Effect of Multistability and Hysteresis on Oral Microbiome Patterns Observed in Clinical Studies

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This work marks a turning point —
not only in my academic path, but in the quiet pursuit of becoming who I aspire to be.

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Lastly, to those walking unfamiliar paths, striving for something better: *may we keep going*.

Kowoon Noh
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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Effect of Multistability and Hysteresis on Oral Microbiome Patterns Observed in Clinical Studies

The oral microbiome of a *healthy cohort* is utilised as a reference point for evaluating clinical cases and interventions. Nonetheless, current cohort-based studies have fallen short in thoroughly considering multistability in microbial communities. Screening analysis has been confined to phenotypic traits that exhibit significant differences among microbial genomic markers. The objective of this study was to assess the temporal stability of the oral microbiome over time in an intervention-free and phenotypically healthy cohort.

33 longitudinal supragingival plaque samples were collected from 11 healthy participants, sourced from the biobank. For each participant, samples were designated as baseline (T0), 1-month (T1), and 3-month (T2) intervals for 16S ribosomal RNA gene sequencing analysis.

Taxonomic profiling consistently exhibited a recurring pattern of predominant genera, specifically *Rothia*, *Prevotella*, and *Haemophilus*, across all observed time points. In the alpha diversity analysis, the Shannon index showed a significant increase with time from T0 ($p < 0.05$). Bray–Curtis dissimilarity (beta diversity) demonstrated substantial variation within the cohort at each time point ($r = –0.02$, $p < 0.01$). The community and stability evaluation at the species level showed a negative correlation with synchrony ($r = –0.739$; $p = 0.009$) and variance ($r = –0.605$; $p = 0.048$). Clustering data based on the species abundance profiles of participants resulted in the formation of three distinct cluster groups, with notable variations in the grouping patterns across the three time points. At all observed time points, the clusters exhibited a markedly distinct array of differentially abundant taxonomic and functional biomarkers.

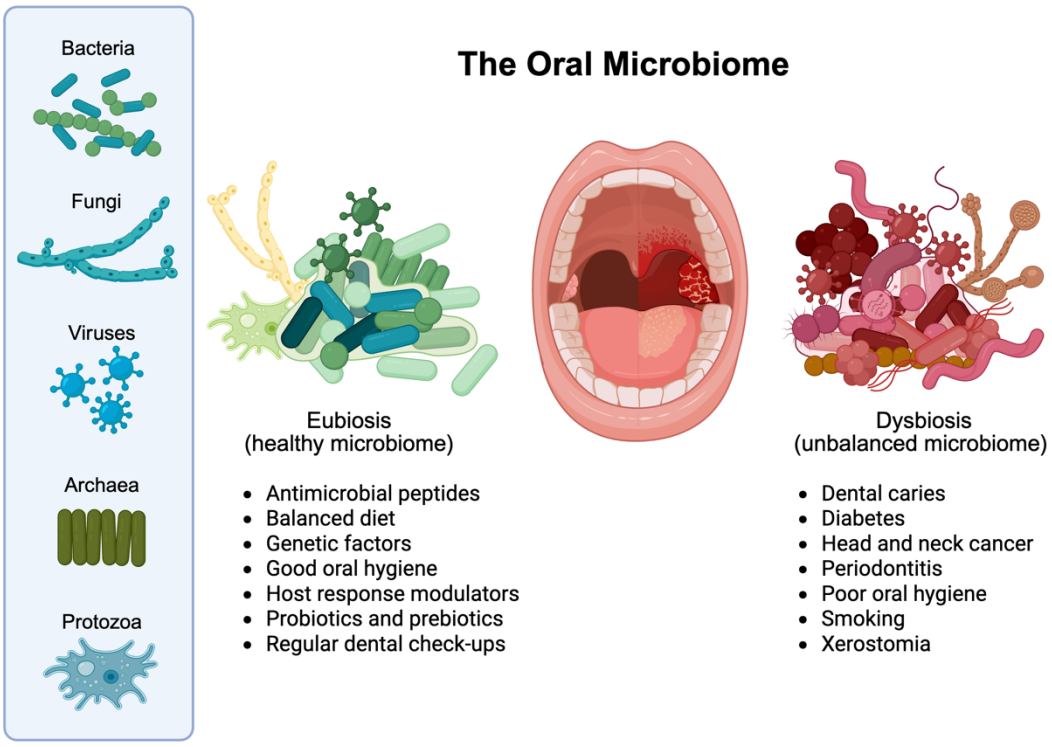
Even in healthy individuals free of intervention, distinct patterns of species turnover and abundance were observed, supporting the concept of multistability and hysteresis within the oral microbiome. Therefore, to establish a definitive and meaningful long-term reference, it is imperative that clinical cohort microbiome research considers the dynamic nature of the oral microbiome and its multi-stable states within the context of personalised therapy. This consideration is crucial for enhancing the accuracy of identifying and classifying reliable markers, ultimately leading to more effective interventions and improved oral health outcomes.

Key words : multistability, oral microbiome, dysbiosis, 16S rRNA gene sequencing, temporal variability

1. Introduction

1.1. Oral microbiome

The oral microbiome is a complex dynamic polymicrobial community that inhabits the oral cavity (Radaic & Kapila, 2021). The oral cavity is the second largest microbial habitat, followed by the gut, harvesting bacteria, fungi, viruses, and archaea (Deo & Deshmukh, 2019). The habitat is unique in its constant exposure to external factors and serves as a primary barricading gateway to the gastrointestinal tract. The microbes within colonise hard tissues such as teeth, and soft tissues such as the oral mucosa, to form a complex symbiotic ecosystem that maintains host homeostasis (Sedghi et al., 2021). Due to the ease of sample collection, the oral microbiome has become the most extensively studied microbiome to date (Deo & Deshmukh, 2019). Studies have shown dysbiosis, an imbalance in oral microflora, as not only the precursor of dento-periodontal diseases but also in its association with systemic disorders (He et al., 2015; Kumar, 2013; Lamont et al., 2018; Van Dyke et al., 2020). The aetiological correlation between microbiome and oral diseases has driven the use of culture and polymerase chain reaction-based techniques to identify the causative organisms.



<Fig 1> Constituents of the oral microbiome. Framework of a healthy versus dysbiotic microbiome. Created with BioRender.com

1.2. Next-generation sequencing

Rapidly advancing technology in metagenomics has enabled improved accessibility and affordability of sequencing-based metabarcoding, such as 16S ribosomal RNA (rRNA) next-generation sequencing (NGS) (Caselli et al., 2020). Traditional methods were self-limited in inferring the *difficult-to-grow* organisms and are considered close-ended (Ng et al., 2021). With the advancement of bioinformatics and the establishment of the 'Polymicrobial Synergy and Dysbiosis' hypothesis (Lamont et al., 2018), researchers preferably utilise the sequence-based approaches to identify difficult-to-grow organisms. Consequently, research on the human oral microbiome has notably enriched in the past decade (Jakubovics & Shi, 2020). The evoking trend is encouraging researchers and clinicians to adopt oral microbiome analyses in clinical studies to incorporate the findings into clinical practice and thus lead a step closer to personalised dental care.

The renewed emphasis on personalised oral health therapy has driven the development of fast advancing microbiomics towards hypothesis-based research (Nibali et al., 2020; Wei et al., 2019). Recent studies have provided detailed insights into the composition and abundance analysis, revealing distinct differences between health and disease states. Current research in clinical dentistry, using conventional study methods, has demonstrated distinctions between carious and non-carious, chronic and aggressive periodontitis, with the help of microbiome sequencing techniques (Pang et al., 2021; Wei et al., 2019). Comparable designs are being rapidly adapted by comparing a phenotypically healthy cohort against a diseased cohort.

1.3. Limitations in current microbiome research

Despite the flourishing publication in the field, concerns have arisen regarding the pitfall of microbiome data interpretation, including the overestimation of clinical relevance (Kim et al., 2017; Zaura et al., 2021). Common confounding factors in oral microbial samples include age, gender, comorbidity, medication, body weight, diet, dental health, and oral health habits and more (Burcham et al., 2020). The risk of overlooking bias is high in microbial studies. Hence, both readers and clinical researchers must understand the sophisticated manner in which metagenomic data are collected and carefully plan clinical studies based on this knowledge to avoid misinterpretation of the results.

From a clinical perspective, numerous hurdles exist in applying appropriate methodologies for microbial intervention studies in surgical settings. Healthcare clinicians are first faced with inexperience when dealing with meta-bioinformatics data and vastly rely on microbial data analysis platform services. Consequently, the risk of failing to identify bias in the data is high. Unlike specialised microbiologists and bench workers in the field, clinicians are restricted both in subject and sample size and thus are much limited when performing a gold standard oral microbiome study. Under such circumstances, thorough attention to experimental design and prior knowledge of inter-individual microbial variability should be arranged to avoid yielding experimental artefacts (Kim et al., 2017). Clinicians must also have insight into common confounding factors in microbial samples, which could substantially impact the outcome of microbiome analyses.

An optimised oral microbiome clinical study design begins with an understanding of the target patient group. Most clinical research takes subject demographic and clinical characteristics into account, yet previous microbial intervention studies have focused on measuring the change in the microbial composition between pre- and post-intervention (Goodrich et al., 2014; Zaura et al., 2021). In addition, the time and duration of intervention observation were selected on the basis of limited evidence (Zaura et al., 2021). The observed subtle changes in the microbial composition may in fact be part of the natural temporal variability of the subject. The global large-scale Human Microbiome Project reported that the oral cavity is highly stable in between-subject diversity, similar to other body sites (Consortium, 2012). However, a subsequent study on repeated observation of the oral microbiome in the dorsum of the tongue revealed that the level of stability varies inter-individually (Flores et al., 2014). Therefore, it is important to understand and assess individuals' homeostatic range of variability in the intervention-free oral microbiome, prior to measuring the impact of clinical intervention.

1.4. Multistability and hysteresis model

Most research designs rely on inferences derived from the incidence of increases or decreases in microbial species levels. This type of description is referred to as bimodal (Gonze et al., 2017). The bimodality concept involves a binary behaviour, where a species associated with healthy state exhibits a reversible and predictable increase or decrease in its population. Before and after oral prophylaxis, the abundance of a known species (e.g., *Porphyromonas* species) will decrease substantially, thereby restoring overall health. However, the given example is self-contradictive when considering the interactions and adaptations of the microbiota over time.

Therefore, a more relevant model was proposed to account for the dynamic balance. This model is referred to as multistability and hysteresis (MSH) (Gonze et al., 2017; Khazaei et al., 2020). The notion of 'multistability' describes the capacity of a microbial community to achieve distinct stable states, even when exposed to similar environmental conditions.‘ Hysteresis’ is a phenomenon where the shift in the microbial composition is path-dependent, where reversing the environmental condition that once triggered to cross a tipping point (threshold factor causing an abrupt shift e.g., oxygen level) is non-reversible (Faust et al., 2015). As a result, the microbial community exhibits distinct stable states. The proposed MSH model incorporates both of these concepts.

Accounting for the MSH model, an optimal oral microbiome clinical study design, whether observational (case-control/cohort) or interventional (clinical trial), should first involve understanding the target subject group. However, the difficulty in establishing a healthy reference group increases when considering variations related to niche-based, time-based, and observer effects. Furthermore, researchers select the timing and duration of intervention or observation based on relatively limited evidence (Zaura et al., 2021). The sampling time points may deviate from the overall cross-sectional and longitudinal patterns. Therefore, it is essential to consider the homeostatic range of variation between participants in the pre-interventional oral microbiome.

1.5. Research objective

In light of the aforementioned considerations, this study sought to elucidate the concept of MSH within oral microbiome samples, focusing specifically on a healthy cohort derived from biobank-sourced specimens. The central hypothesis was that the oral microbiome collected from the cervical third surface of the first molar in young, disease-free individuals would exhibit a random and variable state. This variability was considered a relevant factor for clinicians in selecting participants for control groups in planned clinical interventions.

To simulate a clinically realistic follow-up framework, the study implemented sampling across three time points over a 90-day period, without any intervention. Additionally, a focused comparison between two phenotypically similar participants was conducted to explore intra-cohort variability. The null hypothesis was that there would be no significant change in the oral microbiome composition over time within a disease-free (healthy) community sample.

2. Materials and Methods

2.1. Study population and data collection

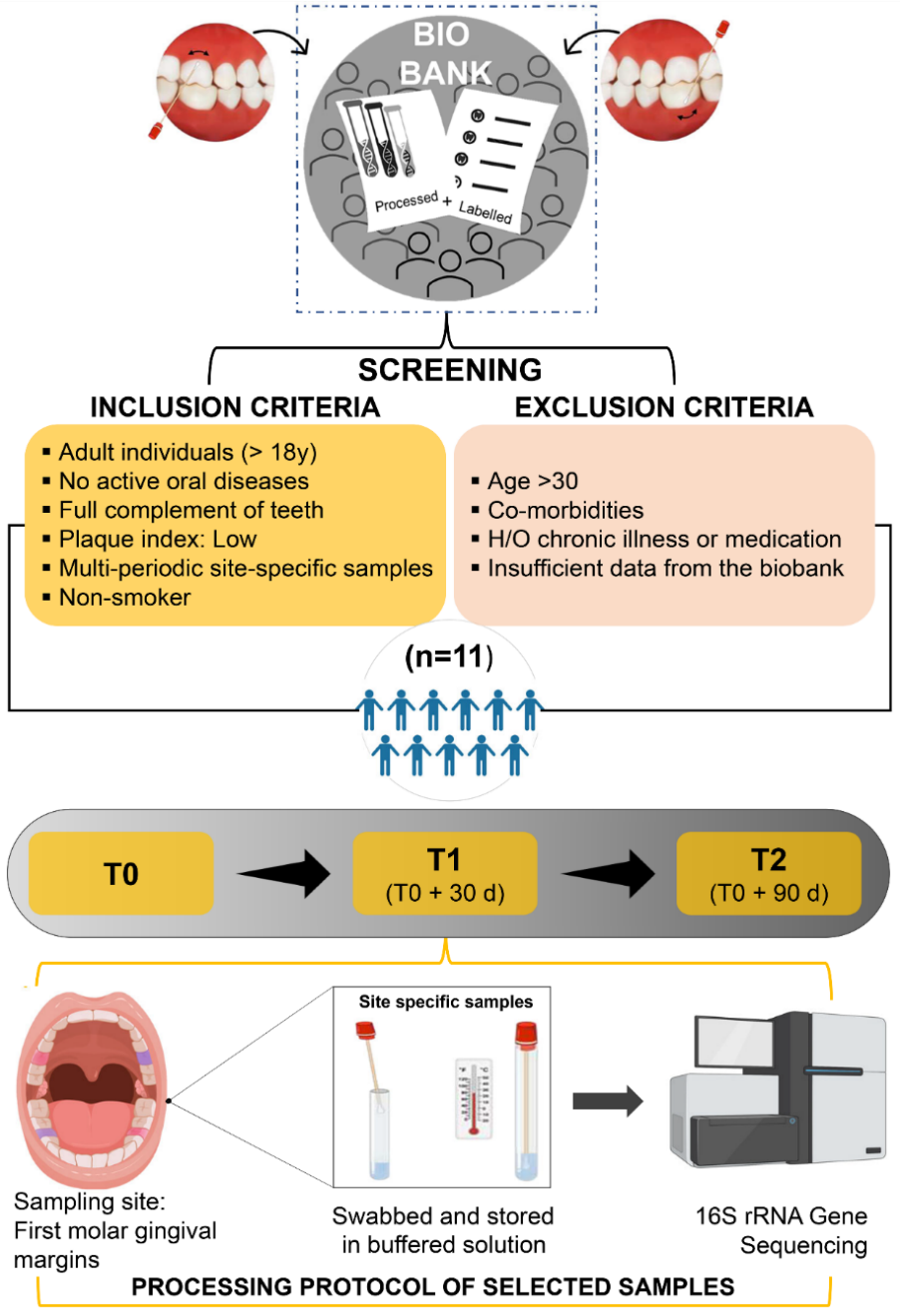
The study was approved and conducted in accordance with the guidelines from the Institutional Review Board at Yonsei University Dental Hospital (Approval number: 2-2021-0050). Genomic samples were collected from the Oral-derived bioresources for the human-derived materials biobank located at the Yonsei University Dental Hospital, South Korea (Korea Biobank Network: KBN4_A04).

The inclusion criteria were as follows: (i) sequential multi-period supragingival plaque samples, (ii) taken from the marginal gingival regions of the posterior teeth, (iii) low plaque index, (iv) individuals over 18 years old at the time of the first sample, and (v) samples collected and stored using a consistent buffering protocol (Hallmaier-Wacker et al., 2018).

The exclusion criteria were as follows: (i) individuals over 30 years old during sample collection, (ii) smoking, or a history of pregnancy or breastfeeding, (iii) loss of natural tooth structure at or near the sampling site, (iv) professional dental treatment within 6 months prior to sampling, (v) a history of chronic medication use (e.g., antibiotic therapy, probiotics) and comorbidities (e.g. gastrointestinal issues), and (vi) inadequate or inconsistent biobank data.

Based on the outlined criteria, 33 samples were selected from 11 participants. The samples were collected and stored using an OMNI-gene OMR-110 kit (DNA Genotek, Ottawa) in line with a consistent processing protocol. The sample intervals were redefined as baseline (T0), one month after (T1 = T0 + 30 days), and three months after the baseline (T2 = T0 + 90 days).

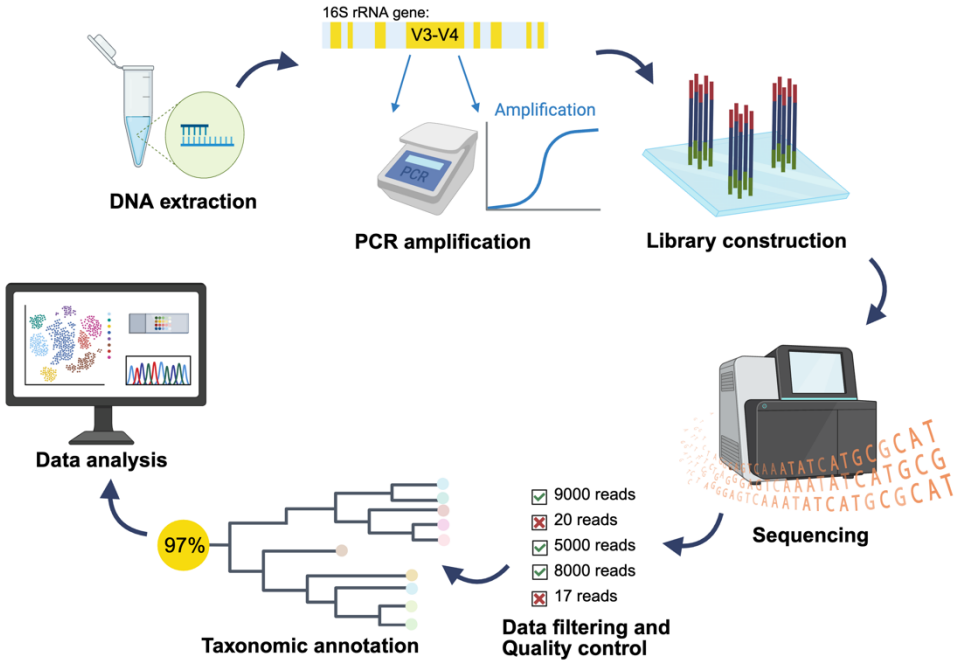
Among the various locations within the oral cavity, samples were collected from the first permanent molar. This decision was based on the fact that it is the earliest permanent tooth to appear, offering a record of an individual's past oral health and containing health information unique to the person (Brickley et al., 2020). Additionally, a recent systematic review highlighted that the first molar has been incorporated into the case definition and examination protocol for diagnosing periodontitis (Albandar, 2014; Bouziane et al., 2020). In the current study, the collection of samples was specifically focused on the first molar regions. Samples were collected from the upper and lower first molars on the contralateral side and subsequently combined for sequencing analysis. Samples from each individual were obtained at three different time points: T0, T1, and T2.



<Fig 2> Study design and flow. Samples obtained from the Biobank that met the specific selection criteria were sequenced and analysed.

2.2. 16S rRNA sequencing and data processing

The sequencing of the 16S rRNA gene was conducted at CJ BioScience Inc. (Seoul, Republic of Korea), following the established protocol (Kim et al., 2021). In brief, DNA samples were extracted, followed by polymerase chain reaction amplification using primers targeting the V3-V4 regions of the 16S rRNA gene. Amplicon sequencing was performed using an Illumina Miseq Sequencing System (Illumina, CA, USA). Raw reads were processed for quality check, and low-quality reads (<25) were filtered, followed by the merge of paired-end sequence data. Primers were trimmed, and 16S rRNA unique reads using a similarity threshold of 97% were isolated for taxonomy allocation based on the EzBioCloud 16S rRNA database (Yoon et al., 2017). The chimeric reads were filtered out.



<Fig 3> General workflow of 16S rRNA sequencing. Created with BioRender.com.

2.3. Abundance profiling and diversity measurements

Downstream analyses were conducted primarily using web-based tools, except where otherwise stated. Using the EzBioCloud platform, the microbiome data were normalised by gene copy number (Yoon et al., 2017). Normalised gene count data were then uploaded onto the MicrobiomeAnlayst tool for meta-analyses (Chong et al., 2020; Dhariwal et al., 2017; Yoon et al., 2017). Relative abundance at the genus level was adjusted at a 1% cut-off, grouping those below this threshold under the 'Others' category.

To observe within-subject species richness and evenness, alpha diversity was visualised using the Chao1, Shannon and inverse Simpson indices. The Wilcoxon signed-rank test was employed for statistical analysis, with a significance threshold of 0.05. To compare the distance matrix between each subject and the time points, beta diversity was performed using the principal coordinate analysis (PCoA) using the Bray-Curtis dissimilarity index (Duran-Pinedo et al., 2021). Dissimilarity distances were then statistically analysed using permutational multivariate analysis of variance (PERMANOVA) and analysis of similarities (ANOSIM). The calculations involved are comprehensively presented in Tables 1–3.

<Table 1> Description of different indices used in analysing the alpha diversity

Index			Clinical implication
Chao1	Estimation of species richness from rare-species counts	A higher Chao1 value suggests higher species diversity.	A significant increase or decrease in alpha diversity implies a shift in species diversity, which, for the oral microbiome, is typically associated with deteriorating dental or periodontal health.
Shannon	Estimation of species evenness relative to species richness	A higher Shannon value indicates greater species diversity, with the maximum value implying equal numbers for each species.	In an individual, a shift in species evenness (relative abundance) may indicate disruption in symbiosis, i.e., suppression of beneficial commensal bacteria and enrichment of pathogenic bacteria. An increase in species richness indicates the appearance of more species within a community, leading to increased species diversity, and vice versa for decreased species richness.
Inverse Simpson	Estimation of species richness and evenness	A higher inverse Simpson value indicates greater species diversity, with 1 representing no diversity.	While focusing on the dominating (highly abundant) species in the given sample, the characteristics and functions of each species should be investigated to understand the overall clinical effect of the shift in alpha diversity.

<Table 2> Description of index used in analysing the beta diversity

Index			Clinical implication
Bray–Curtis dissimilarity	Dissimilarity coefficient based on the distance between communities.	A smaller distance suggests greater similarity between communities.	By observing mutual species between communities, beta diversity is often used to contrast microbial shifts from point A (e.g., before intervention) to point B (e.g., after intervention). Among the beta diversity indices, the Bray–Curtis index uses principal coordinate analysis to visualise community dissimilarity in terms of distance. In a clinical study, beta diversity helps understand intervention-specific shifts in microbial communities and community variance between study subjects or time points.

<Table 3> Description of metrics and statistics used in alpha and beta diversities

	Index	Clinical implication
Wilcoxon signed-rank test	A non-parametric test used to compare samples not adhering to a normal distribution. A low p-value suggests a significant difference between groups.	In microbiome studies, the test compares (i) the median variations in alpha diversity and (ii) the proportion of core taxa or the abundance of specific taxa between paired study groups.
Principal coordinate analysis (PCoA)	A multivariate analysis method based on a distance matrix visualising relationships between samples by reducing the high-dimensional data to a lower space.	PCoA visualises Beta diversity by plotting sample clusters using a selected matrix (e.g., Bray–Curtis index). Closer sample points indicate microbial community similarity based on relative abundance.
Permutational multivariate analysis of variance (PERMANOVA)	Non-parametric test for analysing multivariate data using a distance matrix. F value is the ratio of between- to within-group variation; higher values indicate greater group distinction. R^2 value shows the proportional variation related to the grouping factor, with values near 1 implying more variation between groups.	F and R^2 values compare baseline to post-intervention data. A high F value indicates a strong intervention impact on the microbiome composition. An R^2 value near 1 indicates a strong association between intervention and microbiome changes.
Analysis of similarities (ANOSIM)	Non-parametric test comparing average ranked dissimilarities within and between groups. R values near +1 indicate higher dissimilarities between groups, suggesting strong separation. A value near 0 indicates no separation. Value near -1 is uncommon, indicating higher within-group dissimilarities, suggesting possible sampling design issues.	ANOSIM identifies significant differences in microbial composition among individuals, environments, or treatments and factors influencing group variation. An R value near +1 indicates a significant difference in community composition.

2.4. Microbiome stability analysis

The ‘Codyn’ package in RStudio (version 2021.09.0, MA, USA) was utilised to assess the extent of temporal changes (Hallett et al., 2016). Species turnover was assessed to measure the proportion of species appearance and disappearance, and stability was determined based on the ratio of the temporal mean to the temporal standard deviation. Additionally, the variance ratio was examined to contrast the community’s overall variance with the sum of individual variances, and synchrony was assessed by comparing the variance of total species abundances to the combined variances of each species (Duran-Pinedo et al., 2021). A detailed description of the indices used is provided in Table 4.

<Table 4> Description of the different indices used in analysing species turnover and stability

	Index	Clinical implication
Species turnover	The rate of species composition changes within a community over time, calculated by the proportion of species gained or lost between time points. A higher value indicates greater fluctuations in species composition over time, with more species appearing and disappearing between periods.	The species turnover metric is valuable for examining a microbial community's temporal dynamics and evaluating intervention effects on community structure.
Community stability	The overall stability of a microbial community over time, assessed by calculating the ratio of temporal mean to temporal standard deviation of aggregated species abundance. A higher value suggests a more stable community, where the average species abundance remains relatively constant over time, in contrast to variations in their abundance.	The community stability metric evaluates microbial communities' dynamic behaviour by assessing their stability over time. The metric indicates the community's resilience in preserving its structure and function despite species abundance fluctuations.
Synchrony	Comparison of aggregated species abundances with the summed variances of individual species. The value ranges from 0 to 1, where 0 is absolute asynchrony and 1 is absolute synchrony.	In data showing synchrony despite fluctuations in individual species numbers, total community stability is maintained through the loss of one species compensated by the gain of another. In contrast, asynchrony is associated with community perturbations.
Variance	Patterns of species covariance based on comparing community variance to summed individual species variance. A value less than 1 suggests negative species covariance, whereas a value greater than 1 suggests positive species covariance.	In positive covariance data, despite fluctuations in species numbers, community stability is maintained as species loss by one is offset by another's gain. Conversely, negative covariance is linked to community perturbations.

2.5. Clustering and biomarker comparisons

Temporal dynamics were investigated by clustering the data according to species abundance profiles. The trajectories of the species were organised using the method detailed in Arumugam et al., which formulates the concept of enterotypes for the gut microbiome (Arumugam et al., 2011). The concept can be applied similarly to as stomatotype for the oral microbiome (Willis et al., 2018).

In brief, normalised genus abundance profiles were organised and clustered using the Jensen-Shannon divergence distance in conjunction with the partitioning around medoids clustering algorithm (cluster library) (Maechler, 2019). The Calinski-Harabasz index was employed to determine the optimal number of clusters. Ultimately, the statistical significance of the optimal clustering was verified using the silhouette coefficient. The full algorithm and its explanations are comprehensively outlined in the online tutorial (http://enterotype.embl.de), which was followed precisely without any alterations (Arumugam et al., 2014; Arumugam M, 2014).

Biomarkers were compared across clusters to identify taxa associated with variability between the clustered groups. Functional biomarkers were assessed using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) (Avolio et al., 2015). Significant differences were determined using a Linear Discriminant Analysis Effect Size (LEfSe) with a threshold linear discriminant analysis (LDA) score ≥2.0 (Segata et al., 2011). For brevity, only the abbreviation ‘LDA’ is used within the table cells in Table 6. The results were subsequently mapped against the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database to interpret the metabolic functions of the microbial community. For brevity, only the abbreviation ‘KEGG’ is used within the table cells in Table 6.

Lastly, to demonstrate periodic variation within similar phenotypes, datasets from two representative subjects were compared. A comparable analysis was conducted to emphasise the turnover pattern and depict proportional differences at the genus level using the Statistical Analysis of Metagenomic Profiles (STAMP) with false discovery rate (FDR)-corrected q-values (Parks & Beiko, 2010). For brevity, only the abbreviation ‘FDR’ is used within the table cells in Table 6. The descriptions of the indices and calculations involved are comprehensively presented in Tables 5 and 6.

<Table 5> Description of stomatotype clustering and biomarker analysis

Index		Clinical implication
Clustering	Categorising individuals into groups based on similar oral microbial community profiles (stomatotype).	While numerous clinical studies categorise subjects into groups such as healthy versus diseased or pre- versus post-intervention, categorisation based on microbial similarity is also frequently employed in human microbiome research.
Functional biomarker analysis	Identification of metabolic pathways associated with highly abundant species (or genera) in a community.	The optimal aim of an oral microbiome study is to pinpoint key species associated with the clinical state in question (e.g., healthy periodontium, progressed periodontitis) using functional biomarker analysis.

<Table 6> Description of metrics and indices used in clustering and biomarker analysis

	Index	Clinical implication
Jensen-Shannon divergence distance (JSD)	Symmetric distance metric to compare probability distributions, where 0 indicates an identical distribution and higher values indicates greater differences.	JSD identifies microbial shifts and clusters samples by microbial compositions. Calculating JSD at different time points allows researchers to track community composition changes over time.
Calinski-Harabasz (CH)	Cluster validity index to assess clustering quality, particularly when determining the optimal number of clusters.	CH index is used to cluster samples with similar microbial compositions, potentially reflecting distinct health states. The index also prevents over- or under-clustering.
Silhouette coefficient	Metric evaluating clustering quality. A higher Silhouette coefficient indicates data points are more closely grouped within their clusters and further from other clusters.	Silhouette coefficient evaluates the quality of microbial-based clustering algorithms. When clustering oral microbiome samples, a high value suggests successfully identified distinct and well-separated stomatotypes.
Phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt)	Bioinformatics tool to predict the functional profiles of microbial communities from marker gene data, primarily 16S rRNA sequencing.	PICRUSt provides a functional snapshot of a microbial community based on its taxonomic composition, which is valuable for understanding the metabolic capabilities of uncultivated species. It is commonly used with the KEGG database of genes and metabolic pathways.
Linear discriminant analysis effect size (LEfSe)	Bioinformatics tool to identify biomarkers that significantly characterise differences between groups in microbiome data.	LEfSe identifies statistically and biologically distinct genomic features between sample groups. Features over the threshold score of 2.0 LDA are indicated as significantly different.
Statistical analysis of metagenomic profiles (STAMP)	A software platform for statistically analysing taxonomic and functional profiles of metagenomic data in microbiome analysis.	STAMP compare sample groups and identify significant microbial differences. Due to the high risk of false positives in microbiome studies, an FDR corrected q-value is used to represent the expected proportion of false discoveries among significant features.

3. Results

3.1. Subject demographics

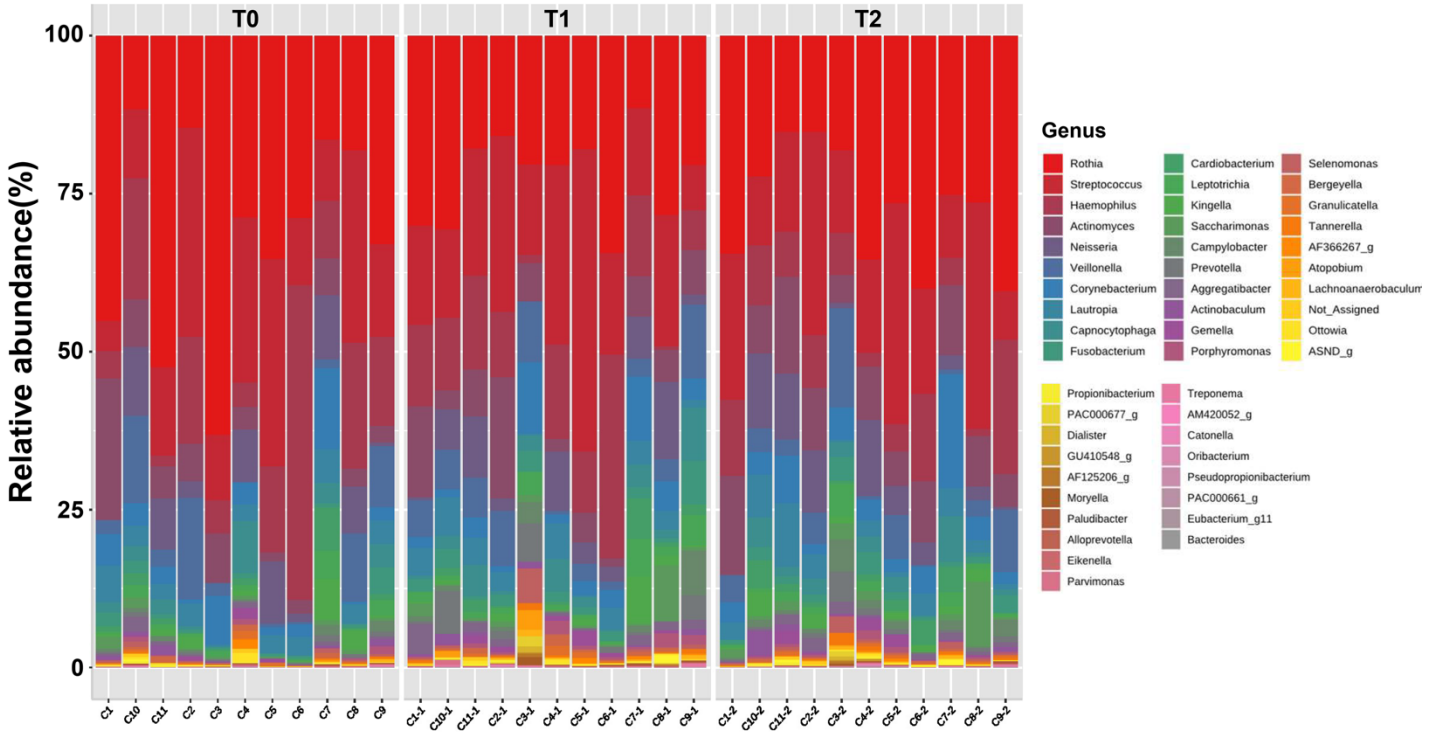
Using the Wilcoxon signed-rank test, a pairwise comparison of the Silness & Löe plaque index data showed no significant differences ($p > 0.05$) between the time points. All participants were fully dentate, with no restorations on the first molar (the area of interest). Likewise, all participants shared the same racial and geographical background.

<Table 7> Subject demographic characteristics

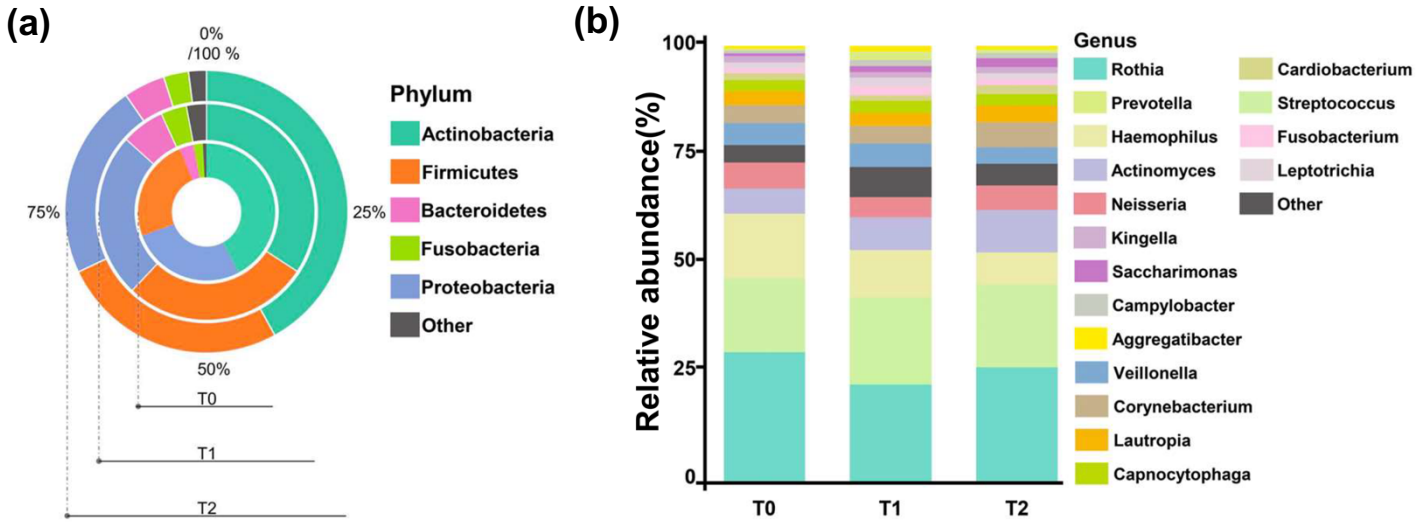
Demographic feature	
Age in years (range)	25.8 ± 1.73 (23-28)
Gender, n (%)	
Male	3 (27.3)
Female	8 (72.7)
Silness & Löe Plaque index	
T0	0.39 ± 0.27
T1	0.39 ± 0.20
T2	0.44 ± 0.19
Pairwise comparison (p-value)	
T0 – T1	0.937
T0 – T2	0.527
T1 – T2	0.240

3.2. Abundance profiling

The abundance distributions for all samples across the three time points, categorised at the genus level, is depicted in Fig 4. The microbial composition of the study population at phylum level (Fig 5a) and genus level (Fig 5b) remained consistent from T0 to T2, suggesting a comparable community composition across the three time points. At the phylum level, Actinobacteria were found to be the most abundant at every sampling point. Initially, at T0, Proteobacteria were more prevalent than Firmicutes, but this trend reversed at T1 and T2, with the difference remaining under 5%. At the genus level, the sequence of the most prevalent taxa remained consistent across the three time points, with *Rothia*, *Prevotella*, and *Haemophilus* leading in abundance.



<Fig 4> Relative abundance distribution. Variations among individuals at three different time points, aggregated at the genus level.

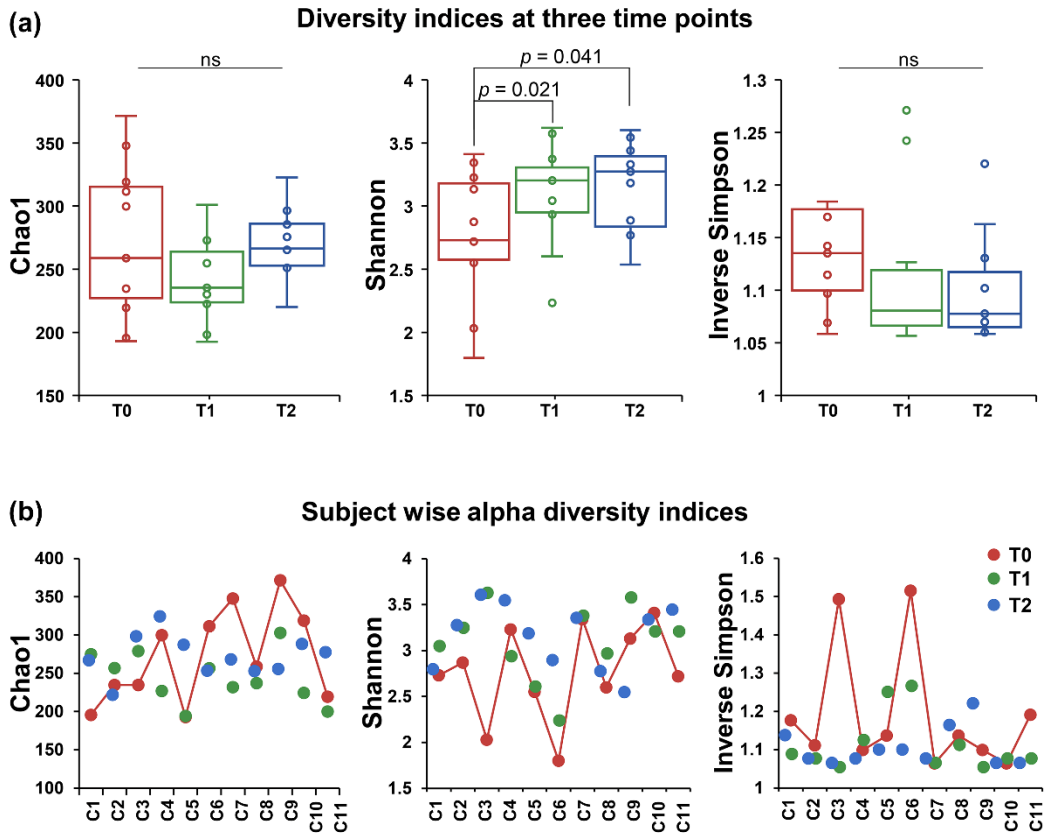


<Fig 5> Relative distribution of (a) phyla and (b) genera across time points. The dot and line marks the values at the initial time point, T0. T1 and T2 indicate the values recorded at 30-day and 90-day intervals from T0, respectively.

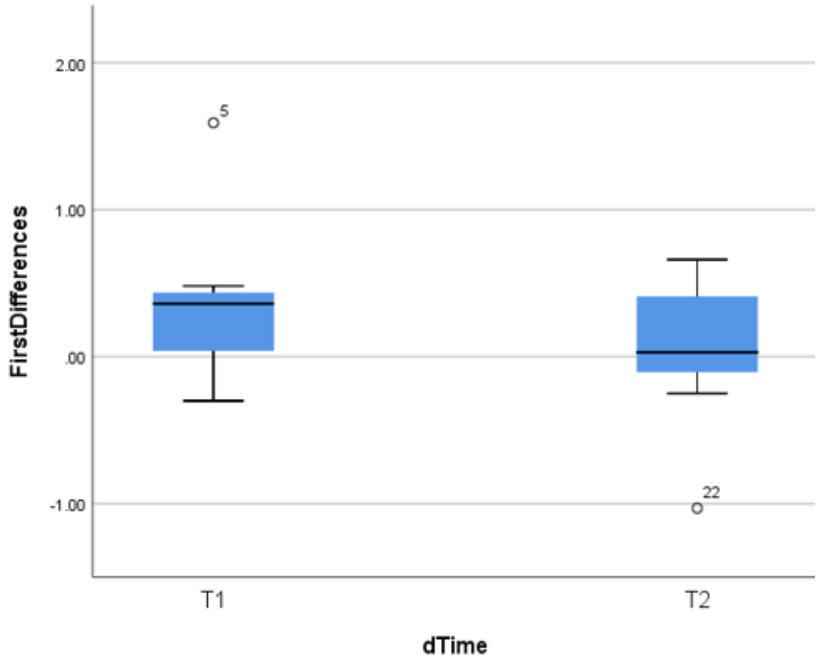
3.3. Alpha diversity measurements

The analysis comparing the Chao1 and the inverse Simpson index over different time points revealed no statistically significant differences. The Shannon index presented a significant increase at both T1 ($p = 0.021$) and T2 ($p = 0.041$) (Fig 6a). Conversely, the initial difference was not statistically significant ($p = 0.306$, Fig 7).

Fig 6b illustrates the subject-level variation by indicating the T0 results with a traced line. Any divergence from this line represents a degree of fluctuation in the within-participant alpha diversity. The comparison at the subject-level revealed differences in the pattern across T0, T1, and T2, with some participants (e.g., C1, C2) exhibiting less variation in diversity compared to others (e.g., C3, C9).



<Fig 6> Alpha diversity metrics. (a) Box plot of Chao1, Shannon, and inverse Simpson indices of all samples in three different time groups. The boxes cover the range from the first to the third quartiles, with the horizontal lines inside indicating the median, while the dots show all samples at each time point. Pairwise comparison is performed using the non-parametric Wilcoxon test, with the p-values provided. (b) Profiles of the three diversity measures for the individual participants.

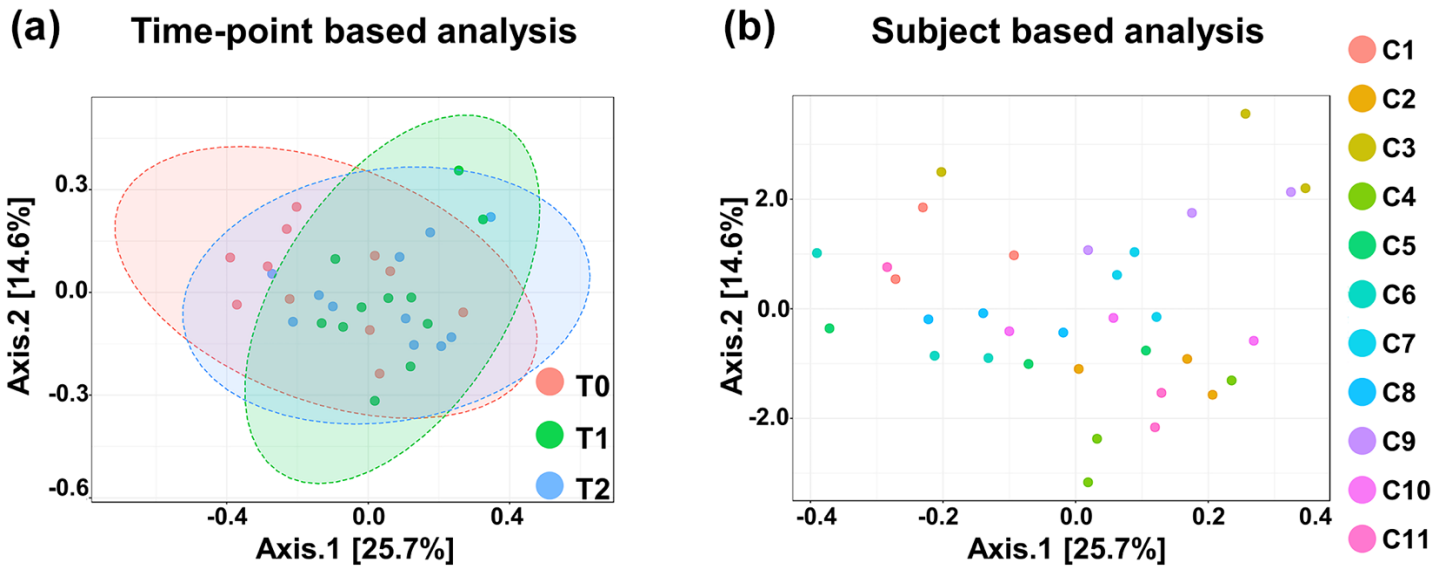


<Fig 7> Rate of change in the Shannon diversity index. No statistically significant difference was observed in the index (expressed as the first difference) between short-term (T0 to T1) and long-term (T0 to T2) changes (Wilcoxon signed-rank test, $p = 0.306$). The error bars represent the standard deviation.

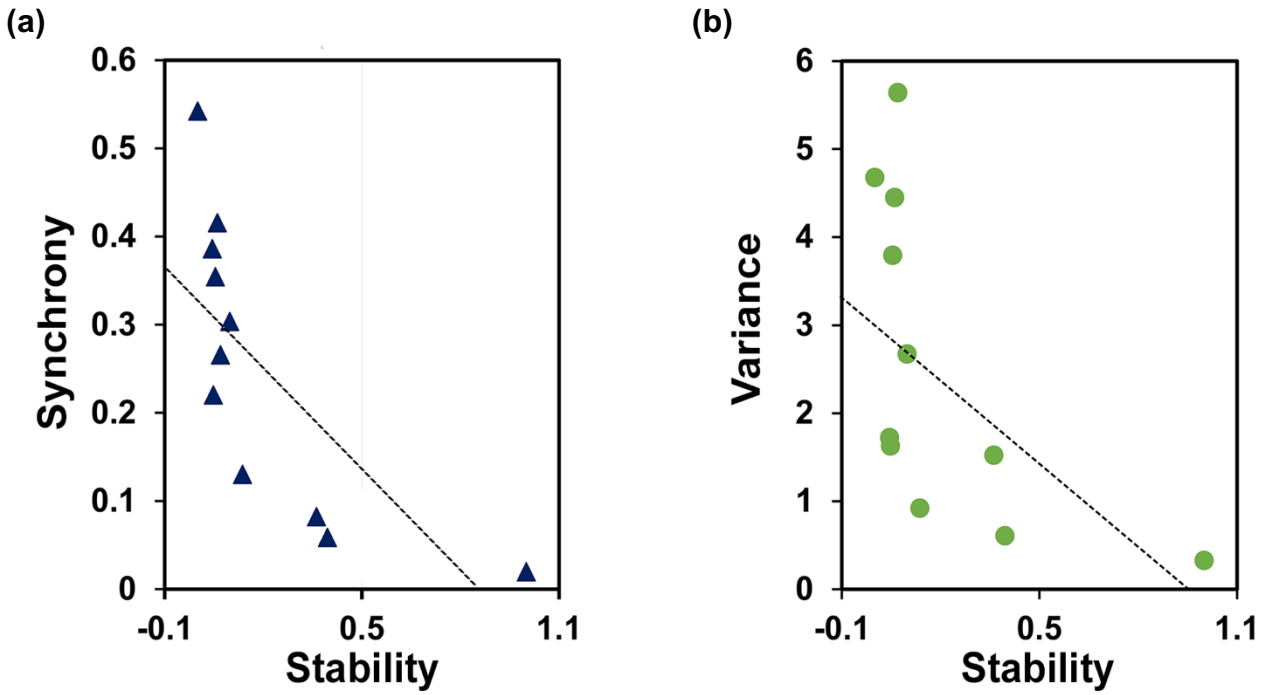
3.4. Patterns of dissimilarity and stability within the cohort

Beta-diversity metrics highlight the dissimilarity in abundance at the community level. The distance matrix, when visualised through PCoA, shows the dissimilarities between two samples as dots. The analyses based on time points (Fig 8a) showed a significant overlap in diversity, suggesting a certain level of similarity. Conversely, when examining the metric at the level of individual participants (Fig 8b), a substantial degree of variation emerged. The Bray–Curtis dissimilarity index demonstrated statistical significance ($p < 0.001$), analysed with multivariate ANOSIM ($R = 0.745$) and ($R^2 = 0.64$, $F = 3.8$).

In the analysis of community stability, a significant negative correlation—ranging from medium to strong—was identified with both synchrony (Fig 9a: $r = –0.739$; $p = 0.009$) and variance (Fig 9b: $r = –0.605$; $p = 0.048$). This indicates that higher community stability is linked to reduced synchrony and more substantial negative covariance.



<Fig 8> Beta diversity of microbial communities. Principal coordinate analysis with the Bray-Curtis dissimilarity distance was utilised to conduct multidimensional ordination for (a) time point-based and (b) participant-based analyses, by calculating the mean distance of individual groups from their respective group centroids.

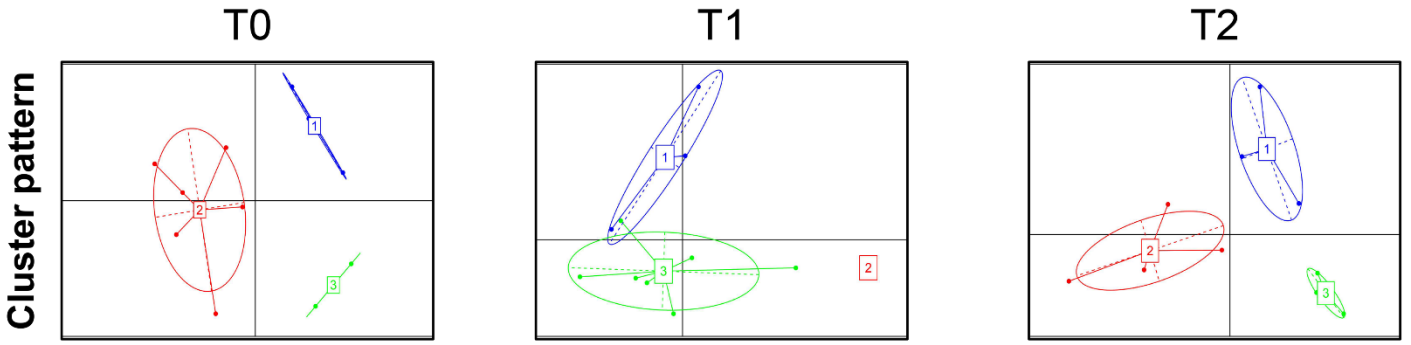


<Fig 9> Community stability metrics of (a) synchrony and (b) variance to the stability.

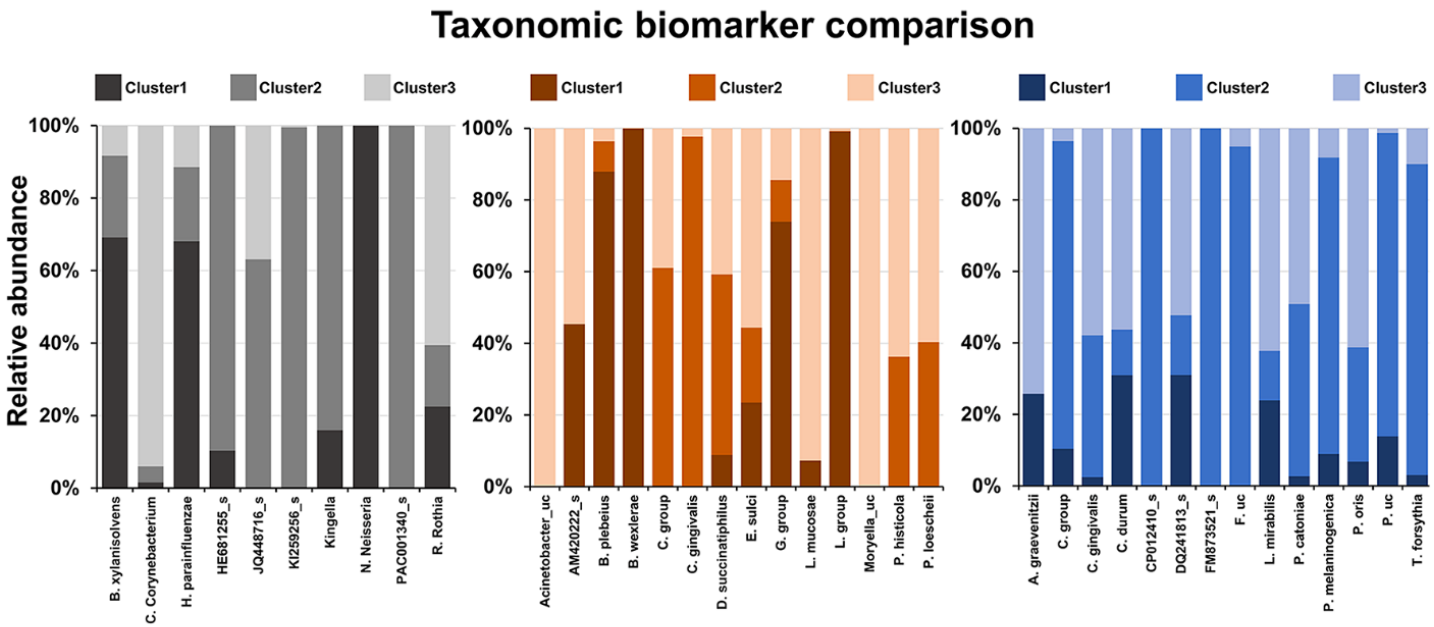
3.5. Stomatotype cluster variation across time

The participant-based clustering approach produced three distinct groups, which were unevenly distributed in terms of participant numbers and showed inconsistency from T0 to T2 (Fig 10). These participant clusters exhibited differences in their microbial compositions, particularly in taxa with high and low abundance.

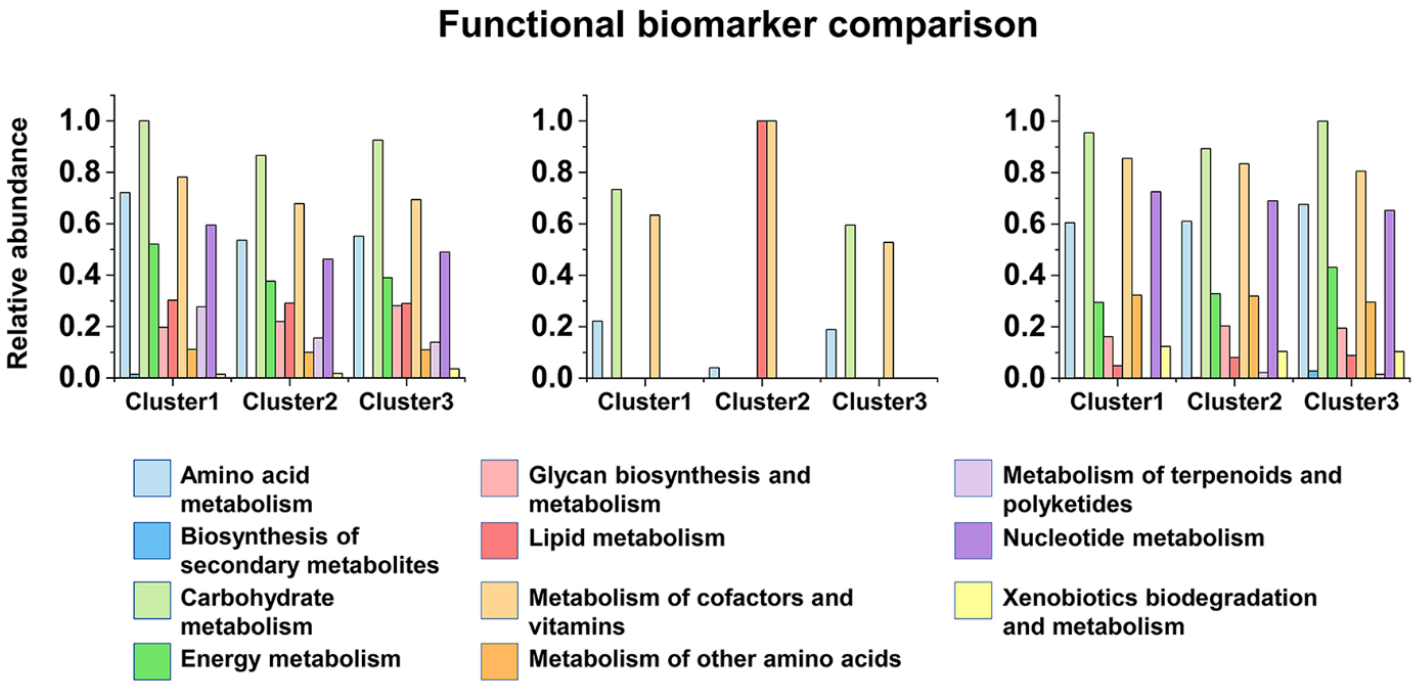
Taxonomic biomarkers were identified to determine the key species or higher taxa that exhibited significant variation (LDA $\geq$ 2.0) among the three clusters (Fig 11). Genera such as *Neisseria*, *Acinetobacter*, and *Moryella*, along with species such as *Blautia* and *Capnocytophaga*, were predominant in one of the clusters. In terms of functional biomarker analysis, 11 metabolic pathways exhibited significant variations between the clusters (Fig 12).



<Fig 10> Participants' microbial-based clustering. Stomatotypes are computed using the Jensen-Shannon Divergence metric at three different time points (T0, T1, and T2).



<Fig 11> Taxonomic biomarkers. The biomarkers were used to ascertain the representative species or higher taxa that varied significantly (LDA ≥ 2.0) between the three clusters. The time points are arranged from left to right, from T0 to T2.



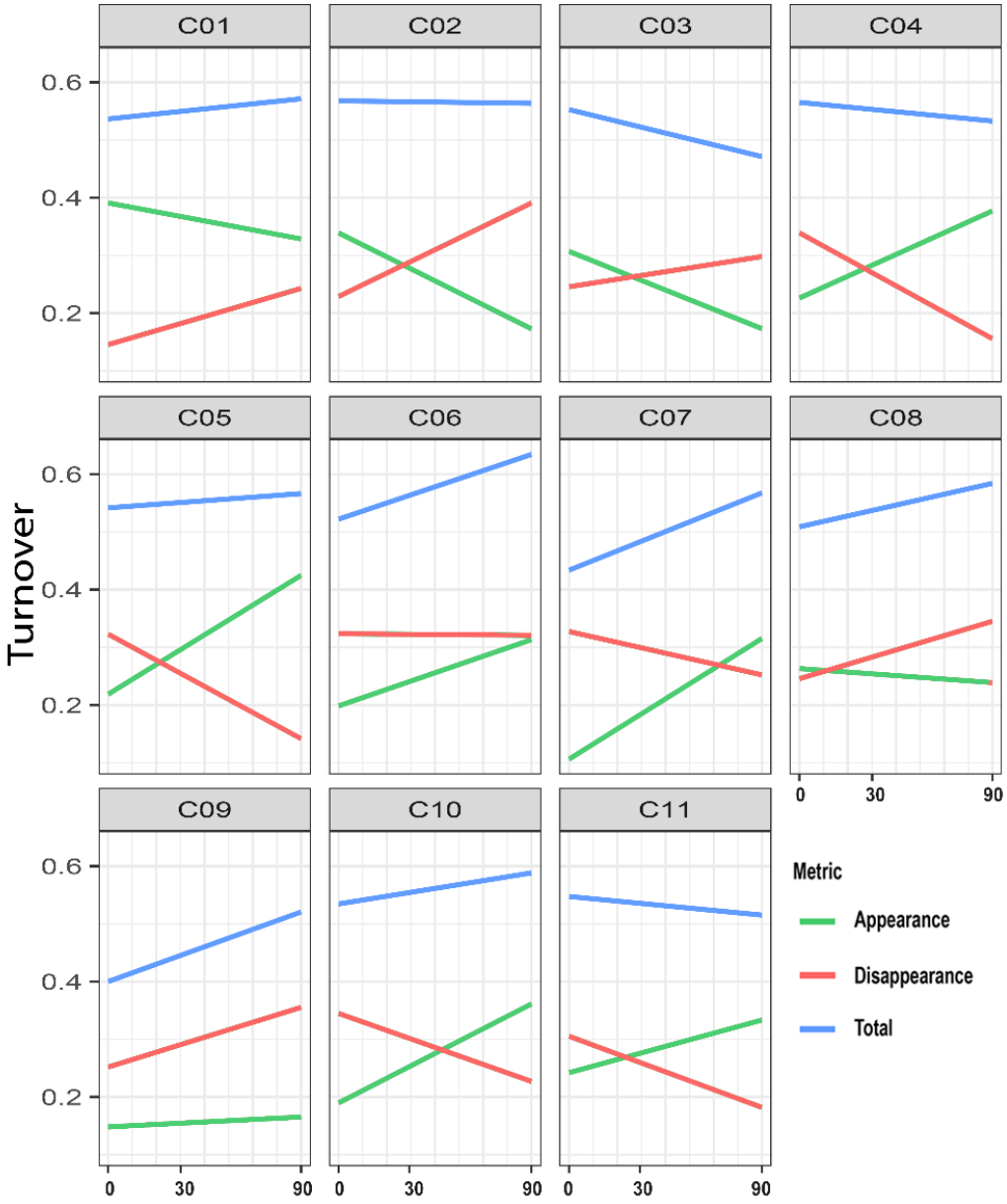
<Fig 12> Functional biomarkers. The biomarkers were used to identify the traits that varied significantly (LDA ≥ 2.0) between the three clusters. The time points are arranged from left to right, from T0 to T2.

3.6. Periodic variation within similar phenotypes

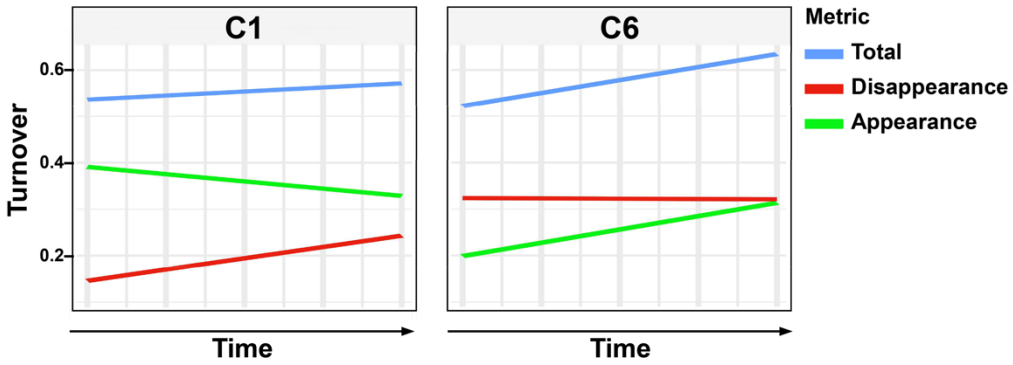
According to the clinical data and species turnover rate depicted in Fig 13, subjects C1 and C6 were similar in terms of overall species turnover rate and community stability metrics, in addition to sharing comparable demographic characteristics clinically. However, the rates of appearance and disappearance varied between the two individuals (Fig 14). Both participants showed an uneven distribution of taxa, with each having unique patterns of dominant taxa (Fig 15).

Through proportional-difference analysis, the appearance and disappearance patterns of significant taxa with fluctuating differences were identified (Fig 16). At T2, the most substantial range of differences is highlighted by notable variations in *Saccharimonas* and *PAC000677_g*, which correspond to the turnover trend depicted in Fig 14.

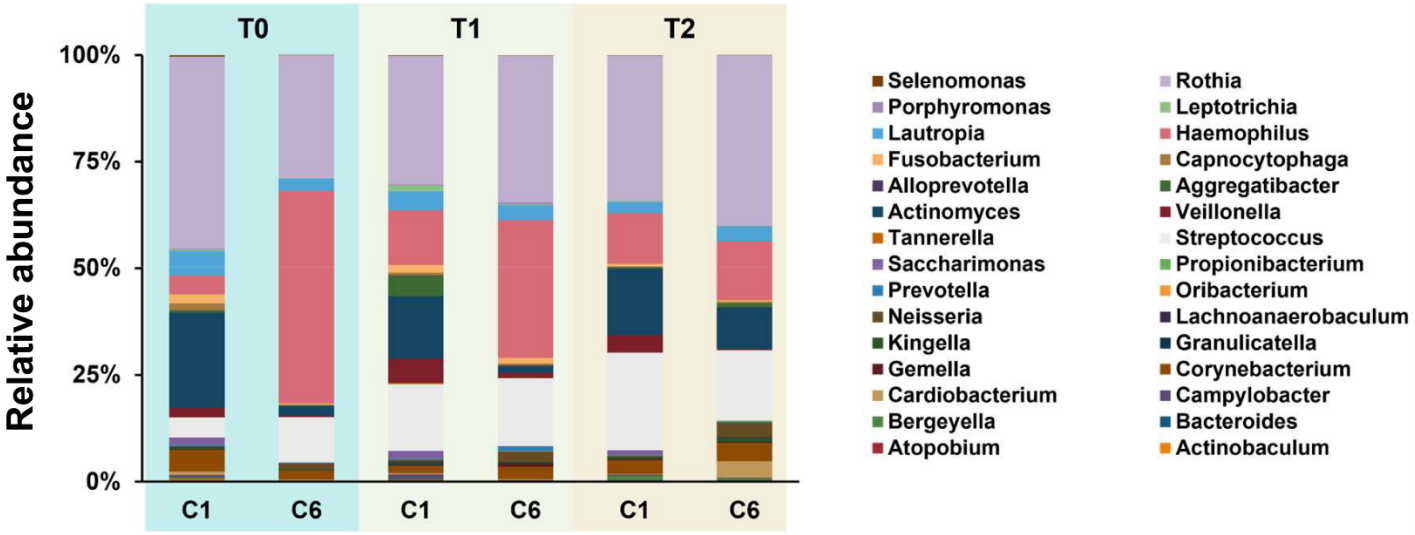
Microbial taxa that significantly contribute to variation in community composition among participants at all time points were identified using similarity percentage (SIMPER) analysis. These taxa collectively contributed to a dissimilarity of 49.11%, with the *Haemophilus parainfluenzae group* and *Rothia dentocariosa* accounting for a significant proportion (Table 8). Variations at the KEGG metabolism level were observed through functional analysis, with markers for carbohydrate and amino acid metabolism pathways being relatively more prominent in C1 compared to C6 (Fig 17).



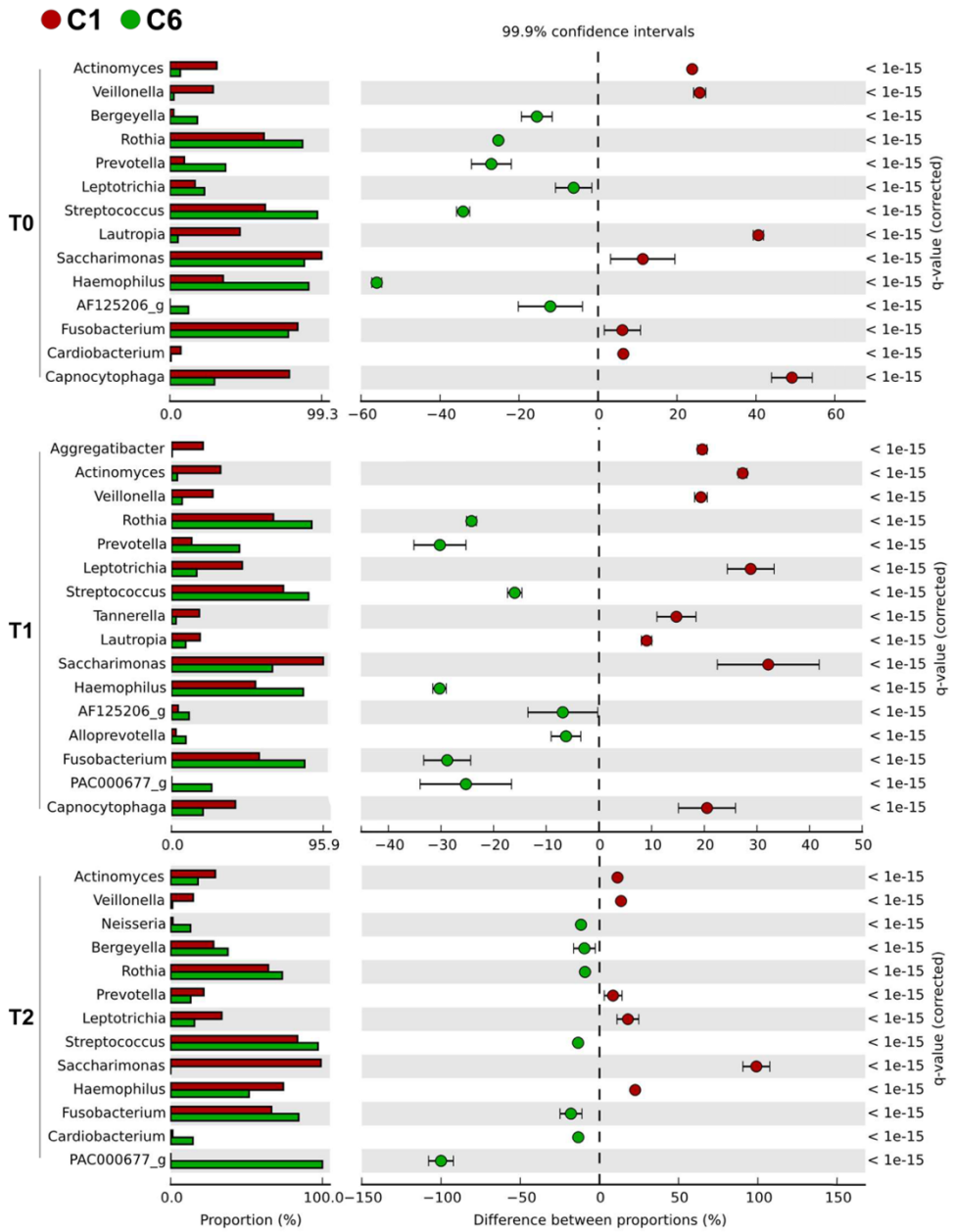
<Fig 13> Community stability turnover metric. The turnover metric is described as the total appearance and disappearance of the species across time.



<Fig 14> Community turnover metrics of participants C1 and C6. The data display contrasting patterns of appearance and disappearance, despite having similar total turnover.



<Fig 15> Comparative analysis of C1 and C6: relative abundance of OTUs. The data were aggregated at the genus level, as observed from T0 to T2.

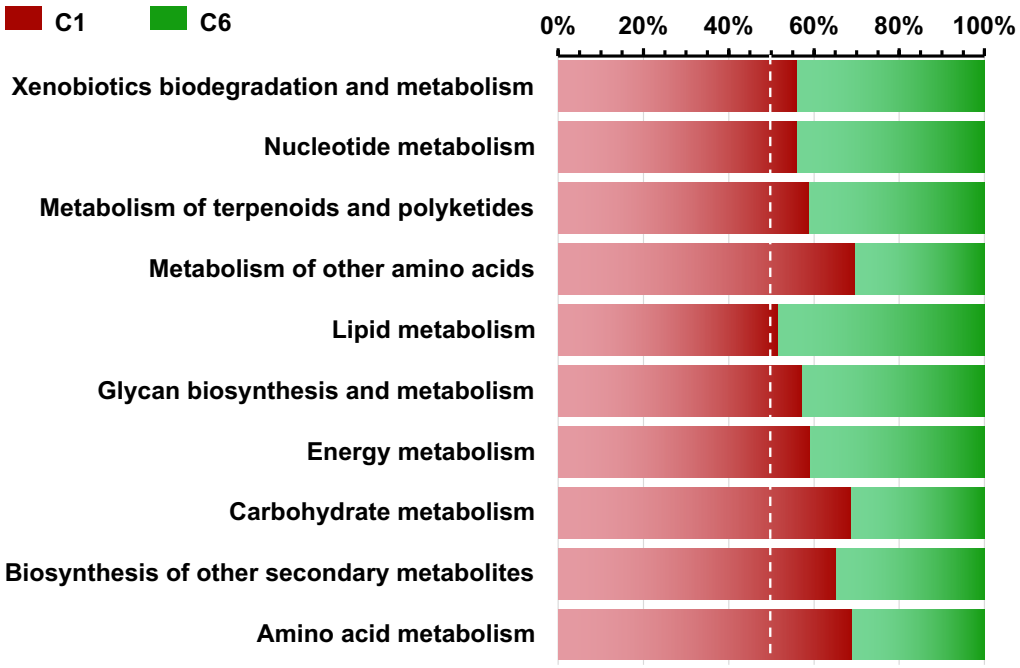


<Fig 16> Comparative analysis of C1 and C6: taxa with fluctuating differences. Significantly different proportions are presented with FDR-corrected p-values (q-value), with the difference expressed as a percentage of the higher groups.

<Table 8 > SIMPER[1)] analysis between the two representative participants. C1 and C6 showing an average dissimilarity of 49.11%.

Taxon	Average dissimilarity	Contribution (%)
Haemophilus parainfluenzae group	12.18	24.79
Rothia dentocariosa	5.076	10.34
Rothia aeria	3.938	8.019
Actinomyces viscosus group	3.447	7.018
Streptococcus pneumoniae group	2.414	4.916
Streptococcus sanguinis group	2.162	4.403
Actinomyces naeslundii	1.924	3.917
Veillonella dispar	1.289	2.625
Lautropia mirabilis	1.089	2.216

[1)] The SIMPER analysis quantifies the relative contribution of each taxon to the overall dissimilarity between groups using the Bray–Curtis dissimilarity index.



<Fig 17> Comparative analysis of C1 and C6: significant KEGG metabolic pathways. The pathways were identified with an LDA effect size ≥ 2.0.

4. Discussion

The concept of a *healthy cohort* has gained importance, largely due to the widely accepted understanding that there are substantial variations among individuals. The empirically established sampling intervals with a schedule of 1-month and 3-month follow-ups were designed to closely mimic standard research setting that includes a healthy reference cohort. The results of this study indicate that the healthy cohort exhibits notable differences in the microbiome profile of samples collected at various times, suggesting variation in the state of microbial stability.

The sampled cohort exhibited similar behaviour in terms of population richness and evenness. Nonetheless, the notable variations in the Shannon index suggest bio-interactions at the species level. The evidence of microbial interaction was further highlighted in the participant-wise index pattern, and a pronounced contrast to the phenotypic (clinical) selection criteria became apparent when examining the changes over three distinct time points. Additionally, a broader range of intra-individual variability was evidenced, particularly concerning the Shannon diversity index measured around T0. The observations described above align closely with the pioneering discoveries from the Human Microbiome Project (Consortium, 2012). Likewise, Sato et al. reported a significant variation in samples collected from healthy adults both on consecutive days and within the same day (Sato et al., 2015). These collective findings conclusively show that the health-associated microbiome exhibits varying degrees of stability over time. Furthermore, these findings are consistent with previous studies confirming the existence of a core microbiome within individuals (Zaura et al., 2009).

These time-dependent fluctuations raise significant concerns when the principle with increased variability within an individual's microbiome serves as an indicator of dysbiosis (Altabtbaei et al., 2021; Zaneveld et al., 2017). In this research, both PERMANOVA and ANOSIM demonstrated statistically significant differences in participant-based analyses, indicating substantial variation within the cohort, i.e., suggesting a higher likelihood of dysbiosis. Consequently, the variability in

the diversity metric found within participants poses a challenge to the concept of a healthy cohort, which is associated with a state of eubiosis.

To identify patterns of variation from T0 to T2 within the study cohort, correlations were examined using a stability metric based on ecological indices (Hallett et al., 2016; Loreau & de Mazancourt, 2008). Regardless of their net abundance, unstable species with high turnover rates have been observed to uphold community states in fluctuating communities (Duran-Pinedo et al., 2021). The stability of a community is influenced by the interaction of its constituent elements. To evaluate this stability, metrics like variance and synchrony are utilised. The variance ratio serves as a tool for comparing community variance with the variance derived from the sum of individual populations (Duran-Pinedo et al., 2021). The synchrony metric evaluates the aggregated abundance of species in relation to the sum of individual species variances (Loreau & de Mazancourt, 2008). Analytically, microbial community states exhibit a pattern of negative covariance or asynchrony when assessed for stability over time (Yachi & Loreau, 1999). In this study, a significant portion of participants demonstrated notably low levels of synchrony (<0.4, asynchrony to synchrony scale ranging from 0 to 1). Furthermore, the study identified a moderate to strong negative correlation pattern, indicating an inverse relationship with stability (Duran-Pinedo et al., 2021). Consequently, these findings suggest the existence of an alternative state of balance among the cohort participants, indicating the presence of unstable species. In the present study, a baseline was established by selecting participants who had not received any oral prophylaxis treatment for at least six months. Nevertheless, it is important to acknowledge that the effects of prior professional oral hygiene treatments cannot be completely overlooked. As a result, the presence of unstable species may have been influenced by these earlier treatments.

A cluster model was implemented to categorise taxa into stomatotypes and explore distribution similarity. As a characteristic feature of a stable community, taxa clustering is expected to result in a reproducible pattern (Duran-Pinedo et al., 2021; Sato et al., 2015). Such characteristic responses to clustering are associated with low inherent variation within an individual. Nevertheless, the concept is contradicted by the observed significant variations in (i) clustering patterns, (ii) biomarkers, and (iii) metabolic indicators. The inconsistency in cluster reproducibility stands in contrast to the findings for a healthy cohort, as concluded by Sato et al (Sato et al., 2015). However,

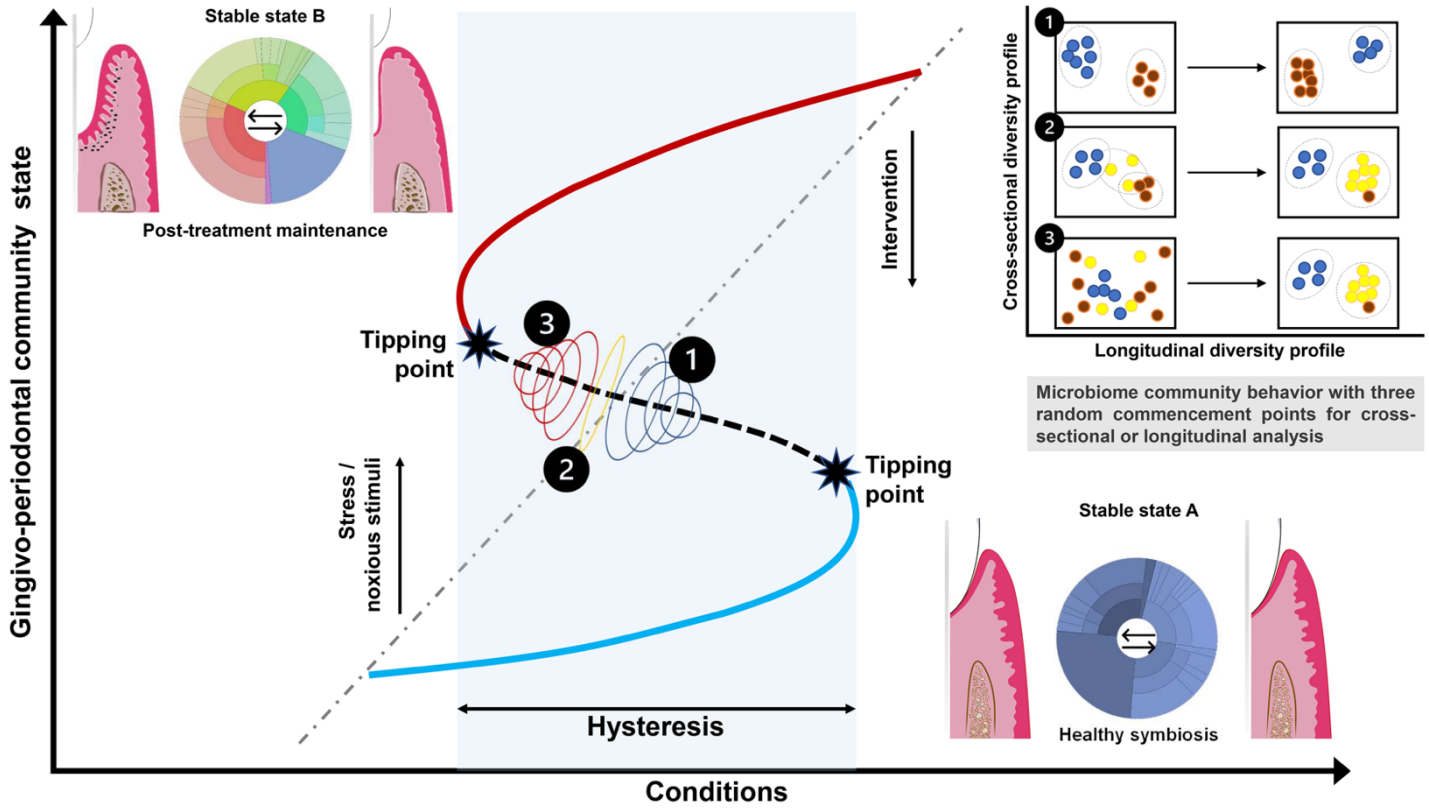
these discrepancies may also stem from the relatively extended sampling intervals examined in this study, thereby underscoring the significant impact of time as a factor influencing the stability of the community state.

Despite the variations over time, it is thought that overall stability can be sustained when the increase in one species balances out the decline in another (Hallett et al., 2016). Earlier research on microbiomes has identified temporal fluctuations as a ubiquitous and essential element in maintaining the stability of the overall community (Hooper et al., 2005; Loreau, 2000; Loreau et al., 2001; McCann, 2000; McNaughton, 1977). In essence, temporal fluctuations and asynchronous patterns observed over time are natural occurrences contributing towards stabilising microbial communities. Yet, significant stress can trigger a change in the stomatotype (clustering pattern), thereby leading to an alternative state (Vandeputte et al., 2021).

The shift in stability is most evidently illustrated through temporal analysis. The example cases highlighted in this study demonstrate a pattern between two individuals who, based on clinical parameters, would typically be classified within a healthy group in a clinical study. Nevertheless, the comparison of the turnover metric between the two individuals revealed a contrasting trend in the number of taxonomic units that gained (appearance) and lost (disappearance). Abundance profiling highlighted the variations, showing a distinct set of dominant genera between the two participants throughout the entire time points. Collectively, they exhibited a dissimilarity of 49.11% (SIMPER, Table 8). In addition, the turnover metrics effect was expressed in a significant disparity in proportions, with an effect size greater than 5. At the initial time point, T0, the 99.9% confidence intervals showed a difference in proportions within a 60% range, which decreased to 40% by T1. While the genus *Leptotrichia* was more abundant in subject C6 at the T0 timepoint, over 20% increase in its proportion was observed in subject C1 at the T1 timepoint. While genera *Rothia*, *Fusobacterium*, and *Saccharimonas* maintain similar proportions at each of the three time points, *Saccharimonas* and *PAC000677_g* exhibit a marked change in variation. In essence, there is a probable pattern of shifts in the community state, where distinct stable states are characterised by varying dominant species.

Alterations in a stable state are influenced by multiple factors, including the role of microbial metabolic interactions. Distinctions at the KEGG metabolism level were detected, with markers for carbohydrate and amino acid metabolism pathways being relatively more pronounced in C1 compared with C6 (Fig 16). A recent investigation utilising genome-scale mathematical modelling yielded definitive findings. Alterations to oxygen and nutrient conditions resulted in a noticeable shift in metabolism. This metabolic shift was then followed by modifications in the expression of related genes, ultimately leading to multiple stable states. Likewise, a recent study utilising genome-scale mathematical modelling has yielded definitive findings. Changes in oxygen and nutrient environments caused a distinct alteration in metabolic processes. This metabolic shift was then followed by modifications in the expression of associated genes, ultimately resulting in the formation of multiple stable states. The necessity of investigating the role of MSH in microbiome dynamics independent of otherwise simplistic causation correlations was emphasised by Khazaei et al (Khazaei et al., 2020). Based on the aforementioned points, it can be inferred that even in a state of health, individuals may exhibit variability, with each person exhibiting distinct stable states with minor differences in the dominant species. These variations within individuals appear to remain consistent longitudinally.

It can be concluded that while the phenotype appears clinically healthy, the intermediate states of the microbiome may be on the verge of transitioning into a disease state. Stable states can encounter triggering events, whether favourable or unfavourable, that may result in a sudden change. Therefore, a single sampling event in a study could reflect a temporary condition, potentially leading to an inaccurate estimation of eubiosis or dysbiosis. In the context of this study, transitioning the baseline point to T1 (instead of T0) could result in a markedly different interpretation, as evidenced by variations in stomatotyping. Thus, a new stable state or reversal between states can only occur if a stimulus exceeds the trigger threshold. In the context of this study, transitioning the baseline from T0 to T1 might lead to a notably different interpretation, as evidenced by changes in stomatotyping. Thus, a new equilibrium or a shift between states can only be achieved if the stimulus surpasses the trigger threshold. Analysis of samples that are in a transitional state, though considered as standard healthy, can introduce bias due to locational effects (illustrated as points 1, 2, and 3 as initiation points in Fig 17) (Zaneveld et al., 2017)



<Fig 18> Multistability and hysteresis perspective. The microbial community undergoes alterations in response to changing conditions, leading to the development of a multi-stable community. Within similar environmental conditions, different stable states are adopted. State changes are precipitated by trigger events, whether environmental or therapeutic, that exert abrupt changes. The system transitions across a tipping point (black stars), and changes may not fully revert (hysteresis). Markers 1, 2, and 3 illustrate three potential scenarios for the initiation of a study, each offering distinct perspectives in cross-sectional and longitudinal analyses.

In summary, the findings of this study imply that when designing an oral microbiome study, relying solely on phenotypic screening may be insufficient as the confounding factors extend beyond the basic characteristics of the subjects (Zaura et al., 2021). Conducting a genomic screening phase will aid in characterising the homeostatic range of the cohort and objectively stratifying participants based on relevant microbial features. This approach will be particularly valuable for identifying disease-associated strains and supporting the development of customised treatment strategies. Genomic screening also plays a crucial role in accounting for interpersonal variation and will become increasingly important in precision analysis of individual genotype–phenotype relationships (Vandeputte et al., 2021). Collectively, these efforts will improve the quality of oral microbiome research to pinpoint diagnostic and therapeutic markers. Another crucial aspect of intervention and follow-up research is incorporating a 'false start'. This strategy not only helps to counteract the observer effect but also supports the development of an adaptational equilibrium over time, which is essential for determining an accurate baseline (Zaura et al., 2021). While there is currently a lack of substantial evidence on the precise number of visits required to achieve equilibrium, it is advisable to collect replicate samples prior to the intervention phase. These replicate samples are valuable for evaluating an individual's natural temporal stability and for making comparisons post-intervention.

While this study offered an in-depth examination of data based on time and participants, the reliance on web-based tools for analysis can present a fundamental limitation. Additionally, sourcing samples from the biobank posed challenges concerning the availability of concurring sample counts and the duration of the assessment period. Lastly, comparison of associative phenotype patterns was not possible due to the limited host metadata available for the samples. While it was possible to screen participant metadata for medication use, factors related to lifestyle, such as the type of toothpaste and dietary components, could not be fully considered (Adams et al., 2017). As a result, the small sample size and the lack of comprehensive host metadata does not allow for a generalisation of the findings from this study. A future investigation incorporating repeated sampling over an extended period will enhance the understanding of MSH in the oral microbiome and aid in the establishment of guiding criteria for selecting healthy cohorts in dental clinical studies.

5. Conclusion

This study evaluated the homeostatic range of stability observed within the oral microbiome of young, disease-free individuals across three time points. Establishing a microbial reference for a healthy cohort is pivotal when designing comparative clinical research exploring health versus disease states or when conducting observational case studies. The findings from this study indicate variations in taxonomic profiles, diversity, and community stability over time within a clinically healthy group.

By applying metrics such as variance and synchrony, this study revealed the existence of temporal variability among participants. Stomatotype clustering analysis further identified meaningful differences in clustering patterns and taxonomic biomarkers across the sampled time points. These findings substantiate the applicability of the multistability hypothesis, which posits that temporal shifts and asynchronous patterns that emerge over time are natural phenomena contributing to the stabilisation of the oral microbiome.

The null hypothesis that there would be no significant difference in the oral microbiome data at each time point, was therefore rejected. The observed temporal differences in microbial community structure and functional potential suggest that even in the absence of clinical intervention or disease, the oral microbiome exhibits variability over time.

In conclusion, for dental clinical research to generate meaningful and generalisable microbial references, the inherent variability and multi-stable nature of the pre-intervention oral microbiome must be carefully considered. The notion of static microbial baseline of health may not adequately capture the natural fluctuations observed even in clinically healthy individuals. Acknowledging such variability has important implications for refining study design and developing a more detailed comprehension understanding of microbial health in both clinical and research settings.

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Abstract in Korean

임상 연구에서 구강 마이크로바이옴 패턴에 미치는 다중 안정성 및 이력 현상의 영향

구강 마이크로바이옴 연구에서 '건강한 코호트(동일집단)'라는 개념은 임상 증례 및 치료적 평가하기 위한 기준점으로 활용되고 있다. 그러나 최근까지의 코호트 기반 연구는 마이크로바이옴의 다중 안정성 측면을 충분히 고려하지 않았다. 임상 연구 시 선별 검사 방법 또한 뚜렷하게 유의미한 차이를 보이는 미생물 유전체 지표와 관련된 표현형 특성들에만 국한되어 있었다. 따라서 본 연구의 목적은 임상적 개입이 없는 표현형적으로 건강한 코호트에서 시간 경과에 따른 구강 마이크로바이옴의 일시적 안정성을 평가하는 것이다.

본 연구는 인체 유래물 은행을 통해 11 명의 참여자로부터 33 개의 치은연상 치태 샘플을 분양받아 후향적으로 수행되었다. 각 참여자의 샘플은 기준일(T0), 1 개월(T1), 3 개월(T2) 간격으로 지정하여 16S 리보솜 RNA 유전자 염기서열 분석을 실시하였다.

마이크로바이옴 계통 분류 프로파일링 결과, *Rothia*, *Prevotella*, *Haemophilus* 속이 모든 시간 범위 그룹에서 지속적으로 우세한 패턴을 보였다. 알파-다양성 지표 중 하나인 샤논 지수(Shannon index)에서는 시간 경과에 따라 T0 으로부터 유의미한 증가를 나타냈다 ($p < 0.05$). Bray Curtis 비유사도 지수에 따른 베타-다양성 지표 분석 결과, 각 시간 범위 코호트마다 개인 간 변이가 높게 관찰되었다($r = -0.02$, $p < 0.01$). 종 수준에서의 미생물 군집 안정성 평가는 동시성(synchrony: $r = -0.739$; $p = 0.009$)과 분산(variance: $r = -0.605$; $p = 0.048$) 분석에서 음의 상관 관계를 나타냈다. 참여자의 종 상대적 풍부도 프로파일을 기반으로 데이터를 클러스터링한 결과, 세 가지 클러스터 그룹이 형성되었다. 이러한 데이터 클러스터링을 각 시간 범위 그룹마다 수행했을 때, 시간대마다 뚜렷한 클러스터 그룹 패턴 차이가 존재하였다. 또한, 각 시간대에서 형성된 클러스터 그룹들은 계통 분류의 다양성 및 기능적 바이오마커 측면에서 뚜렷한 차이를 보였다.

임상적 개입이 없는 건강한 코호트에서도 개인 간 종 교체율과 풍부도에서 차이가 나타나는 것은 구강 마이크로바이옴에서 이력 현상과 다중 안정성의 개념을 뒷받침한다. 따라서 보다 정확하고 의미 있는 장기적인 참조 데이터를 확립하기 위해서는 개인 맞춤형 치료의 맥락에서 임상 코호트 연구가 마이크로바이옴의 역동성과 다중 안정성을 고려해야 한다. 이러한 고려 사항은 신뢰할 수 있는 바이오마커를 식별하고 분류하는 데 있어 정확성을 향상시키고, 궁극적으로 더 효과적인 임상 개입을 통해 구강 건강의 진전을 도모할 수 있다.

핵심되는 말 : 구강 마이크로바이옴, 다중 안정성, 디스바이오시스(미생물총 불균형), 일시적 변동성, 16S 리보솜 RNA 유전자 염기서열 분석