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Original Article

Temporal stability of tongue microbiota in older patients – A pilot study

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KEYWORDS
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Abstract *Background/purpose:* Healthy states of human microbiota depend on a stable community of symbiotic microbes irrespective of external challenges from the environment. Thus, long-term stability of the oral microbiota is of importance, particularly for older patient populations.
Materials and methods: We used next-generation sequencing (NGS) to examine the tongue microbiota of 18 individuals receiving long-term care over a 10-month period.
Results: Beta diversity analysis demonstrated temporal stability of the tongue microbiota, as microbial compositions from all time points were indistinguishable from each other ($P = 0.0887$). However, significant individual variation in microbial composition ($P = 0.0001$) was observed, underscoring the presence of a unique microbial profile for each patient.
Conclusion: The temporal dynamics of tongue microbiota exhibit long-term stability, providing diagnostic implications for oral diseases within older patient populations.

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Introduction

The microbiota encompasses a diverse collection of microorganisms that can exist in commensal, symbiotic, or pathogenic relationships with the human host at various anatomic sites including gut, oral cavity, and skin.[1,2] Comprising bacteria, archaea, protists, fungi, and viruses, these microorganisms are essential for maintaining the immunological, hormonal, and metabolic balance of their hosts.[3] A substantial corpus of research has demonstrated that the composition of microbiota could influence an individual's health state.[4] Among the various microbiota in the human body, the oral microbiota ranks as the second largest.[5] Dysbiosis of the oral microbiota not only influences oral health (e.g. periodontal disease and dental caries) but is also associated with systemic health conditions such as diabetes,[6] cardiovascular diseases,[7] stroke,[8] and Alzheimer's disease.[9] Recent studies further showed that oral cancer is related to the dysbiosis of the oral microbiota.[10,11] Additionally, pneumonia was shown to be associated with high abundance of pathogenic bacteria such as *Pseudomonas* and *Corynebacterium* in patients' tongue microbiota.[12,13]

A healthy state of microbiota generally depends on a complex, stable community of commensal microbes that excludes outgrowth of pathogenic species.[14] Stability of the oral microbiota not only contributes to general health, but also benefits clinical diagnosis for disease prevention. Various factors may cause the dysbiosis of oral microbiota, such as age, stress, lifestyle, feeding methods, dietary habits, and host genetic factors.[15,16] Further, the oral-gut axis theory demonstrates that dysbiosis of the gut microbiota might also influence the oral microbiota through the esophagus,[17,18] further contributing to the variations of oral microbial community. It is therefore important to focus on a more diverse, stable niche in oral microbiota to detect physiologic variations among individuals.

The oral cavity harbors diverse microbiota at distinct anatomical niches such as the tongue, gingival sulcus, and saliva. These niches range from anaerobic to oxygen-rich environments, and may reflect different aspects of the host's physiological conditions. Selecting suitable sampling sites is critical to obtain accurate data for the research objective. As older patients receiving long-term care are often edentulous, sampling the gingival sulcus is not viable in some circumstances. Additionally, xerostomia, a common condition among older individuals, affects the consistency of saliva samples.[19] Although sampling at the buccal and palatal mucosal surface are technically feasible, these sites may present limited colonization of microorganisms due to rapid epithelial shedding and the transient, periodic nature of the bacterial layer.[20] For the salivary sample, such as those found in saliva, may coat the teeth and mucous membrane to facilitate microbial adhesion. Nevertheless, certain salivary proteins also promote the desorption, agglutination, and removal of microorganisms by ingesting saliva, which causes the trace to be unstable and difficult to confirm.[20] In contrast, the tongue dorsum is characterized by multiple layers of bacterial biofilm with diverse microbes, presenting greater abundance and variety of microorganisms in comparison to other mucosal regions.[21] Furthermore, studies have shown that certain microbes in the tongue microbiota are of clinical importance, associated with pneumonia and gut diseases.[22] Consequently, in this study the tongue dorsum was chosen as the primary sampling site to explore the oral microbiota of older, long-term care patients.

In the context of microbiota stability, most studies have predominantly focused on healthy subjects,[23] often overlooking the intricate immunological factors and physical conditions prevalent in the aging population. As a result, a substantial gap exists in the evidence concerning the effectiveness of stability measures in patients under long-term care. By utilizing next-generation sequencing (NGS), healthcare practitioners can efficiently survey patients' health status to trace disease evolution during successive clinical examinations. We hypothesize the long-term stability of tongue microbiota in older patient populations receiving long-term care. Our research extended a period of ten months, focusing on examining the consistency of the tongue microbiota in older individuals.

Materials and methods

Patient recruitment and study design

This study involved 18 participants receiving long-term care in Yilan county, Taiwan. Every participant was followed for 10 months. The time of dental treatment was conducted at the 5th, 7.5th, and 10th month time points, and tongue coatings were sampled at the 0^{th}, 5th, and 10th month time points. Dental treatments were applied after tongue coatings sampling.

The inclusion criteria for the participants were the presence of moderate to severe disabilities accompanied with long-term dental home care. At the beginning, 18 participants were included in the study, but due to the degradation of their general health, 8 participants died and lost follow-up at the end of this study. The study was approved by the Ethic Committee of National Yang Ming Chiao Tung University (ID 2020B002).

Tongue sampling and amplicon sequencing

Tongue samples were obtained using sterilized cotton swabs and immediately placed in a fresh, sterile TE buffer at 4 °C for storage. Within 24 h, the samples were transported to the laboratory at AllBio Science Inc. (Taichung, Taiwan) for genomic DNA extraction. Total genomic DNA was extracted from the samples following the manufacturer's protocol using the AllPure Bacteria Genomic DNA kit, and the concentration and quality of the DNA were assessed using the Equalbit dsDNA HS Assay Kit. The V3 to V4 regions of 16S rRNA gene were targeted for amplification, using the primers 341F (CCTACGGGNGGCWGCAG) and 805R (GACTACHVGGGTATCTAATCC).[24] The DNA library was purified using magnetic beads (AMPure XP), and the concentration and fragment size were measured using the Qubit™ 3 Fluorometer (Thermo Fisher Scientific Inc., Waltham, USA) and agarose gel electrophoresis, respectively. The library was adjusted to 10 nM before undergoing 250-bp paired-end sequencing on the Illumina MiSeq platform.

Sequence trimming and demultiplexing

Our computational pipeline's foundation was Qiime2[25] version 2021.11, coupled with custom-made, specialized numerical analysis using Python. Primer sequences, low-quality bases (Phred score <20) and reads that were too short (<20 bp) were removed from paired-end reads using cutadapt.[26]

Amplicon sequence variant (ASV) feature detection and taxonomic identification

The DADA2 denoise method[27] was used with Qiime2 to generate ASVs, producing a feature table and representative sequences for each ASV. A naïve Bayes classifier that has been previously trained on the SILVA database was used to assign taxon for each representative sequence.[28] ASVs of the same taxon were pooled by adding their counts after each ASV was identified with its anticipated taxon.

Alpha and beta diversity analysis

Using Qiime2, alpha diversities from the feature table were calculated. The "scipy" package was used to run the Wilcoxon rank-sum (i.e. Mann-Whitney U) test. In addition to Qiime2, Python programs such as numpy,[29] sklearn,[30] and seaborn[31] were utilized for beta diversity (Bray-Curtis and weighted Unifrac). The "scikit-bio" Python library was used to perform principal coordinate analysis (PCoA), which was then visualized using "seaborn" software. Analysis of similarity (ANOSIM) was conducted by using "scikit-bio".

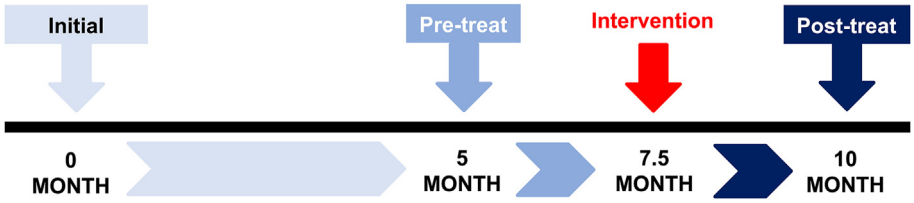

Figure 1 Timeline of study design. This longitudinal study involved three sampling time points: initial, pre-treat, and post-treat at 0, 5, and 10-month time points, respectively, with one oral-hygiene care intervention at the 7.5-month point.

Table 1 Patient characteristics and oral health status.

	Initial (N=18)	Pre-treat (N=18)	Post-treat (N=10)
Sampling time	2021.03-05	2021.08-10	2022.01-03
Male/Female	7/11	7/11	5/5
Mean age (SD)	70.6 (23.9)	70.6 (23.9)	68.1(24.5)
Bedridden	18 (100%)	18 (100%)	10 (100%)
Feeding methods			
Oral-feeding	8 (44%)	8 (44%)	5 (50%)
Nasogastric-tube	10 (56%)	10 (56%)	5 (50%)
Diseases			
Hypertension	8 (44%)	8 (44%)	4 (40%)
Diabetes mellitus	5 (28%)	5 (28%)	3 (30%)
Cardiovascular disease	8 (44%)	8 (44%)	8 (80%)
Dementia	9 (50%)	9 (50%)	5 (50%)
Pneumonia history			
Yes	9 (50%)	9 (50%)	7 (70%)
No	9 (50%)	9 (50%)	3 (30%)
Dental caries			
Decay	2 (11%)	2 (11%)	2 (20%)
Missing	5 (28%)	5 (28%)	4 (40%)
Periodontal status			
Healthy	0 (0%)	0 (0%)	0 (0%)
Gingivitis	0 (0%)	0 (0%)	0 (0%)
Periodontitis	18 (100%)	18 (100%)	10 (100%)
Calculus			
Mild	2 (11%)	2 (11%)	0 (0%)
Moderate	1 (6%)	1 (6%)	0 (0%)
Severe	15 (83%)	15 (83%)	10 (100%)
Dental prosthetics			
Partial	0 (0%)	0 (0%)	0 (0%)
Full mouth	0 (0%)	0 (0%)	0 (0%)
Crown / Bridge	9 (50%)	9 (50%)	5 (50%)

AI-assisted writing

ChatGPT (GPT-4 May 24 Version) was used for semantic and grammatical correction. For each paragraph, we created a first draft based on our findings and reasonings. Based on ChatGPT's results, we made semantic corrections on our original draft to prevent any hallucination. This very paragraph describing the AI-assisted writing process was completely written by humans without using any AI technology.

Results

A long-term study involving 18 participants spanning 10 months

Each participant was monitored over a span of 10 months, during which tongue plaque samples were collected by dentists at three time points: the Initial (0 month), Pre-treatment (5 months), and Post-treatment (10 months) (Fig. 1). Among the participants, a majority suffered from multiple systemic diseases. Specifically, nine of them exhibited varying levels of dementia, while eight patients had cardiovascular disease and hypertension. Additionally, half of the total patients had a history of pneumonia. Notably, the oral health conditions of these patients were generally poor. All 18 patients presented with periodontitis, with most of them experiencing severe calculus buildup (Table 1).

Consistent taxonomic composition and alpha diversity across three time points regardless of oral hygiene treatment

For taxonomic characterization, each ASV was labeled at the species level by using a pre-trained naïve Bayes classifier on the SILVA database. The Venn diagram showed that a total of 236 species were shared across three time points, while 118, 52, and 24 species were exclusive to the initial, pre-treatment, and post-treatment periods, respectively (Fig. 2A). Alpha diversity analysis, including Shannon entropy and observed ASV features, revealed no significant changes across the three time points (Fig. 2B). Together, alpha diversity and species composition analysis demonstrated temporal stability of tongue microbiota, regardless of oral hygiene intervention.

Major bacterial compositions identified across three time points

To explore potential differences in microbial composition across three time points, we performed unsupervised

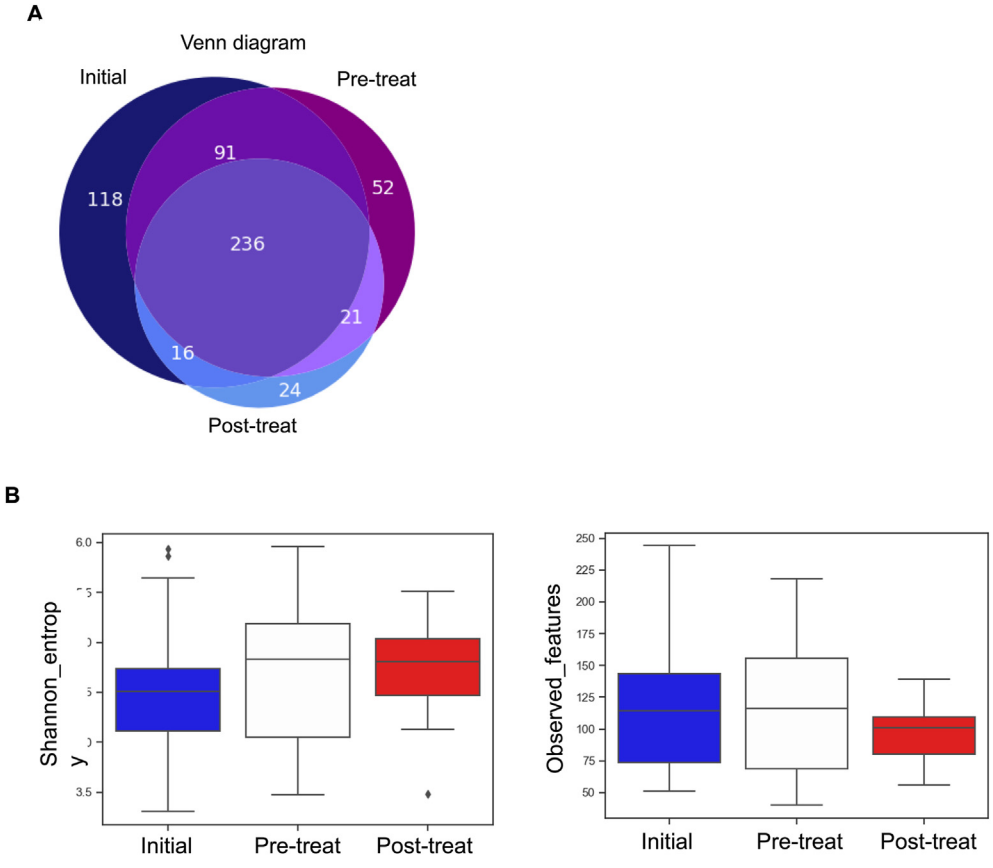

Figure 2 Comparison of the microbiota composition between three time points. (A) Venn diagram of species composition among the three time points. (B) Alpha-diversity analysis showing Shannon entropy (left panel) and observed features (right panel) for the three time points. Wilcoxon rank-sum tests showed no statistical significance (P value $>$ 0.05) between any given pair of time points.

hierarchical clustering. The resulting heatmap revealed that while the genus compositions at these time points showed patient-specific distinctions, they were similar across all time points (Fig. 3). Specifically, the 20 most prevalent genera, including *Streptococcus*, *Neisseria*, *Actinomyces*, *Veillonella*, *Corynebacterium*, *Pseudomonas*, *Fusobacterium*, and *Porphyromonas*, were consistent with findings from previous investigations.[12,13,32] Four groups (I–IV) of genera were assigned based on their collective patterns observed across all samples (Fig. 3). In general, group I and IV genera exhibited a higher prevalence of Gram-negative bacteria and a heterogeneous composition of aerobic, facultative, and anaerobic organisms. Conversely, group II and III primarily comprised Gram-positive anaerobic bacteria.

Beta diversity reveals temporal stability of tongue microbiota with patient-specific distinctions

To investigate the disparities of microbiota composition among patients, beta-diversity analysis was conducted. Two inter-sample distance metrics, Bray-Curtis and weighted UniFrac, were computed. Regardless of the utilization of different metrics, samples from the three time points consistently overlapped with each other in the PCoA plots (Fig. 4A and C) ($P = 0.0887$), suggesting temporal stability of the tongue microbiota. Conversely, significant differences were observed between individuals, demonstrating that each patient possessed a unique microbial composition (Fig. 4B and D) ($P = 0.0001$). In summary, beta-diversity analysis revealed distinctive individual patterns of tongue microbiota which were temporally stable.

Stability of tongue microbial composition for each patient

To examine the relative abundance of bacterial species in each patient's tongue plaque sample collected at three time points, taxonomic compositions were calculated at the genus level. This analysis revealed that the majority of patients' tongue microbiota displayed consistent compositional patterns across the three time points. Notably, among patients (F, J, M, N, and P) who were deceased at the third time point, significant alterations in relative abundance and tongue bacterial composition were observed between their initial and pre-treatment time points, suggesting that deterioration of general health condition was reflected by tongue microbiota. The dysbiosis of a patient's microbiota may have unidentified effects on their general health. Consequently, these findings emphasize the significance of maintaining microbiota balance and the stable nature of tongue microbiota (Fig. 5).

Discussion

To our best knowledge, this study was the first long-term investigation to identify the stability of the tongue microbiota in the Taiwanese older population. Importantly, the

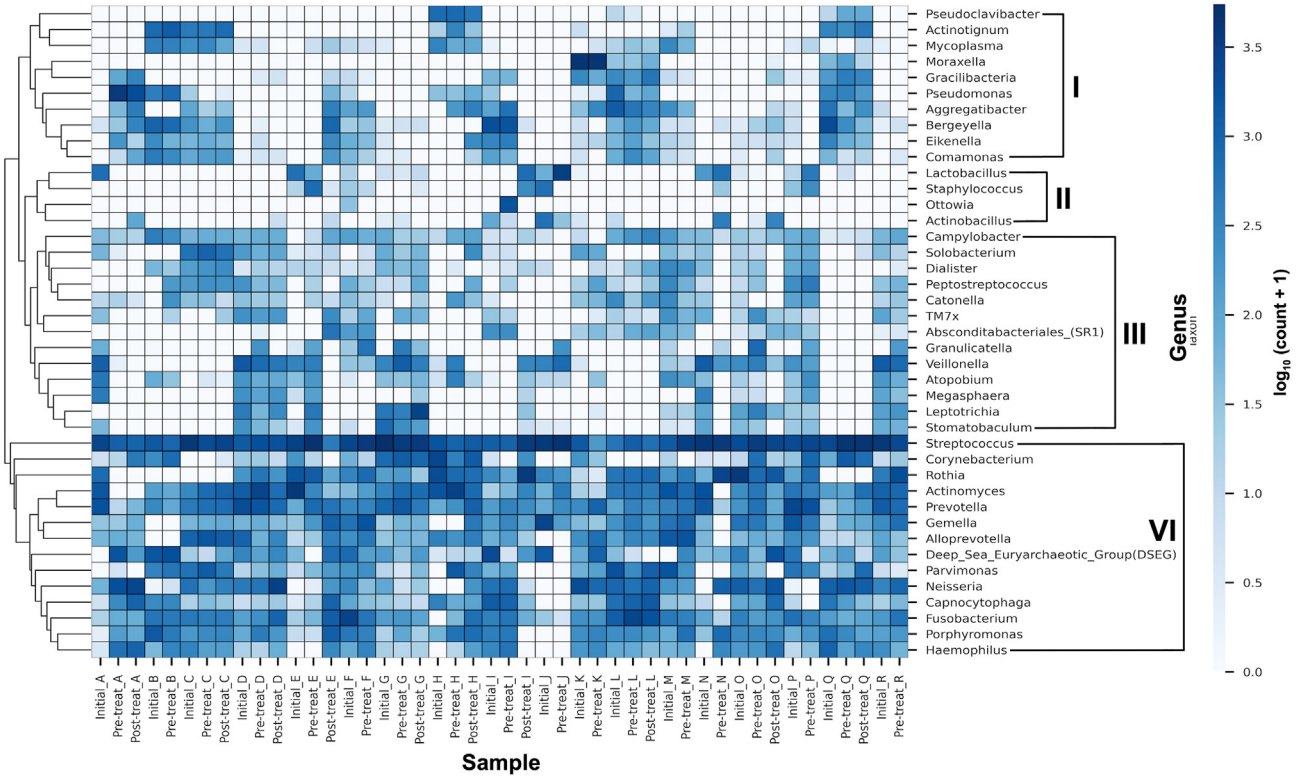

Figure 3 Unsupervised hierarchical clustering shows major groups of tongue bacterial genera. Columns and rows represent samples and genera, respectively. 46 tongue samples from 18 patients (denoted by alphabets "A" to "R") were collected at 3 time points: initial, pre-treat, and post-treat. The 41 most abundant genera covering 95 % of total reads were shown. Four groups of genera were denoted from "I" to "IV". Color shades of the heatmap indicate log10 pseudocounts.

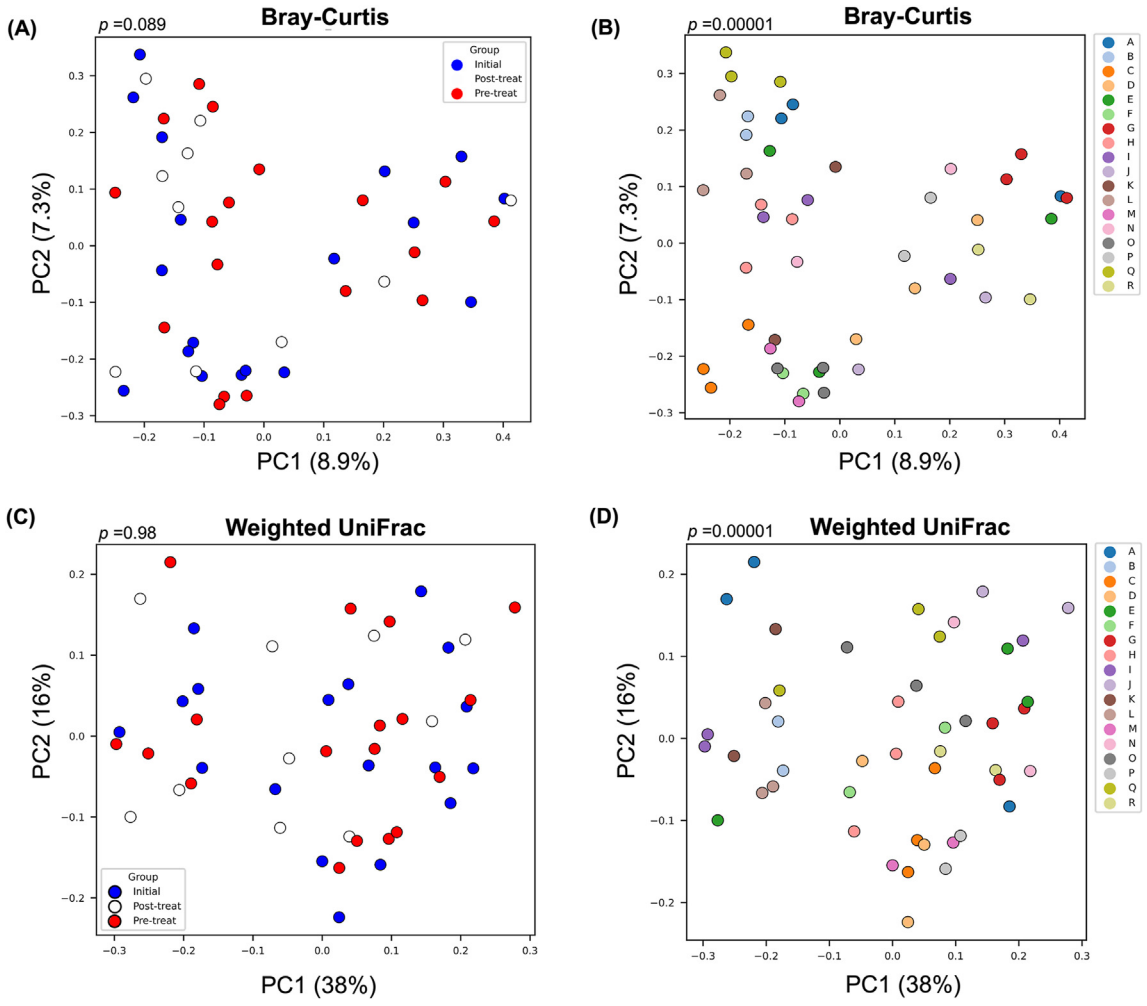

Figure 4 Beta-diversity analysis indicates temporal stability and inter-patient variations of tongue microbiota. Each point represents a tongue sample, with inter-point distance showing Bray-Curtis (A, B) and weighted UniFrac (C, D) distance after PCoA dimensionality reduction. Points were colored by sampling time points (A, C) or patients (B, D). Percentages in the parentheses indicate contributions of the two principal components, PC1 and PC2. P values were determined using ANOSIM with 99,999 random permutations.

tongue microbiota from each patient had a pattern that was similar across their three-time point sample collections, while the bacteria component revealed notable variations between individuals. Chen et al.[46] monitored 338 participants for 4 years to investigate their gut microbiota and found that the gut microbiota stability differs across bacterial species and that individual-specific, temporally stable microbial profiles can fingerprint the host.[33] Oh et al.[47] gathered the skin microbiota of 12 individuals for approximately 3 years and discovered that the skin microbiota may vary by place and person, but is stable at the strain level, despite external exposures.[34] The stability of the oral microbiota in mouthwash samples has been identified in several studies,[35,36] reminiscent of our findings as the first to describe the tongue microbiota stability in Taiwan. Furthermore, considering individual precision medicine, it is important to define a suitable tool that can be used easily and non-invasively, and the sample niche should also reflect oral and general health. Traditionally, Chinese medicine has been shown to diagnose diseases based on the color of the tongue coating[37] Some research also found that the bacterial pathogens may cluster on the tongue plaque, then pass through the esophagus or bloodstream to the gastrointestinal or other organs and cause diseases.[38] Based on our findings, tongue microbiota could serve as a detection tool that reliably reports oral health condition without being altered by external interference (e.g., dental prosthetic treatment), meaning that tongue microbiota can reflect the general condition of the bacterial component in the oral microbiota. On the other hand, it is noted that a variety of pathogens exist in the tongue microbiota. Insufficient cleaning of the tongue plaque as part of oral care can potentially lead to general diseases, particularly with dysbiotic microbiota or underlying immunological deficiency.

In our previous study, we identified an increased abundance of *Corynebacterium* in the tongue microbiota among patients using nasogastric tubes.[32] In the present study, we further observed a higher prevalence of the species *Corynebacterium simulans* in deceased patients. This

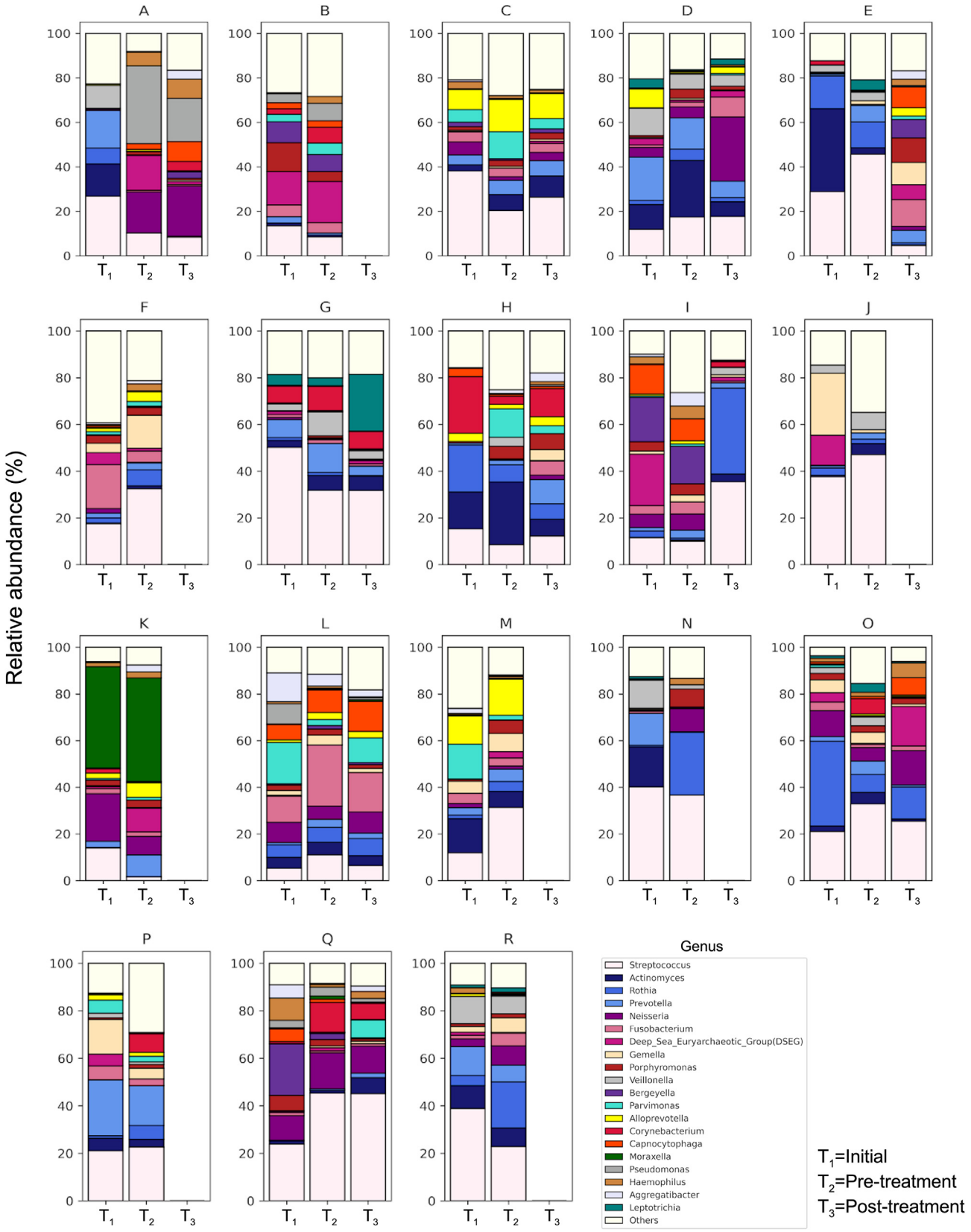

Figure 5 Relative abundance of genus composition of each individual. Each box shows the genus composition of an individual (denoted by alphabets "A" to "R") across three time points, with T_1, T_2, and T_3 indicating initial, pre-treat, and post-treat, respectively. For visual clarity, the 20 most abundant genera across all samples were shown, with the remaining minor genera combined in the group "Others".

observation implies a potential link between nasogastric tube usage and the dominance of specific bacterial species, such as *C. simulans*, which could contribute to pneumonia and reduce patients' life expectancy.[39] Additionally, our previous research demonstrated elevated richness of the genus *Veillonella* in long-term care patients who were physically more active and do not require home care.[12] In the current study, we found a greater abundance of *Veillonella parvula* in living patients, contrasting with decreased levels in deceased patients. In summary, our findings align with previous research[12,32] indicating that specific bacterial species may play a role in oral microbiota dysbiosis, exerting adverse effects on overall health.

This research underscores the importance of oral microbiota in the context of not only effective treatment, but also continuous health monitoring, a consideration vital to medical practitioners, caregivers, and researchers. Our findings suggest that the detection of tongue microbiota offers a reliable technique for monitoring an individual's health status. Notably, the distinct bacteria observed in both living and deceased patients could potentially serve as early indicators for disease identification and prevention. We envision a future medical service model that extends beyond treatment provision, enabling disease prevention and enhancing longevity, particularly for older patients. It should be noted that several subjects involved in this study were in poor health and died before the last sampling time point. Acknowledging high attrition in this patient population, we designated our work as a pilot study, emphasizing the need for larger sample sizes for future research. Despite these challenges, our findings still provide values particularly on individual tongue microbiota's utility and stability. In conclusion, we identified the tongue microbiota as a stable oral microbial niche suitable for long-term monitoring, especially for the older population. Notably, a health-to-frailty trend in dysbiosis was evidenced by the divergence in bacterial species and the tongue microbiota composition between living and deceased individuals. This suggests a potential use of tongue microbiota for the early detection for frailty and associated diseases.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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