a higher risk of developing cardiovascular events in AAA patients. To characterize the signatures of salivary microbiota in participants with AAA, we performed 16S rRNA gene sequencing on salivary samples from AAA patients and controls. Permutational multivariate analysis of variance (PERMANOVA) was used to assess the effect of clinical characteristics on the overall microbial composition (Table 2). It did not show a significant impact of blood lipids on the composition of the microbiota. In addition, the effect of AAA (group) was the most critical contributor to the microbial dissimilarity between groups, suggesting that an abnormal salivary microbiota is strongly associated with AAA.

3.2. Altered diversity of salivary microbiota in participants with AAA

We further examined salivary microbiota diversity in AAA and CTL samples. The rarefaction curves, derived from the Chao1 index and observed species index, showed that the richness in both groups approached saturation and that the sequencing depth was sufficient to cover most bacteria (Fig. 1A). Additionally, they indicated that the richness of AAA samples was lower than that of CTL samples. As estimated by the Shannon index and Simpson index, the microbial diversity was slightly decreased in the AAA samples versus the CTL samples, although the difference did not reach statistical significance (Fig. 1B). Analysis of phylogenetic diversity, which incorporates phylogenetic differences between species, revealed significantly lower diversity of AAA samples (Fig. 1B). A phylogenetic method (UniFrac distance) and a non-phylogenetic method (Jaccard distance) were used to analyze beta diversity. The principal coordinate analysis based on unweighted UniFrac distance revealed separate microbiota profiles in the two groups (Fig. 1C).

Table 2
PERMANOVA results of associations between microbial composition and characteristics of participants.

Characteristic	Bray-Curtis (P)	Jaccard (P)	Unweighted UniFrac (P)	Weighted UniFrac (P)
Demographics				
Age	0.377	0.606	0.597	0.741
Gender	0.567	0.491	0.331	0.472
Height	0.98	0.761	0.865	0.95
Weight	0.262	0.214	0.503	0.621
BMI	0.279	0.19	0.839	0.541
BSA	0.185	0.194	0.578	0.476
Periodontitis	0.373	0.184	0.492	0.714
SBP	0.896	0.977	0.61	0.668
DBP	0.549	0.967	0.927	0.79
Smoking history	0.404	0.35	0.153	0.792
Antihypertensive treatment	0.559	0.892	0.58	0.901
Lipid-lowering treatment	0.757	0.469	0.173	0.798
Group	0.001	0.001	0.001	0.033
Blood test				
TG	0.98	0.979	0.402	0.981
TC	0.389	0.271	0.311	0.849
LDL	0.469	0.394	0.091	0.949
HDL	0.655	0.273	0.082	0.781
GLU	0.621	0.748	0.842	0.403
UA	0.618	0.617	0.568	0.439
ALT	0.816	0.668	0.846	0.634

Bray-Curtis, Jaccard, unweighted UniFrac and unweighted UniFrac distances were used to measure the pairwise dissimilarity in the microbiome profiles.

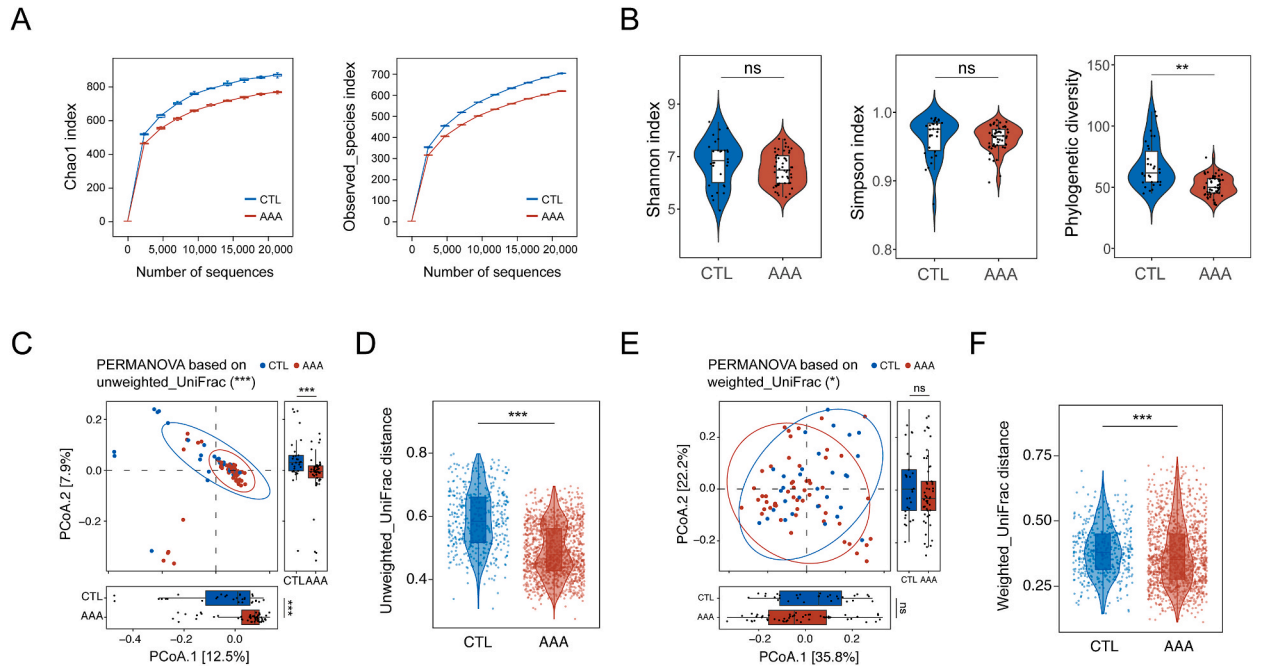

Fig. 1. Shifts in microbiota diversity in the saliva of participants with AAA. (A) Rarefaction curves based on the Chao1 index or observed_species index in control (CTL) and AAA participants. **(B)** Shannon index, Simpson index, and phylogenetic diversity of salivary microbiota. **(C)** Analysis of beta diversity using principal coordinate analysis (PCoA) based on unweighted_UniFrac distance. **(D)** Analysis of similarity (ANOSIM) based on unweighted_UniFrac distance showing the differences in microbial community structures. **(E)** Analysis of beta diversity using PCoA based on weighted_UniFrac distance. **(F)** ANOSIM based on weighted_UniFrac distance showing the differences in microbial community structures. The Wilcoxon rank-sum test was used for statistical analysis in B, D and F and to determine the differences in PCoA.1 and PCoA.2 between CTL and AAA in C and E; the PERMANOVA test was used to test the significance of beta diversity in C and E. *, **, *** indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

ANOSIM based on unweighted UniFrac distance revealed a significant difference in microbial community structures between the two groups (Fig. 1D). Similar results were obtained when weighted UniFrac distance or Jaccard distance was examined (Fig. 1E–F, Fig. S1).

3.3. Taxonomic alterations of salivary microbiota in participants with AAA

We further analyzed compositional changes in the salivary microbiota of AAA participants. A total of 98.60 % and 97.27 % of all reads were assigned to families and genera, respectively (Fig. S2). Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria, and Fusobacteria were the predominant phyla in saliva (Fig. S3A). Firmicutes was significantly enriched, whereas Fusobacteria substantially declined in the saliva of the AAA group when compared with the CTL group (Fig. 2A). Neisseriaceae, Prevotellaceae, Streptococcaceae, Veillonellaceae and Pasteurellaceae were the top 5 families in saliva (Fig. S3B). We further compared the relative abundance of the top 30 families and found that there were 11 families with significant differences between the two groups, among which Streptococcaceae and Gemellaceae were markedly increased in the saliva of the AAA group (Fig. 2B). Stacked bar charts of relative abundances at the genus level displayed obvious differences in the salivary microbiota of the two groups (Fig. S3C). The top 50 genera comprised approximately 97.15 % of the total genera. Specifically, there were 13 genera with significant differences in the top 50 genera, and 4 genera were significantly increased in the saliva of the AAA group (Fig. 2C). LEfSe analysis also systematically revealed differentially enriched taxa between the two groups (Fig. S4).

We next identified the most important microbial features using random forest analysis, which predicts disease status based on an ensemble of decision trees [27]. The results revealed that genera such as Streptococcus, Staphylococcus, Gemella, Peptostreptococcaceae_[XI][G-7] and Peptidiphaga were predictive of the disease status (Fig. 2D). Receiver operating characteristic (ROC) analysis demonstrated sufficient predictive power using the top 5 genera selected from the random forest analysis and the 13 differentially enriched genera (Fig. 2E).

3.4. Changes in the symbiotic networks in the salivary microbiota of AAA participants

Host–microbe symbiosis can be disrupted by an altered microbiota that is potentially linked to diseases. We analyzed the co-occurrence and co-exclusion relationships of the top 13 differentially abundant genera. There were significant positive correlations among Granulicatella, Gemella and Streptococcus, whose abundances were significantly increased in the saliva of AAA participants,

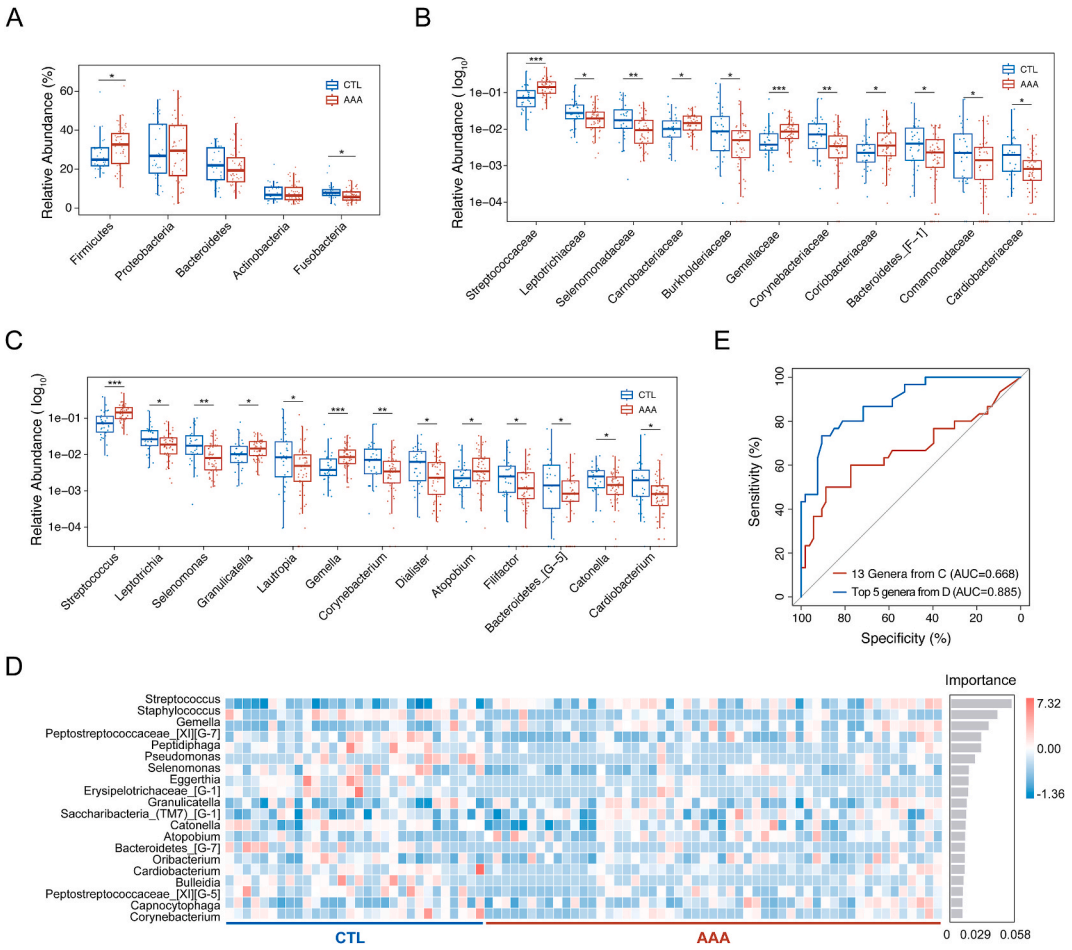

Fig. 2. Alterations of salivary microbial taxa in patients with AAA. (A) Relative abundances of the top 5 phyla of salivary microbiota in CTL and AAA participants. **(B)** Relative abundances of the top 11 most abundant families with significant differences between CTL and AAA participants. **(C)** Relative abundances of the top 13 most abundant genera with significant differences between CTL and AAA participants. **(D)** Random forest analysis indicating the importance scores of genera in the saliva of CTL and AAA participants. **(E)** Receiver operating characteristic (ROC) curves for AAA prediction by the top 13 most abundant genera with significant differences or the top 5 genera selected from random forest analysis. Wilcoxon rank-sum tests were used in A, B and C to calculate significance. *, **, *** indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

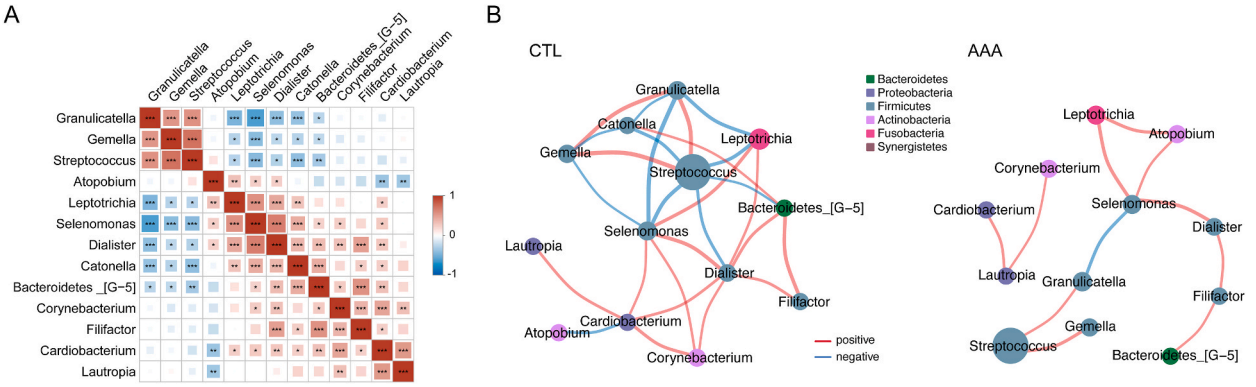

Fig. 3. Correlations among differentially abundant microbial genera in saliva. (A) Heatmap of Spearman's correlation coefficients among the relative abundances of the top 13 differentially abundant salivary genera. **(B)** Co-occurrence networks of the top 13 differentially abundant genera in saliva. Node size is proportional to the average relative abundance of the corresponding genus. Colors of the nodes represent the phyla to which the genera belong. Line thickness denotes the correlation strength. Red and blue lines indicate positive and negative correlations, respectively. *, **, *** indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

indicating synergistic interactions between the potentially harmful bacteria (Fig. 3A). Moreover, these genera were significantly negatively correlated with Leptotrichia, Selenomonas, Dialister, Catonella and Bacteroidetes_[G−5], whose abundances were significantly decreased in the saliva of AAA participants, implying a mutually antagonistic relationship among the genera. The co-occurrence network showed much fewer correlations among genera in the AAA group than in the CTL group (Fig. 3B). Specifically, as the most abundant genus, Streptococcus was negatively correlated with Leptotrichia, Selenomonas, Dialister, Bacteroidetes_[G−5] and Catonella in the CTL participants, suggesting a mutually exclusive relationship. However, these relationships disappeared in the saliva of the AAA group, which may underlie the enrichment of Streptococcus in the AAA group. Similarly, the absence of restriction of Gemella and Atopobium with other genera may explain the enrichment of the two genera.

3.5. *Functional characterization of salivary microbiota associated with AAA*

To gain functional insights into the AAA-associated salivary microbiota, enriched pathways were investigated using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Fourteen enriched pathways with the most significant differences between the two groups were identified (Fig. 4A). Three pathways were significantly enriched in the microbiota of AAA participants, namely, ko00311 (penicillin and cephalosporin biosynthesis), ko01501 (beta-lactam resistance) and ko05150 (*Staphylococcus aureus* infection). However, the other eleven pathways, including ko02040 (flagellar assembly), ko02030 (bacterial chemotaxis) and ko00340 (histidine metabolism), were significantly decreased in the AAA group. We further investigated the associations between the functional changes and the relative abundance of the top 50 genera (Fig. 4B). Streptococcus, Rothia, Gemella, and Granulicatella clustered together, indicating a similarity in functional correlations. The relative abundances of these four genera were highly correlated with ko00311, ko01501 and ko05150, which were significantly enriched in the AAA group. We then verified the relevance and robustness of the identified differentially enriched pathways in the prediction of AAA by ROC analysis. The results showed that the AUC value based on the differentially enriched pathways was 0.874. Moreover, the combination of the differentially enriched pathways with the top 5

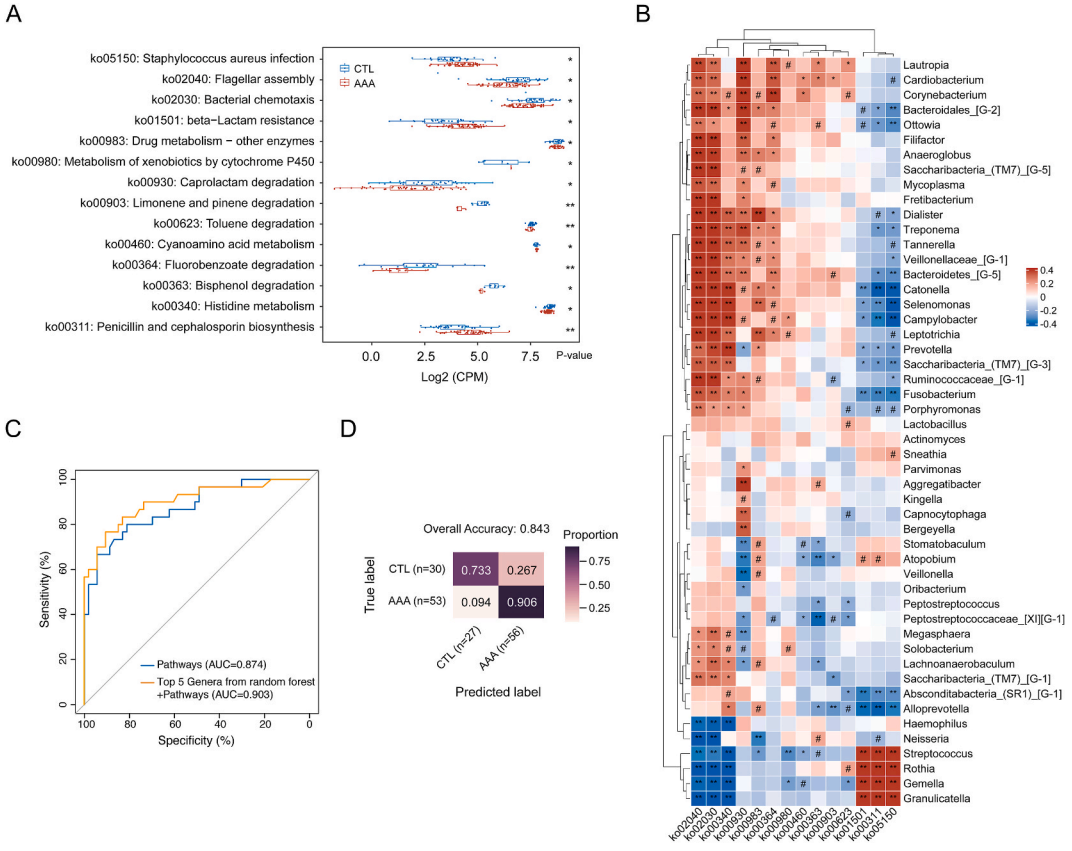

Fig. 4. Functional changes of salivary microbiota in AAA participants. (A) Pathway enrichment analysis showing significantly enriched or depleted microbial KEGG pathways in CTL and AAA. CPM: copies per million. **(B)** Heatmaps of Spearman's correlation coefficients between the differentially enriched KEGG pathways and relative abundances of the top 50 genera in the salivary microbiota. **(C)** ROC curves for AAA prediction by the differentially enriched pathways or the combination of the differentially enriched pathways with the top 5 genera selected from random forest analysis. **(D)** Confusion matrix displaying the ROC model accuracy of the combination of the differentially enriched pathways with the top 5 genera selected from random forest analysis, with an overall accuracy of 0.843, baseline accuracy of 0.639, and an accuracy ratio of 1.321. The Wilcoxon rank sum test with Benjamini-Hochberg correction was used in A. #, *, ** indicate $P < 0.1$, $P < 0.05$ and $P < 0.01$, respectively.

genera selected from random forest analysis improved the performance, which yielded an overall accuracy of 0.843 (baseline accuracy, 0.639; accuracy ratio, 1.321) with an AUC value of 0.903 (Fig. 4C and D). These results imply that the combinatorial effects of microbial species and functions may be associated with AAA.

3.6. Associations between salivary microbiota and clinical parameters of AAA participants

To evaluate the clinical significance of salivary microbiota in AAA progression, Spearman's correlation analyses were performed to assess the associations between the clinical characteristics of participants and the features of microbiota. We first evaluated AAA by two one-dimensional measurements (aortic maximal diameter, AMD; infrarenal aortic length, IAL) and a three-dimensional measure (infrarenal aortic volume, IAV), as demonstrated schematically in Fig. 5A. We analyzed the relationship between clinical characteristics and the relative abundances of the top 50 genera in saliva (Fig. 5B). The results showed that 16 genera were significantly positively correlated with AAA morphological parameters. In particular, Ottowia, Treponema, Fretibacterium, Tannerella, Bacteroidetes_[G-5], Sneathia, Parvimonas, and Bacteroidales_[G-2] showed strong positive correlations with both AMD and IAV, which are two key morphological markers of AAA remodeling [28]. Additionally, other genera, such as Filifactor, Dialister, Fusobacterium, Cardiobacterium, Capnocytophaga, Corynebacterium, Peptostreptococcus and Gemella, showed significantly positive

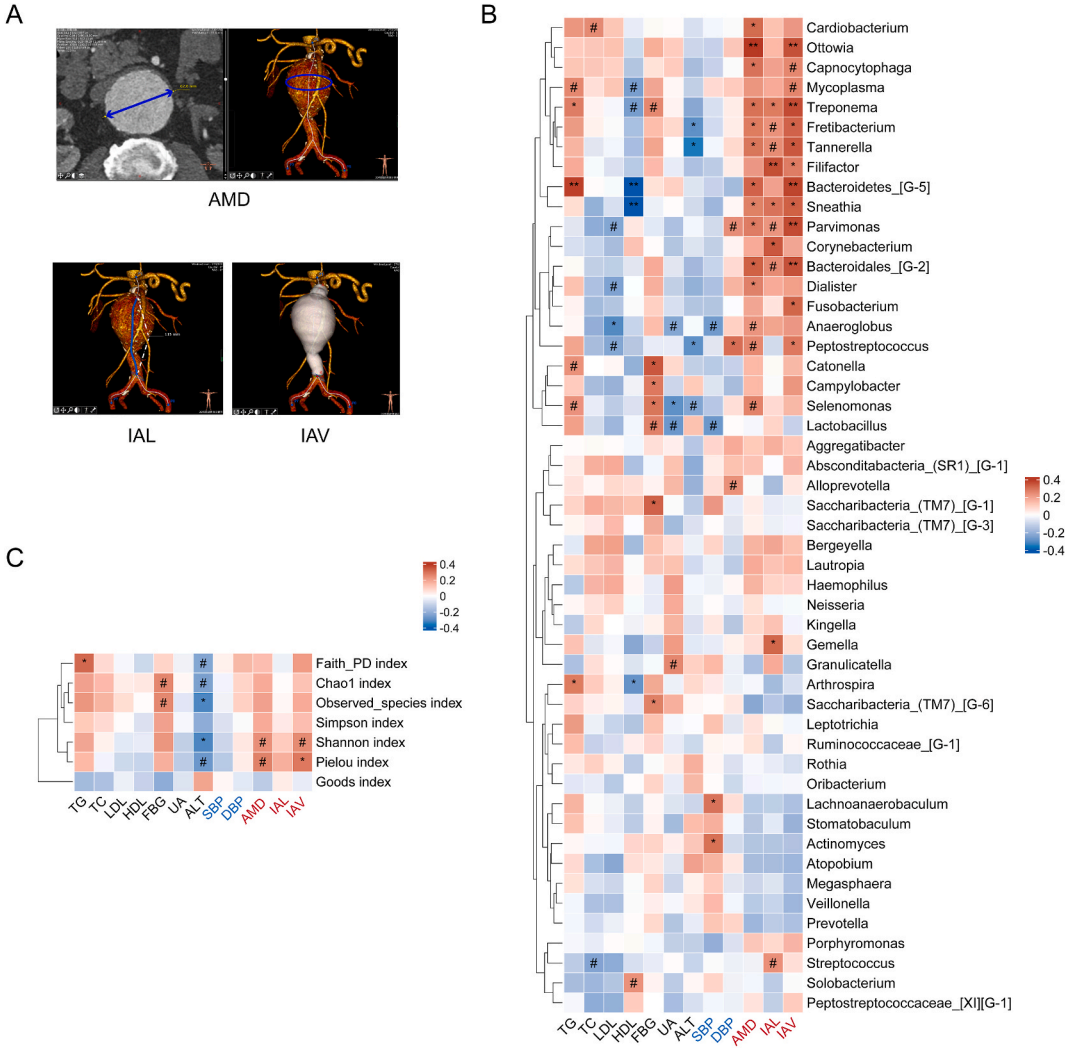

Fig. 5. Associations between salivary microbiota and AAA parameters and other clinical characteristics. (A) Illustrations of measuring AAA parameters. AMD, aortic maximal diameter; IAL, infrarenal aortic length; IAV, infrarenal aortic volume. **(B)** Heatmaps of Spearman's correlation coefficients between clinical parameters (blood test, blood pressure, AAA parameters) and relative abundances of the top 50 genera in the salivary microbiota. **(C)** Heatmaps of Spearman's correlation coefficients between clinical parameters and alpha diversity indexes of salivary microbiota in AAA patients. #, *, ** indicate $P < 0.1$, $P < 0.05$ and $P < 0.01$, respectively. TG, triglyceride; TC, total cholesterol; LDL, low-density lipoprotein; HDL, high-density lipoprotein; FBG, fasting blood glucose; UA, uric acid; ALT, alanine aminotransferase; SBP, systolic blood pressure; DBP, diastolic blood pressure.

correlations with AMD, IAL or IAV. Further analysis revealed that the majority of the alpha diversity indexes had positive correlations with AMD and IAV (Fig. 5C). The positive correlations between these potentially pathogenic bacteria and AAA parameters as well as alpha diversity and AAA parameters indicated that the severity of AAA may be associated with an enrichment of harmful salivary bacteria.

4. Discussion

Our study for the first time revealed compositional and functional alterations in the AAA-associated salivary microbiota, established correlations between the salivary microbiota and clinical parameters of AAA patients, and identified specific microbial biomarkers for the progression of AAA. These results provide new insights into our current knowledge of AAA etiology, treatment and prevention from a microbial perspective.

To our knowledge, studies of the oral microbiota and aneurysms are scarce, albeit sequencing technologies for microbial profiling are rapidly advancing. In our study, 16S rRNA gene analysis of salivary samples was performed in AAA and non-AAA participants. The difference in demographic parameters between AAA and CTL was minimized as much as possible according to the inclusion/exclusion criteria. The AAA participants had higher circulating levels of TC and LDL and lower levels of HDL, although the proportion of people receiving lipid-lowering treatment was comparable between the two groups. These disorders of lipids have been considered causal factors for the increased risk of AAA [29,30]. For instance, HDL can promote macrophage cholesterol efflux, which prevents cholesterol accumulation and macrophage infiltration into the aortic wall [31]. Lipid disorders did not exert a significant impact on the salivary microbial composition, as revealed by PERMANOVA. Thus, the characteristics of the salivary microbiota shaped by AAA were further investigated.

We identified significant alterations in the microbial diversity and taxa distribution in the saliva of AAA participants. The salivary microbiota characteristics remain relatively constant in healthy people [32]. It has been shown that age and health deficits are the strongest influential factors of bacterial diversity in saliva [33]. The relationship between host health and salivary alpha diversity is in opposition with one another, presenting an inverse correlation, as illustrated by our data and other studies [19,34]. The salivary taxa of our study were distributed in 12 bacterial phyla, and the six major phyla, Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria, Fusobacteria and Spirochaetes, constituted approximately 98 % of the taxa, which was in accordance with previous studies [35]. In saliva, Neisseria, Streptococcus, Prevotella, Veillonella and Haemophilus are among the major core genera [36]. The Wilcoxon rank-sum test and LEfSe analysis revealed that Streptococcus, Gemella, Granulicatella and Atopobium were significantly increased in the saliva of AAA participants.

As early and abundant colonizers, Streptococcus can create a microenvironment rich in CO_2, lactate, and acetate and low in oxygen, which facilitates the survival of other species [37]. Studies have shown that Streptococcus species are related to stroke, infective endocarditis and other cardiovascular diseases [38,39]. Bacterial detection of cardiovascular specimens has revealed that *Streptococcus mutans* is the most frequently detected bacteria, with detection rates of 42.7 % and 62.8 % in heart valve and aneurysm wall specimens, respectively [40]. Streptococcus contains many virulence factors to evade the immune system and damage host tissues [41]. Significantly enriched in the oral cavity of patients with rheumatoid arthritis, Streptococcus is a source of peptidoglycan-polysaccharide polymers that can induce inflammation and exacerbate arthritis [42]. Specific strains of Streptococcus with strong collagen-binding properties can accumulate on exposed collagen layers of vessels, thus aggravating bleeding and stroke [38]. Based on our result showing Streptococcus over-representation, we speculate that an interaction between Streptococcus and the aorta may promote injury and dilation of the aorta. Further investigations are required to test this hypothesis.

Emerging evidence indicates that the oral microbiota is closely associated with the onset, progression and recurrence of a series of diseases [43]. Salivary microbiota may influence the microbiota in the distal gut under pathological states and lead to a systemic microbiota-immune change [44,45]. Studies show that saliva may provide prognostication of cirrhosis and tumorigenesis [46,47]. Given the undiscovered role of saliva in AAA disease, we investigated the microbial biomarkers related to AAA prediction. Our results implied that a combination of Streptococcus, Staphylococcus, Gemella, Peptostreptococcaceae_[XI][G-7] and Peptidiphaga was able to discriminate AAA from non-AAA participants. On the other hand, uncovering the functionality of the microbial community is necessary to characterize its biological processes [48]. Functions related to endotoxin biosynthesis are significantly higher in the saliva of cirrhosis patients and may be associated with subsequent liver-related hospitalizations [49]. Our functional analysis showed that the AAA participants' saliva was enriched with pathways mainly pertaining to antibiotic resistance, and the set of differentially enriched pathways also had the potential to be biomarkers for AAA. These characteristics of the functional analysis and the fewer correlations among genera in the AAA group implied that the ecology of microbiota in the AAA group was under a more stressed state [50].

Currently, it remains largely unknown which microbial factors are associated with AAA expansion and rupture risk. Significantly reduced in the gut of AAA patients, *Roseburia intestinalis* and its metabolite butyrate markedly mitigate AAA formation in mouse models [51]. In saliva, we found that Cardiobacterium, Ottowia, Capnocytophaga, Treponema, Fretibacterium, Tannerella, Filifactor, Bacteroidetes_[G-5], Sneathia, Parvimonas, Bacteroidales_[G-2], Dialister, Fusobacterium and Peptostreptococcus showed significant positive correlations with AMD or IAV. Some species under these genera have been associated with chronic periodontitis or aggressive periodontitis [52–55]. Periodontal infections triggered by pathogenic biofilms contribute to the pathogenesis of cardiovascular diseases by way of endothelial dysfunction, molecular mimicry, platelet aggregation, direct arterial invasion and systemic inflammation [56]. The two genera, Streptococcus and Gemella, selected from random forest analysis as predictive markers of AAA presence, also presented positive correlations with IAL. The reason why they were not best correlated with aneurysm size might be that random forest analysis was conducted on the basis of microbial differences in both the AAA and non-AAA groups, and the two genera might have less effect on AAA enlargement. The other three genera in the random forest analysis were not among the top 50 genera in the AAA group,

so they were not displayed in the correlation analysis.

There were several limitations in our study. Firstly, our cohort was single-centered and the sample size was limited. It would be more persuasive to recruit an independent cohort from a different medical center for validation of the predictive ability of salivary microbiota in AAA. Secondly, although we used PERMANOVA to exclude the effect of multiple clinical variables on the overall microbial composition, we did not adjust for potential confounding variables in multivariate modeling. Finally, most of our results were descriptive and correlative. More studies are needed to better elucidate the function of the postulated oral pathogens in AAA in order to establish potential causation.

5. Conclusion

Our work compared the taxonomic and functional salivary microbiota profiles of AAA participants with those of control subjects. We demonstrated a taxonomically and functionally distinct salivary microbiota in AAA participants with a classification accuracy of 90.3 %. More importantly, we identified specific salivary genera that had positive correlations with the progression and severity of AAA. Further investigations are warranted to confirm the function of oral dysbiosis in the pathogenesis of AAA and to explore the effectiveness of targeting oral dysbiosis in the prevention and management of AAA.

Ethics statement

This study was conducted with the approval of the Institutional Review and Ethics Board of Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine (Approval No. SH9H-2019-T6-2) and kept compliance with the Declaration of Helsinki. All participants provided written informed consent at the time of enrollment.

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CRediT authorship contribution statement

Wen-Zhen Lin: Writing – review & editing, Writing – original draft, Visualization, Project administration, Formal analysis, Data curation. **Bo-Yan Chen:** Writing – review & editing, Writing – original draft, Project administration, Methodology, Data curation. **Peng Qiu:** Writing – review & editing, Visualization, Project administration, Methodology, Formal analysis, Data curation. **Lu-Jun Zhou:** Writing – review & editing, Methodology, Investigation. **Yu-Lin Li:** Writing – review & editing, Project administration. **Lin-Juan Du:** Writing – review & editing, Methodology, Investigation. **Yuan Liu:** Writing – review & editing, Investigation. **Yong-Li Wang:** Writing – review & editing, Methodology, Investigation. **Hong Zhu:** Writing – review & editing, Methodology, Investigation. **Xiao-Yu Wu:** Writing – review & editing, Methodology, Investigation. **Xiaobing Liu:** Writing – review & editing, Supervision, Resources, Conceptualization. **Sheng-Zhong Duan:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Ya-Qin Zhu:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e23040.

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