Evaluation of periodic stability of the oral microbiome from a healthy cohort using 16S ribosomal RNA gene sequencing analysis

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Abstract

Objectives

The use of 16S ribosomal RNA gene sequencing analyses has rapidly increased in clinical oral studies. However, cohort-based clinical research has not sufficiently accounted for the periodic stability in oral microbiota. Herein, we aimed to assess the stability of the oral microbiome across time from an intervention-free “healthy” cohort.

Materials and Methods

We obtained 33 supragingival samples of 11 healthy participants from the biobank. For each participant, we processed one sample as baseline (T0) and two samples spaced at monthly (T1) and quarterly (T2) intervals for 16S ribosomal RNA gene sequencing analysis.

Results

We observed that taxonomic profiling had a similar pattern of dominant genera, namely *Rothia, Prevotella,* and *Hemophilus*, at all-time points. Shannon diversity revealed a significant increase from T0 (p<0.05). Bray Curtis dissimilarity was significant (R=-0.02, p<0.01) within the cohort at each time point. Clustering revealed marked differences in the grouping patterns between the three-time points. For all time points, the clusters presented a substantially dissimilar set of differentially abundant taxonomic and functional biomarkers.

Conclusion

Our observations confirmed presence of periodically different stable states within the oral microbiome in an intervention-free healthy cohort. Accounting for multi-stability will improve the understanding of future research and facilitate identifying and classifying the reliable markers of diseased, healing, healed, and healthy states.

Clinical relevance

The high periodic variation within a healthy cohort demonstrated the presence of multiple stable states within an individual. Clinical research using RNA gene sequencing for comparison should adopt microbiome specific selection criteria for careful classification of a health-associated group.

1. Introduction

The etiological correlation to oral diseases has facilitated the use of culture and polymerase chain reaction-based techniques to identify the causative organisms. However, traditional methods are self-limited in inferring the “difficult-to-grow” organisms and are considered close-ended [1]. With the establishment of the “Polymicrobial Synergy and Dysbiosis” hypothesis [2], researchers have preferably

used the sequence-based approach to identify difficult-to-grow. Research in the human oral microbiome has markedly enriched in the past decade [3, 4]. The majority of advancement is accredited to the rapid improvement in sequencing costs and downstream bioinformatics. Greater convenience and improved access to high throughput 16S ribosomal RNA (rRNA) gene amplicon sequencing have facilitated exploratory studies and led to voluminous growth in oral microbiome data [5].

Renewed focus on personalized oral health therapy has promoted the growth of fast-evolving microbiomics toward hypothesis-driven research [6, 7]. Notwithstanding the existing publications, there are widespread concerns over the pitfall of microbiome data interpretation, including the overestimation of clinical relevance [4, 8]. An optimized oral microbiome clinical study design, namely observational (case-control/cohort) or interventional (clinical trial), begins with understanding the target subject-group. Both study designs commonly rely on a comparative metric to conclude a hypothesis-driven concept with the study participants meeting a defined selection criterion. In recent years, multiple oral microbiome studies have presented comparisons between cohorts categorized by the presence of a disease (e.g., carious versus non-carious[9]) and disease severity (e.g., chronic or aggressive periodontitis[7]). Commonly, the cohort is considered either a “healthy” or “control” group, thus resulting in an inferential basis. However, the complexity in defining a healthy cohort increases upon considering niche-based, time-based, and observer effect variations. In addition, researchers select the time and duration of the intervention or observation based on limited evidence [4]. These warrant considering the *homeostatic range of variability* in intervention-free oral microbiome before measuring the impact between cohorts. Another commonly found approach is the use of biobank data for hypothesis-driven study designs. While the accessibility to a biobank may aid in data collection for study design, use of the traditional selection criteria may not accurately ensure the integrity of results between cohorts.

To illustrate the points above, we explicitly address the concept of a *healthy cohort*, biobank sourced samples. We aimed to examine the periodic stability of oral microbiome collected from the first molar free gingival margin in young and disease-free participants at three time points. Additionally, we replicate the periodicity of sampling with clinically common follow up schedule. Lastly, using a head-to-head comparison between two participants, we highlight the significance of taxonomic and functional biomarkers and draw a conclusion under the multi-stability concept.

2. Materials And Methods

Study population and data collection

The study was approved by and performed according to the Institutional Review Board at the Yonsei University Dental Hospital (Approval number: 2-2021-0050). The samples were obtained from the Oral-derived bioresources for the human-derived materials biobank based at the Yonsei University Dental Hospital, South Korea. The inclusion criteria were as follows: (i) sequential multi-period supragingival plaque samples, (ii) collected from the marginal gingival regions of the posterior teeth, (iii) low plaque-index, (iv) age > 18 years at the first sample, and (v) samples collected and stored with similar buffering

protocol [10]. The exclusion criteria were as follows: (i) age > 30 years during sample collection, (ii) smoking or a history of pregnancy or nursing, (iii) the loss of natural tooth structure at/near the sampling site, (iv) professional dental therapy within 6 months from sampling, (v) a history of chronic medication and co-morbidities (viz. gastrointestinal), and (vi) insufficient or non-coherent biobank data.

Based on the above-mentioned criteria, we selected 33 samples from 11 participants, collected and buffered with the OMNI-gene OMR-110 kit (DNA Genotek Ottawa, Canada) based on a concordant processing protocol. The sample periodicity was redefined as baseline (T0), monthly (T1 = T0 + 30d), and quarterly (T2 = T0 + 90d). We limited the analyzed samples to the first molar regions only. For a time-point, samples belonging to similar participants were pooled for the analysis (Fig. 1).

Sequencing and data processing

16S rRNA gene sequencing was performed at CJ BioScience Inc. Seoul, South Korea, with the published protocol [11]. Briefly, the extracted DNA samples were amplified via polymerase chain reaction with primers targeting the V3–V4 regions of the 16S rRNA gene. Amplicon sequencing was delivered using the Illumina Miseq Sequencing System (Illumina, USA). We processed the raw reads for quality check; low-quality reads (< 25) were filtered, followed by the merging of paired-end sequence data. The primers were trimmed, and 16S rRNA unique reads with a similarity threshold of 97% were isolated for taxonomy allocation based on the EzBioCloud 16S rRNA database [12].

Diversity measurement

We performed downstream analyses using web-based tools, unless otherwise specified. The gene copy number-normalized records were extracted from “EzBioCloud” [12] and uploaded to the “MicrobiomeAnlayst” tool for meta-analyses [12–14]. We formatted the relative abundance at the genus level at 1% cut-off for categorization into the “Others” group. Alpha diversity (richness and evenness) was visualized using the Chao1, Shannon, and Inverse Simpson indices, and statistically compared using the Wilcoxon Signed rank test (significance level 0.5). Beta-dissimilarity distances were calculated using the principal coordinate analysis (PCoA) with the Bray-Curtis dissimilarity index [15] and statistically compared with the permutation analysis of variance (PERMANOVA) and similarity analysis (ANOSIM).

Analysis of microbiome stability

We used the R package “Codyn” on RStudio (ver. 2021.09.0) to investigate the degree of change with time [16]. We measured the species turnover (appearances, disappearances), stability (the temporal mean divided by the temporal standard deviation), variance ratio (community’s variance relative to the sum of individual variances), and synchrony (the variance of aggregated species abundances with the summed variances of individual species) [15].

Clustering and biomarker comparison

We explored temporal dynamics by clustering the data based on the species abundance profiles. The species trajectories were clustered based on the method described by Arumugam et al. by formulating the concept of enterotypes (gut microbiome) [17], also referred to as stomatotype for the oral microbiome [18]. Briefly, we tabulated and clustered normalized genus abundance profiles with the Jensen-Shannon divergence distance and the portioning around medoids clustering algorithm (cluster library) [19]. The optimal number of clusters was determined using the Calinski-Harabasz index. Eventually, we validated the statistical significance of optimal clustering with the silhouette coefficient. The complete algorithm and explanations have been described in the web-based tutorial (http://enterotype.embl.de) and were followed without modification [20, 21].

We contrasted the biomarkers between the clusters and to predict the taxa and metagenomic functional pathways which correlate to the variability between the clustered groups. Functional biomarkers were analyzed using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) [22]. Significant differences were computed using a linear discriminant analysis (LDA) effect size analysis, with a threshold LDA score ≥ 2 [23]. Finally, we illustrate the periodic variability within similar phenotype by comparing datasets of two participants. Performing the similar analysis series, we highlighted the turnover pattern and illustrated the magnitude of difference in a summarized taxonomic and functional biomarker profile. Figure S1 depicts the comprehensive analytical flow, and the observations are elaborated in following sections.

3. Results

Pairwise comparison of the Silness and Löe plaque index records showed no significant differences ($p > 0.05$) with Wilcoxon signed-rank test. Additionally, all subjects had a full complement of teeth, no restoration on the first molar (region of interest), and adjacent teeth in the same arch; and belonged to the same race from the same geographical region (Table S1).

Abundance profiling

The microbial composition of the study population at Phylum (Fig. 2a) and Genus levels (Fig. 2b) were comparable throughout the T0 to T2 time points, indicative of a similar community composition across time points. At the Phylum level, we observed the highest abundance for *Actinobacteria* at all time points. At T0, *Proteobacteria* were relatively more than *Firmicutes*, which was reversed at T1 and T2; however, the difference was < 5%. The Genus level trend displayed similar compositions of the most abundant taxa across the three-time points in the following order: *Rothia, Prevotella*, and *Hemophilus*.

Alpha diversity measurements

A comparison of Chao1 (overall species richness index) and inverse Simpson (average proportional abundance) across time points did not demonstrate any statistical significance. The Shannon index presented a significant increase at both T1 ($p = 0.021$) and T2 ($p = 0.041$) (Fig. 2c). However, the first difference did not present any significance ($p = 0.306$, Figure S2). Subject-level comparison revealed

variations in the trend throughout the T0, T1, and T2, with certain participants showing lower fluctuation in diversity (e.g., C1, C2) than others (e.g., C3, C9). Figure 2d plots the variation at a participant level by indicating the T0 findings traced with a line. The deviation from the line indicated a level of fluctuation in within-participant alpha diversity.

.The taxonomic profiling for all participants is presented as a factor of time, namely T0, T1, and T2, at (**a**) Phylum and (**b**) Genus level data. (**c-d**) Alpha diversity metrics diagrams. **(c)** Box plot of Chao1, Shannon, and Inverse Simpson α-diversity of all samples in three different time groups. The boxes span the first to third quartiles; the horizontal lines inside the boxes represent the median, and the dots represent all samples at each time point. Pairwise comparison is performed using the non-parametric Wilcoxon test, and the *p*-values are provided. **(d)** Profiles of the three diversity measures for individual participants. The dot and line plot traces the values at the initial time point T0. T1 and T2 represent the values observed at 30 d and 90 d intervals from T0.

Patterns of dissimilarity and stability within the cohort

Beta-diversity metrics reveal the dissimilarity in community-level abundance. The distance matrix visualized via PCoA represents the dissimilarities between two samples expressed as dots. The time-point-based (Fig. 3a) analyses revealed considerable diversity overlap, indicative of a certain magnitude of similarity. In contrast, substantial variation was observed when the metric was tested at the individual participant level (Fig. 3b), where the Bray-Curtis dissimilarity index was statistically significant ($p < 0.001$), analyzed with multivariate ANOSIM (R = 0.745) and PERMANOVA ($R^2 = 0.64$, F = 3.8)

On analyzing the correlation of community stability, we observed a significant negative correlation (medium to strong) for both synchrony (Fig. 3c, r =-0.739; $p = 0.009$) and variance (Fig. 3d, r =-0.605; $p = 0.048$), indicating higher community stability had low synchrony and more significant negative covariance.

Stomatotype cluster variation across time

The participant-based clustering method resulted in three cluster groups, which were unevenly distributed in the number of participants and inconsistent from T0 to T2 (Fig. 4a). The clustered participant groups considerably varied in their microbial composition in both high and low abundance taxon.

We identified the taxonomic biomarkers to ascertain the representative species or higher taxa that significantly (LDA > 2) varied between the three clusters (Fig. 4b). Genera, such as the *Neisseria*, *Acinetobacter*, *Moryella*, and species such as the *Blautia* and *Capnocytophaga* dominated one cluster. On functional biomarker analyses, 11 metabolic pathways were significantly variable between the clusters (Fig. 4c).

Head-to-head participant analyses

Based on the available clinical information and species turnover rate (Figure S3), C1 and C6 were comparable in the total species turnover rate and community stability metrics, besides being clinically alike in the demographic characteristics. Nevertheless, the appearance and disappearance turnover rates contrasted the two individuals (Fig. 5a). Both participants presented an uneven taxonomic distribution, with different patterns of dominant taxa (Fig. 5b).

Figure 5. Analytical flow (Figure S1) in the context of two participants (C1 and C6) with comparable features (**a**) The turnover metric displaying contrasting patterns of appearance and disappearance with similar total turnover. (**b**) A comparison of the relative taxonomy abundance at T0, T1, and T2 at three-time points. (**c**) Significantly different (C-**i**) taxonomic unit and (C-**ii**) KEGG metabolic pathways. KEGG; Kyoto Encyclopedia of Genes and Genomes

4. Discussion

The role of a healthy cohort has gained significance owing to the general concept of high differences between individuals. The empirically designed sampling intervals were based on a monthly and quarterly follow-up schedule to closely mimic the common research scenario, comprising a “healthy” reference cohort. The findings from the present study show that the “healthy cohort” has significant variations in the microbiome profile of periodically collected samples, implying a difference in the state of microbial stability.

According to the population richness and evenness results, the sampled cohort behaved similarly. However, the significant Shannon index changes indicated species level bio-interaction. These findings of microbial interaction were further evident in the participant-wise index pattern, and the sharp contrast to the phenotypic (clinical) selection criteria was markedly observed on visualizing the changes across three-time points. Moreover, intraindividual variations were evidenced over a broader range of variability, particularly in the Shannon diversity metric traced about T0. The observations mentioned above were mainly in accordance with pioneering results from the human microbiome project [24]. Similarly, in the findings of Sato et al., a significant difference was reported in a consecutive day and intraday samples of healthy adults [25]. These results are collectively conclusive of varying stability as a factor of time within the microbiome classified as health-associated.

The observed time-dependent variations become particularly concerning when interpreted in light of the Anna Karenina principle (AKP), which hypothesizes that increased within-participant variability in the microbiome as a good marker for dysbiosis [26, 27]. In the present study, both PERMANOVA and ANOSIM displayed statistically significant differences in participant-based comparisons, thus indicating a significant variation within the cohort, *i.e.*, a higher predilection for dysbiosis. Therefore, the observed within-participant variability of the diversity metric challenges the “healthy cohort” concept, synonymous with a state of eubiosis.

To reveal the pattern of variations observed from T0 to T2, within the study cohort, we evaluated a correlation to stability metric from ecology indices [16, 28]. Independent of the net abundance, in a

fluctuating community, unstable species (species with high turnover) have been reported to maintain community states [15]. Analytically, such states present with a negative covariance or asynchrony pattern against stability when observed over time [29]. A moderate to strong negative correlation pattern observed in the present study also indicated an inverse relation to stability. Therefore, our findings would also imply the presence of an alternative state of balance amidst participants of the cohort, suggesting the presence of unstable species.

Enterotypes have been defined as the state where samples get assigned when binning different individuals into classes that share some similarity in microbiota composition.[17, 30] We adopted a cluster model that bins taxa into somatotypes to explore the distribution similarity. As a characteristic of a stable community, clustering of taxa would result in a reproducible pattern. [15, 25]. This characteristic response to clustering is attributed to low inherent variation within an individual. However, our findings challenged the concept with the observation of marked differences in the aspects of: (i) clustering pattern, (ii) biomarkers, and (iii) metabolic indicators. The lack of cluster reproducibility contrasts the outcomes for a healthy cohort from the conclusions of Sato et al. [25]. However, such differences can be attributed to comparatively longer sampling intervals analyzed in this study, thus highlighting a notable effect of temporal dynamics on the oral microbiome.

We performed the PICRUSt analysis to estimate the metagenomic function. Our findings were in contrast to earlier research, which predicted greater stability of the metagenome across time [31]. However, the interpretations were consistent with the finding that single estimates (cross-sectional) do not effectively describe the equilibrium abundance [30]. These findings become increasingly relevant while considering diagnostic or intervention-related changes. Consequently, identifying stable somatotypes within a cohort across time points can markedly contribute to identifying bioindicators [32].

Eventually, we nominated two individuals to illustrate differences due to microbiome stability. The turnover metric revealed an inverse trend upon considering the number of taxonomic units gained (appearance) and lost (disappearance). Abundance profiling of the groups resonated the differences with a distinct group of dominant genera between the two participants at all time points. Comprehensively, they presented 49.11% (Table S2) dissimilarity of significant taxonomic units (Fig. 5c-i). Subsequently, we could identify differences at the KEGG metabolism level with significant variations in secondary metabolite synthesis, carbohydrate, and nucleotide metabolism (Fig. 5c-ii), further indicative of a metabolically different microbial community composition due to a difference in stable states.

Despite fluctuations over a period, it is believed that net stability can be achieved when an increase in one species compensates for a decreased abundance in another [16]. Previous microbiome studies have described temporal fluctuation as a ubiquitous and vital factor in the stability of the aggregate community [33–37]. In other words, temporal fluctuations and asynchronous patterns observed over a time series are natural processes toward the stability of the microbial community. However, substantial distress can elicit a switch of the stomatotype (clustering pattern), thereby resulting in an alternative state

[30]. While this step may not express significantly between niches, researchers cannot overlook the impact of time on stability.

The aforementioned points are further validated under the multi-stable ecosystem concept, which outlines the need to consider the prevalence of more than one stable system within a particular condition [38]. The stable states can experience trigger events (favorable or unfavorable) that can lead to an abrupt change. Therefore, only a single sampling instance for a study may have a transient state, potentially wrongfully estimating eubiosis or dysbiosis. With context to the present study, a shift in the baseline point to T1 instance from T0 may lead to a significantly different inference, as vindicated by stomatotyping variations. Figure 6 schematically depicts the multi-stable state of microbial community ecology while also considering the effect of AKP.[26, 39, 40] Changes in conditions can affect the microbial community, where a multi-stable community adopts different stable states within similar environmental conditions. However, the system states vary across a tipping point (black stars), and changes between stable states are not entirely reversible. Such effect is referred to as hysteresis [40]. Therefore, a novel stable state or reversal between states can occur only with a stimulus beyond the trigger threshold. Studying samples in a transitional state, when regarded as standard “healthy,” may result in bias seeding owing to the locational effect (exampled as points 1, 2, and 3 as points of study commencement) [26].

To summarize, the prestudy findings suggest that phenotypic screening alone may fall short as the confounding factors extend beyond basic subject features while planning an oral microbiome study [4]. A genomic screening step will facilitate understanding the cohorts’ homeostatic range and the sub-selection of objectively relevant groups. Taken together, they will enhance the quality of oral microbiome studies to identify the diagnostic and treatment markers. Another point of emphasis for intervention and follow-up studies is to include a “false start,” and allow an adaptational equilibrium (with time), and help in finding a true baseline [4].

The present study provided a comprehensive analysis of time-and participant-based data; however, the use of web-based tools for analysis may be an inherent limitation. Furthermore, the sample sourcing from biobank was a drawback, in terms of the assessment period being limited to a quarterly interval. Third, we could not compare associative phenotype patterns owing to limited host metadata for the available samples.

5. Conclusion

The microbiome community shows variations associated with time within a clinically “healthy” cohort. Gradual variations in stomatotype clustering patterns supported the applicability of the multi-stability hypothesis in the oral microbiome. By cautiously accounting for the role of periodic variability, investigators can perform effective hypothesis-driven research designs and target identification in clinical microbiome research.

Declarations

CRediT authorship contribution statement

U. Mangal: Data curation, Formal analysis, Visualization, Writing – original draft, Investigation. *K. Noh:* Data curation, Formal analysis, Methodology, Writing – original draft, *S. Lee:* Data curation,Investigation, Methodology*, J-K Cha* and *J-S Song:* Supervision, Resources, *K-J Lee*, *J-Y Cha and K-M Kim:* Supervision, Resources, Investigation. *J-S Kwon:* Resources, Conceptualization, Methodology, Supervision, Writing - Review & Editing, Funding acquisition. *S-H Choi:* Conceptualization, Methodology, Supervision, Writing - Review & Editing, Project administration, Funding acquisition. All authors reviewed the manuscript. *U. Mangal and K. Noh have co-first authorship based on equal contributions*

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Declarations

Ethical approval

The subject selection and sample gathering process was approved by and carried out according to the Institutional Review Board at Yonsei University Dental Hospital. The human-derived materials biobank, which provides de-identified samples, at the Yonsei University Dental Hospital is approved by the Institutional Review Board at Yonsei University Dental Hospital (IRB) to use samples donated by participants specifically for research after receiving an informed consent (IRB reference number: 2-2021-0050). The BioBank protocols are in accordance with the ethical standards of our institution and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent

Informed consent was obtained in writing from each participant for all points of sample collection. Participants were invited to donate the plaque samples after being given a written information consent.

Conflicts of interest

There are no conflicts of interest in connection with this article.

Availability of data and materials

All data generated or analysed during this study are included in this published article (and its supplementary information files)

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Figures

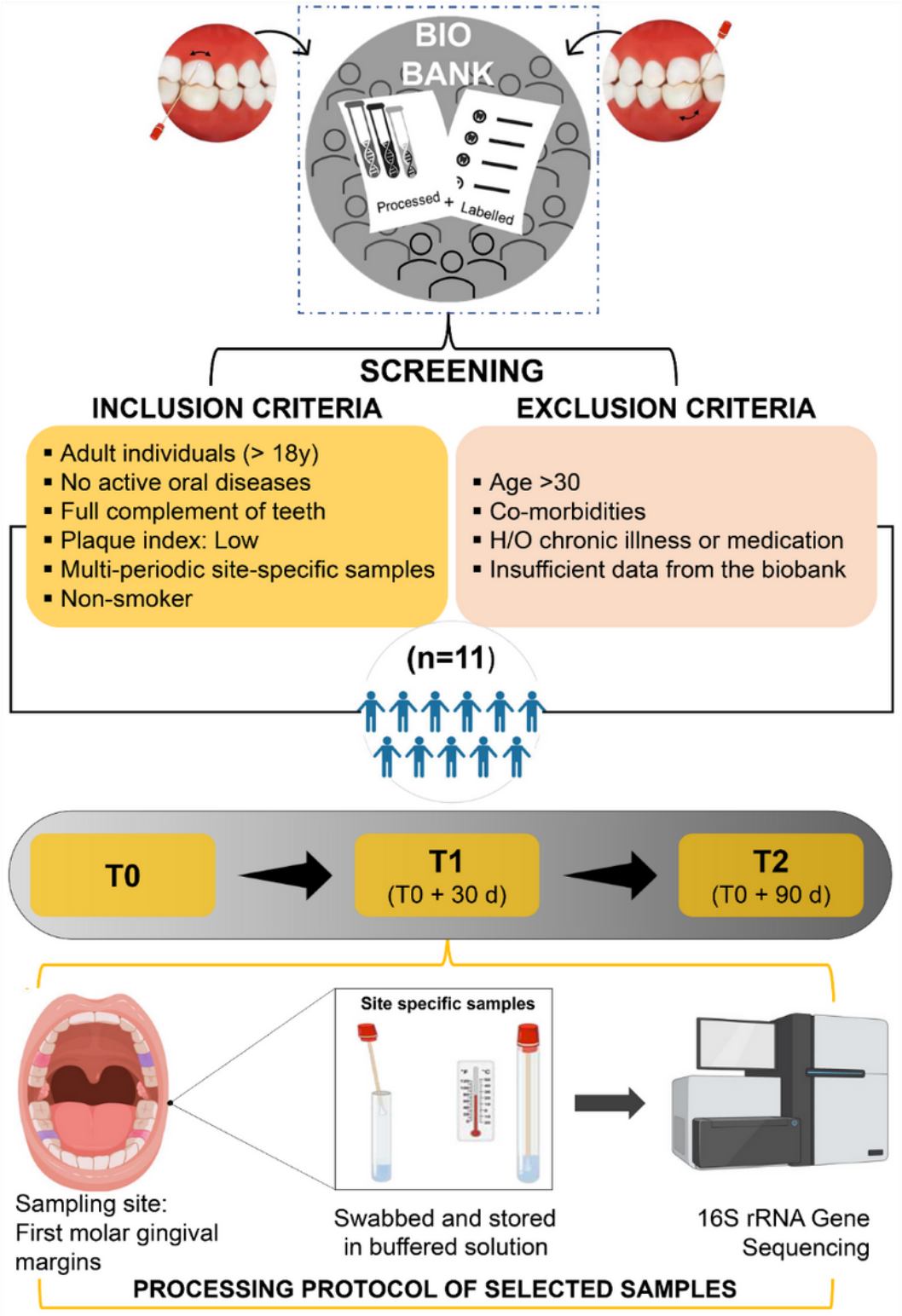

Figure 1

Study design and flow. Samples sourced from the biobank resources meeting the specific selection criteria have been sequenced and analyzed.

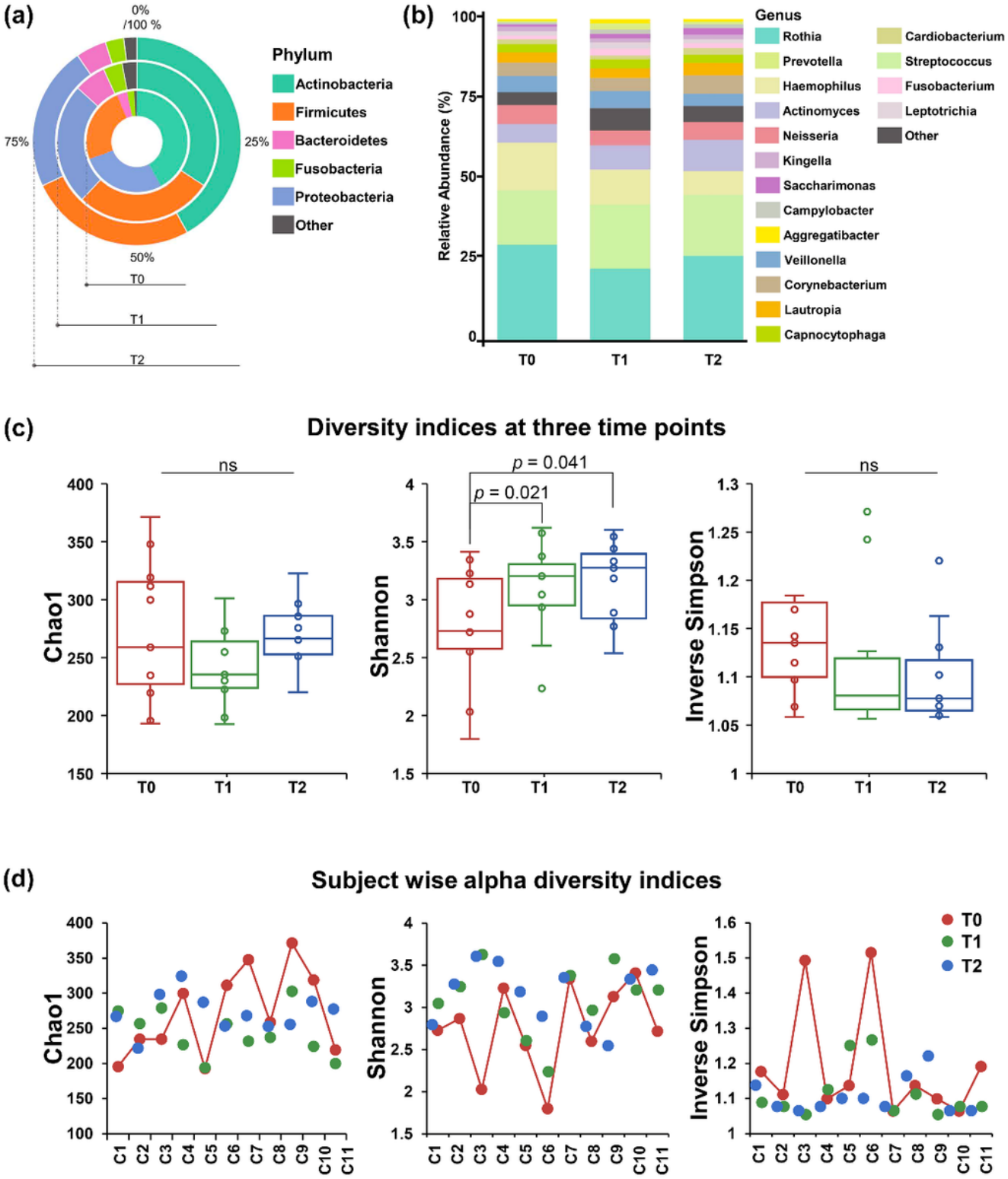

Figure 2

The relative abundance and diversity indices of different Operational Taxonomic Units

.The taxonomic profiling for all participants is presented as a factor of time, namely T0, T1, and T2, at (**a**) Phylum and (**b**) Genus level data. (**c-d**) Alpha diversity metrics diagrams. **(c)** Box plot of Chao1, Shannon, and Inverse Simpson α-diversity of all samples in three different time groups. The boxes span the first to

third quartiles; the horizontal lines inside the boxes represent the median, and the dots represent all samples at each time point. Pairwise comparison is performed using the non-parametric Wilcoxon test, and the *p*-values are provided. **(d)** Profiles of the three diversity measures for individual participants. The dot and line plot traces the values at the initial time point T0. T1 and T2 represent the values observed at 30 d and 90 d intervals from T0.

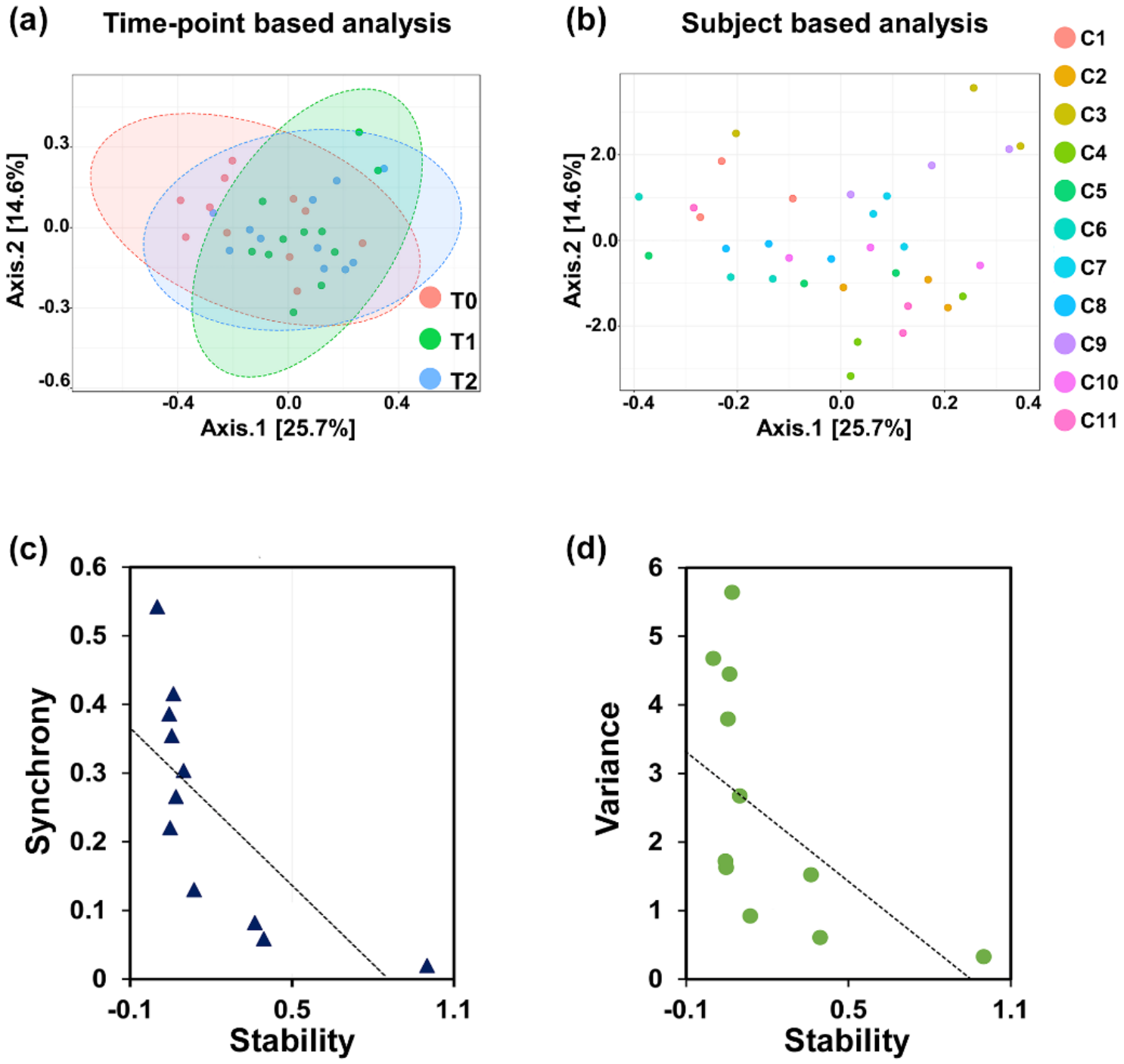

Figure 3

Beta diversity of microbial communities. Using PCoA with the Bray-Curtis dissimilarity distance, we have performed multidimensional ordination for (**a**) time point-based and (**b**) participant-based analysis by

computing the average distance of individual groups to the group centroid. (**c-d**) Community stability metrics show subject-wise patterns of (**c**) synchrony and (**d**) variance to the stability.

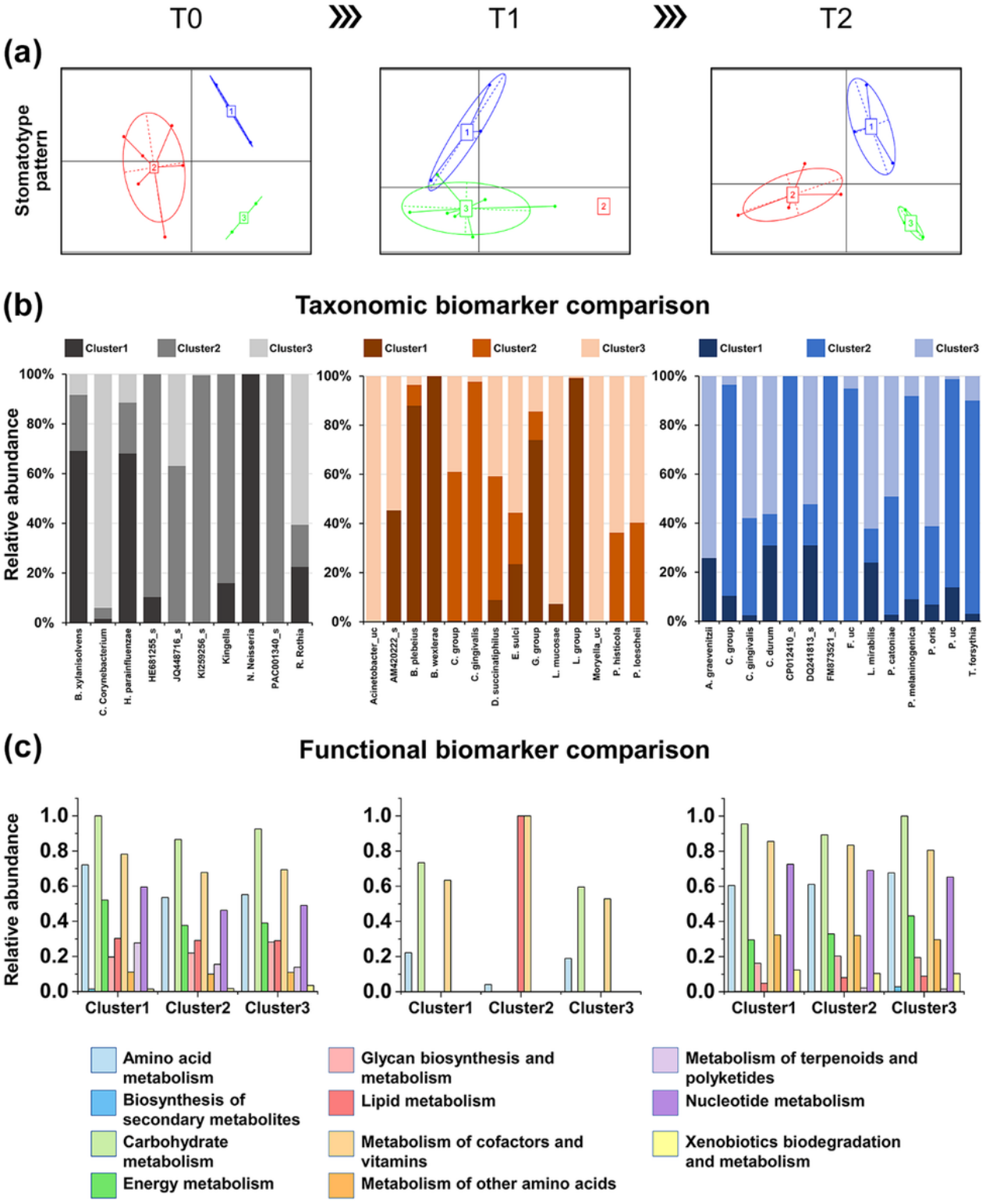

Figure 4

Stomatotypes and significant biomarker differences. (**a**) Stomatotypes are computed using the Jensen-Shannon Divergence (JSD) metric at three different time points (T0, T1, and T2). Significantly different

(p<0.05) (**b**) taxonomic and (**c**) functional metabolic biomarkers have been observed.

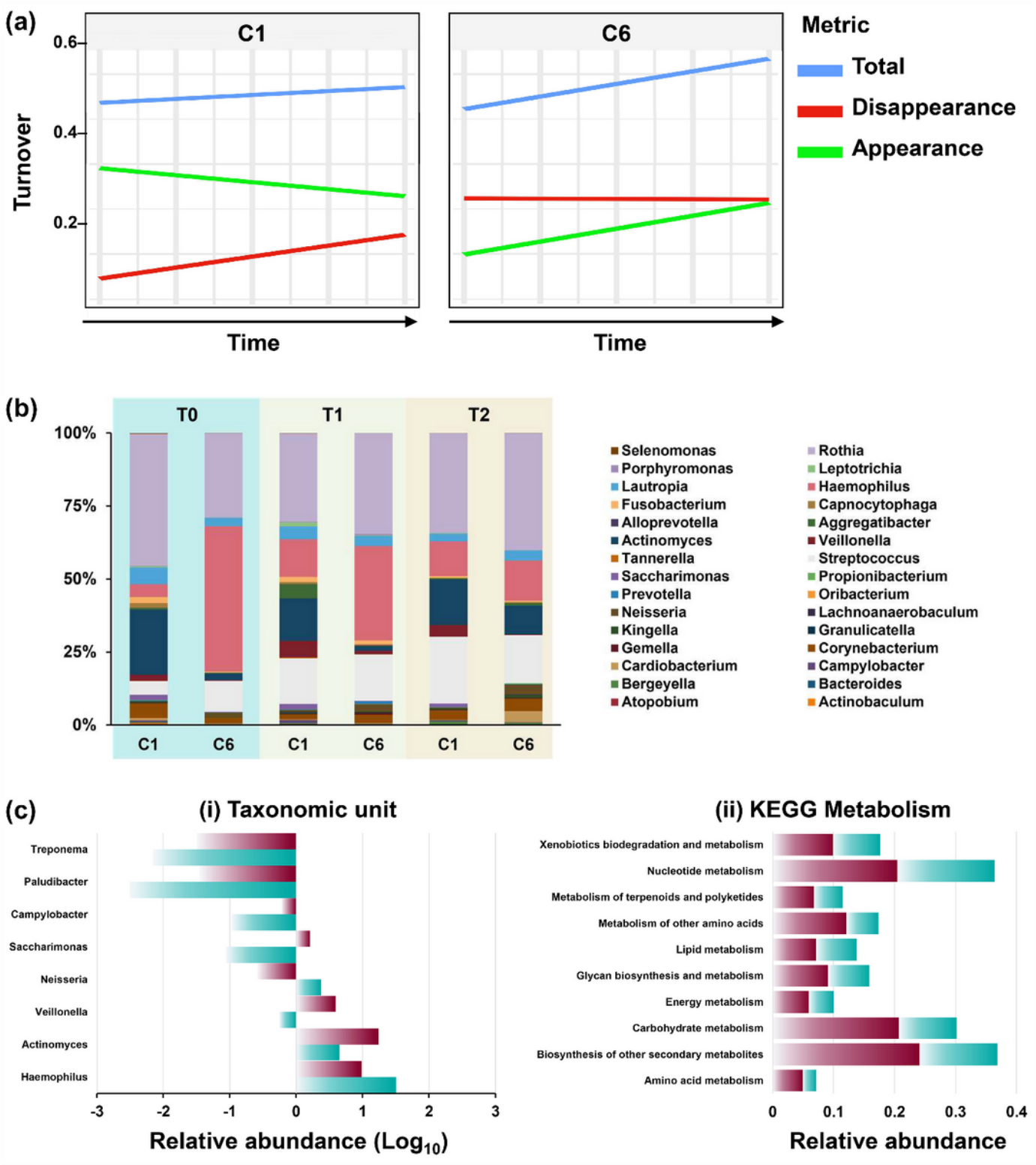

Figure 5

Analytical flow (Figure S1) in the context of two participants (C1 and C6) with comparable features (**a**) The turnover metric displaying contrasting patterns of appearance and disappearance with similar total turnover. (**b**) A comparison of the relative taxonomy abundance at T0, T1, and T2 at three-time points. (**c**)

Significantly different (C-**i**) taxonomic unit and (C-**ii**) KEGG metabolic pathways. KEGG; Kyoto Encyclopedia of Genes and Genomes

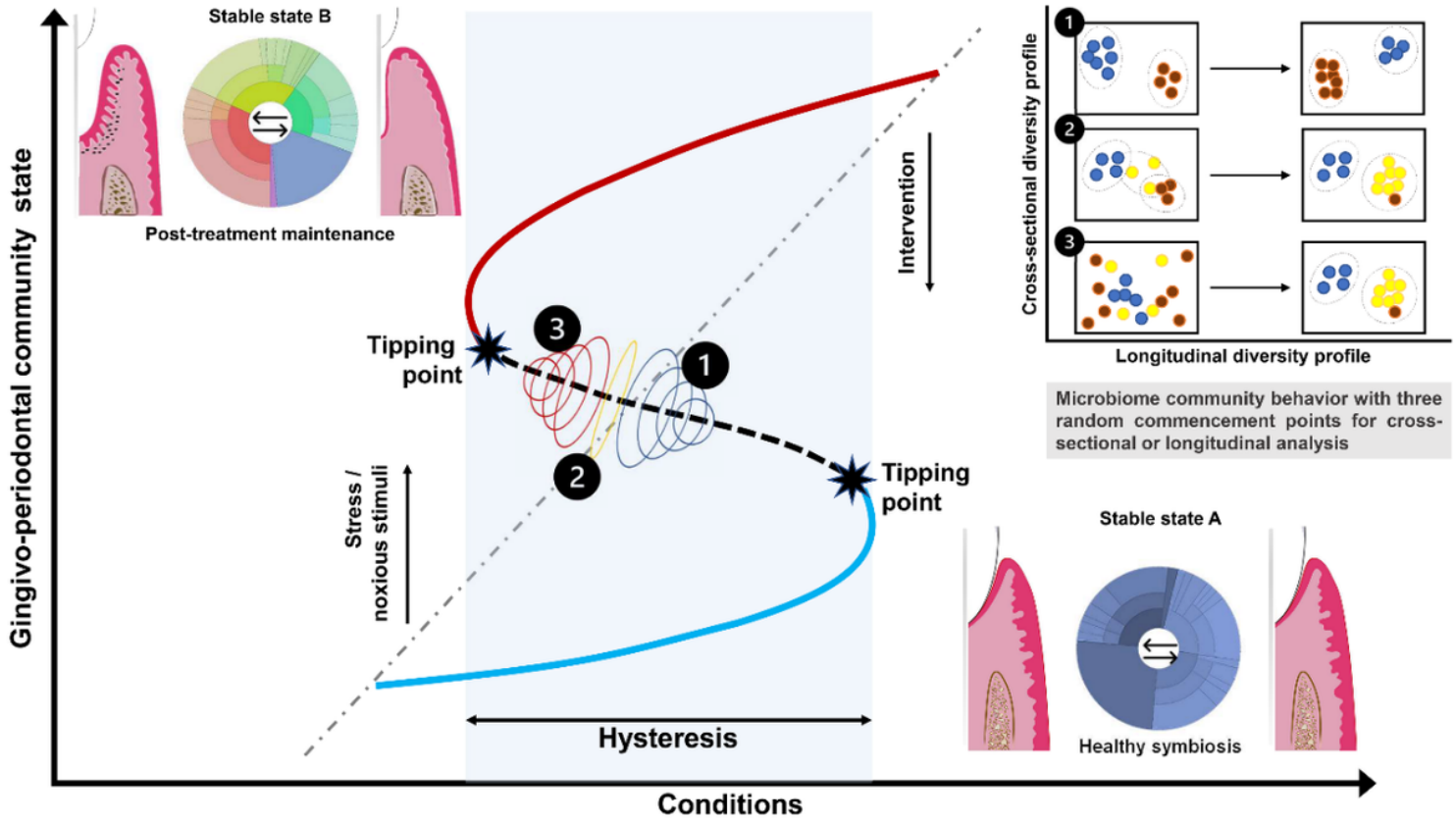

Figure 6

Multi-stable and hysteresis perspective. Changes in conditions can affect the microbial community, where a multi-stable community adopts different stable states within similar environmental conditions. The change in states is precipitated following trigger events(environmental /therapeutic) exerting abrupt change. The system varies across a tipping point (black stars), and changes are not entirely reversible (hysteresis).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- OTUabundanceandmetadata.xlsx
- SupplementarymaterialCOI.docx