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Intraindividual Variation and Personal Specificity of Salivary Microbiota

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Abstract

Salivary microbiota is a typical habitat of the human microbiome. This study intended to use salivary microbiota as a model aiming to systematically address the influence of collection methods and temporal dynamics on the human microbiota compared to personal specificity. We carried out a supervised short-term longitudinal study to evaluate the influence of the change of collection methods and sampling time point on salivary microbiota in 10 systemically and orally healthy individuals with certain confounding factors (sex, oral and general health state, medication history, physical exercise, diet, and oral hygiene behavior) controlled before and during the sampling period. The microbial profiles were analyzed by *16S rDNA* V3 to V4 hypervariable region amplicon sequencing. The taxonomic structure represented by the dominant species and the weighted UniFrac distance algorithm were used to demonstrate the individual specificity and the intraindividual variation introduced by the change of collection method and sampling time point. The findings suggested individual specificity existed in salivary microbiota from individuals with similar oral and general health status. The intraindividual variation brought by the change of collection method or sampling time point might introduce remarkable perturbation with the personal specificity. Insights into the intraindividual variation and personal specificity of salivary microbiota will enhance our understanding in salivary microbiota-related research. We recommend keeping collection conditions consistent within a study to avoid interference brought by the sampling. The strategy of repeated sampling at multiple time points as representative samples, as well as thorough interpretation of the complex relationships and causality between microbiome composition and disease without the interference of temporal dynamics, is optimal for research exploring the relationship between the salivary microbiome and disease.

Keywords: saliva, microbiome, individual specificity, within-individual variation, collection method, temporal dynamics

Introduction

Saliva is attractive to researchers on account of its properties, such as noninvasive, painless, and convenient collection procedures; easily disposable for population-based screening; and low biological risk. In recent decades, large numbers of valuable salivary biomarkers have been unveiled to identify oral and systemic diseases early, evaluate disease prognosis and risk, and monitor the response to treatment (Pfaffe et al. 2011; Zhang et al. 2016; Dawes and Wong 2019). In this process, oral microbiomes are getting more and more important in the oral cavity and the whole body since promising molecular technologies have greatly expanded our understanding of their interactions with variable diseases (Gao et al. 2018). Saliva samples have been routinely taken as a representative average of the entire ecosystem of the oral cavity (Aas et al. 2005).

For saliva collection on a patient basis, mixed whole saliva is the optimal option that is practically feasible in clinical circumstances (Navazesh 1993; Dodds et al. 2005). Saliva can be collected under unstimulated or stimulated conditions. Although several studies have performed comparative evaluations on microbiota obtained from different types of saliva (unstimulated vs. stimulated), contradictions still exist (Belstrom et al. 2016; Lim et al. 2017; Gomar-Vercher et al. 2018). In addition to the collection condition, the question of which segment to collect is also debatable. Considering the cleanliness and stability of the saliva samples, it is preferred to preserve midstream saliva for subsequent analysis by discarding or swallowing the forepart segment (Naito et al. 2003;

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Tarkkila et al. 2012; Chen et al. 2018). So far, the issue of segment selection of saliva sampling is still a "dark matter" overlooked by previous studies. These kinds of conflicts and underexplored issues lead to a lack of consensus and standardization.

Humans interact with the environment and their own physiologic state all the time. Consequently, the human microbiome has proven to be highly dynamic (Gilbert et al. 2018). Characterizing the temporal dynamics of the human microbiome is crucial to improve the sampling strategy. However, the degree of perturbation introduced by the change of the sampling time point across a short-term time scale to personal specificity remains undiscovered. To sum up the research gaps mentioned above, no previous study has systematically addressed the intraindividual variation introduced by the change of collection method and sampling time point compared to personal specificity. The salivary microbiome provides an easy and convenient window into this problem. Salivary components could be affected by the environment (Brito et al. 2016), general and oral health status (Graves et al. 2019), diet (Brandao et al. 2014), physical exercise (Gillum et al. 2014), sex (Gillum et al. 2014), medication (Aps and Martens 2005), oral hygiene behavior (Addy et al. 1997), and so on. It is essential to control the influence of these confounding factors in the exploration of sampling methods or time points for salivary analytes.

Therefore, we used salivary microbiota as a model aiming to systematically address the perturbation of sampling methods and the temporal dynamics on the human microbiota compared to the individual specificity. We carried out a supervised short-term longitudinal study on salivary microbiota in 10 systemically and orally healthy individuals with certain confounding factors balanced or controlled (sex, oral and general health state, medication history, physical exercise, diet, oral hygiene behavior) before and during the sampling period. Saliva samples were collected on 3 consecutive sampling days using different collection methods with the order changed in the crossover design. Microbial profiles were analyzed by *16S rDNA* V3 to V4 hypervariable region amplicon sequencing using the Illumina MiSeq PE300 platform.

Materials and Methods

Ethics Approval and Informed Consent

All procedures followed were in accordance with the Peking University School and Hospital of Stomatology Ethics Committee (PKUSSIRB-201944061) and with the Declaration of Helsinki. This study conformed to the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines, and all the donors signed informed consent.

Study Population and Design

Ten individuals (5 females and 5 males) were enrolled after a review of medical history and oral clinical examination. The specific inclusion criteria are supplied in the Appendix. The flowchart of this study is shown in Appendix Figure 1.

Homogenization and Control of Confounding Factors

Oral hygiene behavior, diet, and physical exercise were homogenized or controlled before and during the sampling period. The specific homogenization and control of confounding factors are supplied in the Appendix.

Sampling and Processing of Saliva

The sampling process is also shown in Appendix Figure 1. The whole saliva samples (n = 120) were categorized as the forepart segment of unstimulated whole saliva (UWS.F), the midstream segment of unstimulated whole saliva (UWS.M), the forepart segment of mechanically stimulated whole saliva (MSWS.F), and the midstream segment of mechanically stimulated whole saliva (MSWS.M). The detailed protocols for sampling and processing of saliva are supplied in the Appendix.

Sequencing Analysis

Microbial DNA extraction was performed and DNA amplicons of the V3 to V4 hypervariable region of the bacterial *16S rDNA* were generated. The sequencing was performed using the Illumina MiSeq PE300 platform. The detailed DNA extraction, sequencing, and analysis methods are supplied in the Appendix.

Statistical Analysis

Data analysis was performed using SPSS 23.0 software (SPSS, Inc.). The comparison of intraindividual variation and temporal dynamics on salivary flow rate, microbial diversity index, and taxonomic relative abundance was evaluated using repeated-measures analysis of variance (ANOVA). Details of the repeated-measures ANOVA are supplied in the Appendix. $P < 0.05$ was regarded as statistical significance (2-sided).

Data Availability

The raw sequencing data of this study are available in the NCBI Sequence Read Archive with accession number SRP193600.

Results

Comparison of Salivary Flow Rates

A total of 10 donors were enrolled in this study, and the sociodemographic background and oral health status are shown in Appendix Table 1. The comparative analysis of salivary

flow rates is shown in Appendix Figure 2. There were significant differences as expected in the flow rates between unstimulated and stimulated saliva in both the forepart and midstream segments ($P < 0.001$, $P < 0.001$, respectively) (Appendix Fig. 2A, $n = 30$ vs. 30, similarly hereinafter). No significant difference was found between the forepart and midstream segments under the unstimulated condition (Appendix Fig. 2A, $P = 0.345$), while significant difference was found under the stimulated condition (Appendix Fig. 2A, $P = 0.002$). The intraindividual temporal dynamics of salivary flow rate across the 3 sampling days within each collection method exhibited a steady trend except UWS.M (Appendix Fig. 2B, $P = 0.172$, $P = 0.010$, $P = 0.167$, $P = 0.431$, respectively) ($n = 10$ vs. 10 vs. 10, similarly hereinafter).

Overview of the Sequencing Analysis

A total of 4,312,638 sequences were generated after quality filtering, with an average of 35,939 (range, 27,048 to 56,063) sequences per sample. The species richness of the salivary microbiota of each sample was estimated by rarefaction analysis (Appendix Fig. 3). We also constructed specaccum curves for each sampling day and collection method using OTUs detected to assess the current state of sampling size (Appendix Fig. 4). The number of shared and unique operational taxonomic units (OTUs) for each sampling day and each collection method is shown in Appendix Figure 5.

The comparative evaluation of the Shannon index (Appendix Fig. 6) indicated unstimulated and stimulated saliva samples have a significantly different microbial diversity index both in forepart and midstream segments (Appendix Fig. 6A, $P = 0.002$, $P = 0.005$, respectively), while forepart and midstream saliva have an equal-level microbial diversity index regardless of unstimulated and stimulated conditions (Appendix Fig. 6A, $P = 0.508$, $P = 0.461$, respectively). As for the intraindividual temporal dynamics of the Shannon index, the UWS showed a steady trend and the MSWS showed a highly dynamic trend ($P = 0.696$, $P = 0.463$, $P = 0.005$, $P = 0.001$, respectively). By analyzing the salivary microbiota of all samples, a total of 11 phyla, 20 classes, 34 orders, 71 families, 149 genera, and 371 species were detected.

Interindividual Variation of Salivary Microbiota

The interindividual variation of salivary microbiota was accessed by the microbial community structure and the weighted UniFrac distance algorithm. The microbial community structure of the salivary microbiota from phylum to species levels is shown in Appendix Figures 7 to 12. The feature of the salivary microbiota structure charted that individual specificity existed at all taxonomic levels.

A schema diagram showing the interindividual distance according to the present study was established (Fig. 1A). The principal coordinate analysis (PCoA) based on weighted UniFrac distance in Figure 1B demonstrated the interindividual variation of salivary microbiota in each collection method and on each sampling day. Then the corresponding interindividual weighted UniFrac distances were extracted (Fig. 1C), suggesting individual specificity existed in salivary microbiota from individuals with similar oral and general health status.

Intraindividual Variation of Salivary Microbiota Introduced by the Change of Collection Method

The intraindividual variation of salivary microbiota introduced by the change of collection method was also accessed by the microbial community structure and distance algorithm. The histogram in Figure 2A demonstrated the salivary microbiota community structure via the relative abundance of the dominant species (mean relative abundance >1%) according to sampling order on each sampling day ($n = 40$). The microbiota community structure was similar between different segments, while obvious differences could be observed between different conditions. Similar phenomena were observed in the analysis based on the rank of dominant species (Appendix Fig. 13). The unweighted pair group method with arithmetic mean (UPGMA) clustering analysis based on the relative abundance of the dominant species according to collection methods on each sampling day was also conducted (Fig. 2B). There were cluster trees according to the collection condition (unstimulated or stimulated) from different donors on all the sampling days, suggesting the unstimulated and stimulated saliva have a significantly different microbiota community structure. The forepart and midstream segments from the same individual under the same collection condition were closely clustered together, suggesting the forepart and midstream saliva have a comparable microbiota community structure.

A schema diagram showing the intraindividual distance introduced by the change of collection method according to the present study was established (Fig. 3A). The PCoA based on weighted UniFrac distance was conducted on each sampling day (Fig. 3B), indicating a separated trend between different conditions and an unseparated trend between different segments. The corresponding intraindividual weighted UniFrac distances of the 4 determined comparisons on each sampling day were extracted (Fig. 3C). The intraindividual weighted UniFrac distances of the 4 determined comparisons and the weighted UniFrac distances between a single donor and 9 other donors using 4 different collection methods were extracted according to each donor and are displayed in Appendix Figure 14. For most of the donors, the intraindividual variation brought by the change of collection condition could challenge the individual specificity, while the individual specificity could tolerate the intraindividual variation brought by the change of collection segment. Similar findings were verified by UPGMA hierarchical clustering analysis based on weighted UniFrac distance (Fig. 3D). The findings in the weighted UniFrac distance algorithm were also verified via the Bray-Curtis distance algorithm (Appendix Fig. 15).

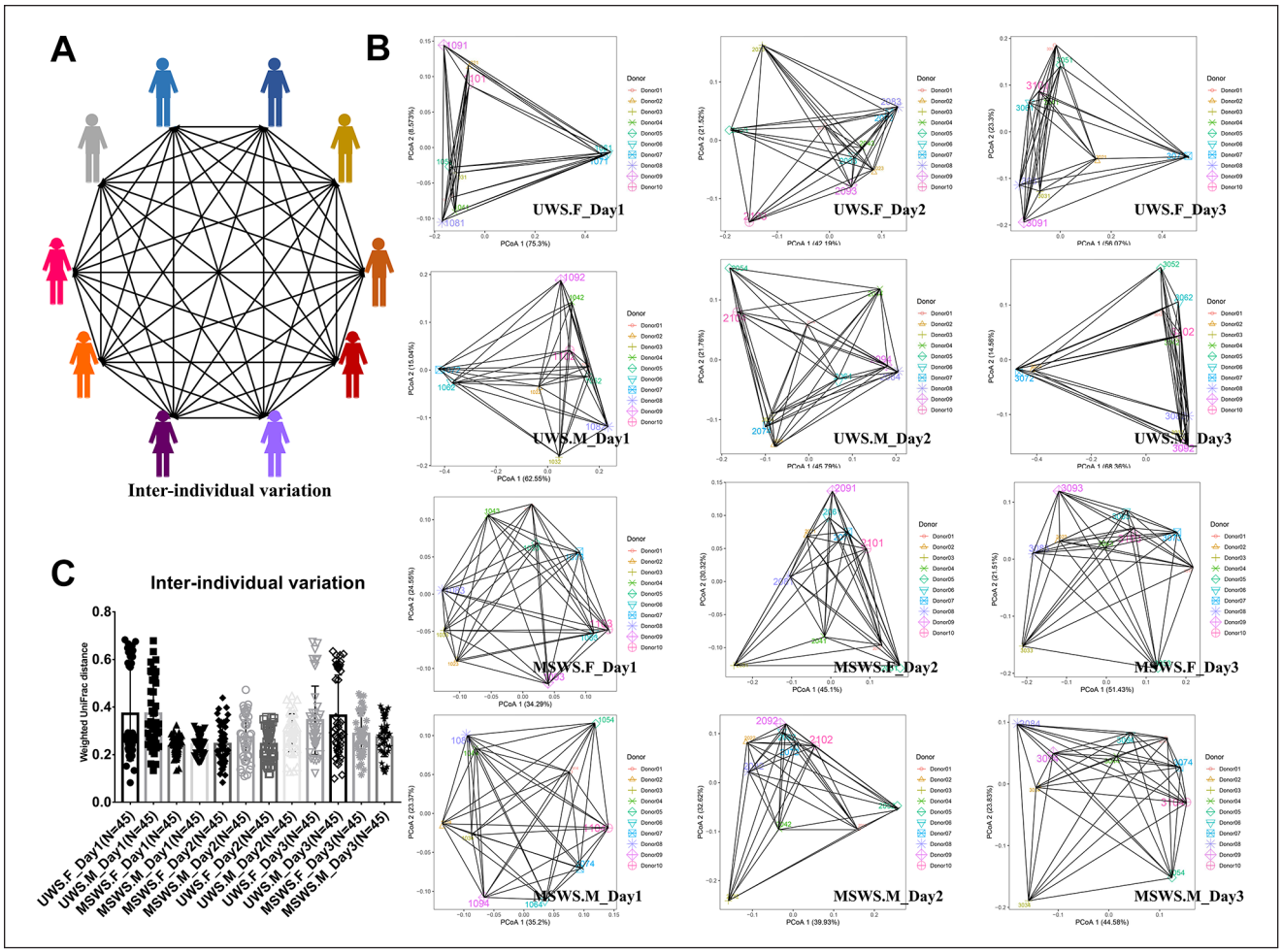

Figure 1. The individual specificity demonstrated via the distance algorithm. (**A**) The schema diagram showing the interindividual variation. (**B**) The principal coordinate analysis (PCoA) based on weighted UniFrac distance showing the interindividual variation of salivary microbiota for each collection method and each sampling day. (**C**) The corresponding interindividual weighted UniFrac distances for each collection method and each sampling day.

Intraindividual Temporal Dynamics of Salivary Microbiota Introduced by the Change of Sampling Time Point across a Short-Term Time Scale

The intraindividual temporal dynamics of salivary microbiota introduced by the change of sampling time point across a short-term time scale was also accessed by the microbial community structure and distance algorithm. The histogram in Figure 4A demonstrates the temporal dynamics of the salivary microbiota community structure via the relative abundance of the dominant species ($n = 30$). Statistically different species were found in all the collection methods, suggesting the salivary microbiota community structure was highly variable regardless of collection methods. The highly dynamic variations regardless of collection methods were also found in the analysis based on the rank of dominant species (Appendix Fig. 16). The UPGMA clustering analysis (Fig. 4B) also revealed that more than half of individual specificity was interrupted by the intraindividual temporal dynamics in all the collection methods.

A schema diagram showing the intraindividual distance between any 2 sampling days within each collection method according to the present study was established (Fig. 5A). The PCoA based on weighted UniFrac distance was conducted within each collection method (Fig. 5B), and the intraindividual dynamics of all the donors were labeled. The corresponding intraindividual weighted UniFrac distances between any 2 sampling days within each collection method were extracted (Fig. 5C). The intraindividual weighted UniFrac distances between any 2 sampling days within each collection method and the weighted UniFrac distance between a single donor and 9 other donors using 4 different collection methods were extracted according to each donor and are displayed in Appendix Figure 17. For most of the donors, the intraindividual dynamics could challenge the individual specificity. The inference was verified by the UPGMA hierarchical clustering analysis based on weighted UniFrac distance (Fig. 5D). These findings in weighted UniFrac distance algorithm were also verified via the Bray-Curtis distance algorithm (Appendix Fig. 18).

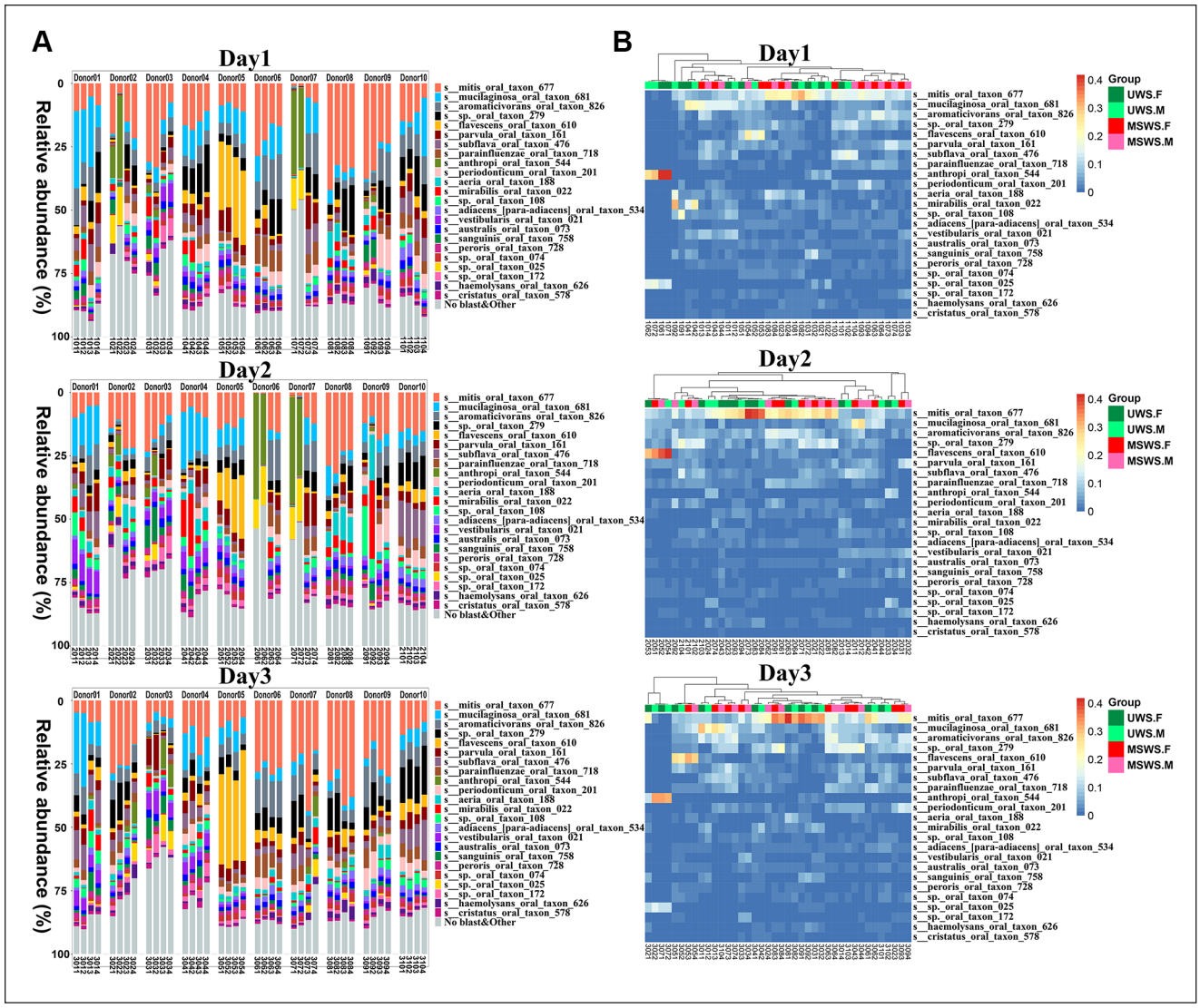

Figure 2. The intraindividual variation introduced by the change of collection method demonstrated via the dominant species (mean relative abundance >1%). (**A**) The histogram demonstrated the community structure via the relative abundance of the dominant species according to sampling order on each sampling day (n = 40). (**B**) The unweighted pair group method with arithmetic mean (UPGMA) clustering analysis based on the relative abundance of the dominant species according to collection methods on each sampling day.

Discussion

The oral cavity harbors a second most diverse ecosystem that plays a critical role in oral and general health-related studies. Salivary microbiota provides an advantaged model for studying the human microbiome. The purpose of the present longitudinal study was to elucidate the perturbation of the collection method and temporal dynamics on salivary microbiota to help determine its effect on host health and disease. Moreover, this study is a supervised study with the confounding factors balanced or controlled before and during the sampling period, and we believe this can provide more reliable information.

In the present sequencing analysis for the salivary microbiota, the rarefaction curves and the specaccum curves confirmed that the sequencing depth and sample size were at a reasonable level (Appendix Figs. 3 and 4). The alpha diversity, as measured using the Shannon index in this study, was significantly higher than previously reported in healthy adults (the mean Shannon index is around 6.25 in this study, while the mean Shannon index is around 5.0 in previous studies) (Piccolo et al. 2015; Yang et al. 2018; Wang et al. 2019). This is most likely owing to the QIAamp DNA Microbiome kit used in this study, which could optimize mechanical and chemical cell lysis linked to more efficient DNA extraction and optimal DNA yield (Guo and Zhang 2013; Walker et al. 2015; Stinson et al. 2018). We believe the microbiota community structure generated from these samples is more accurate and unabridged. Considerable contribution owing to the individual specificity

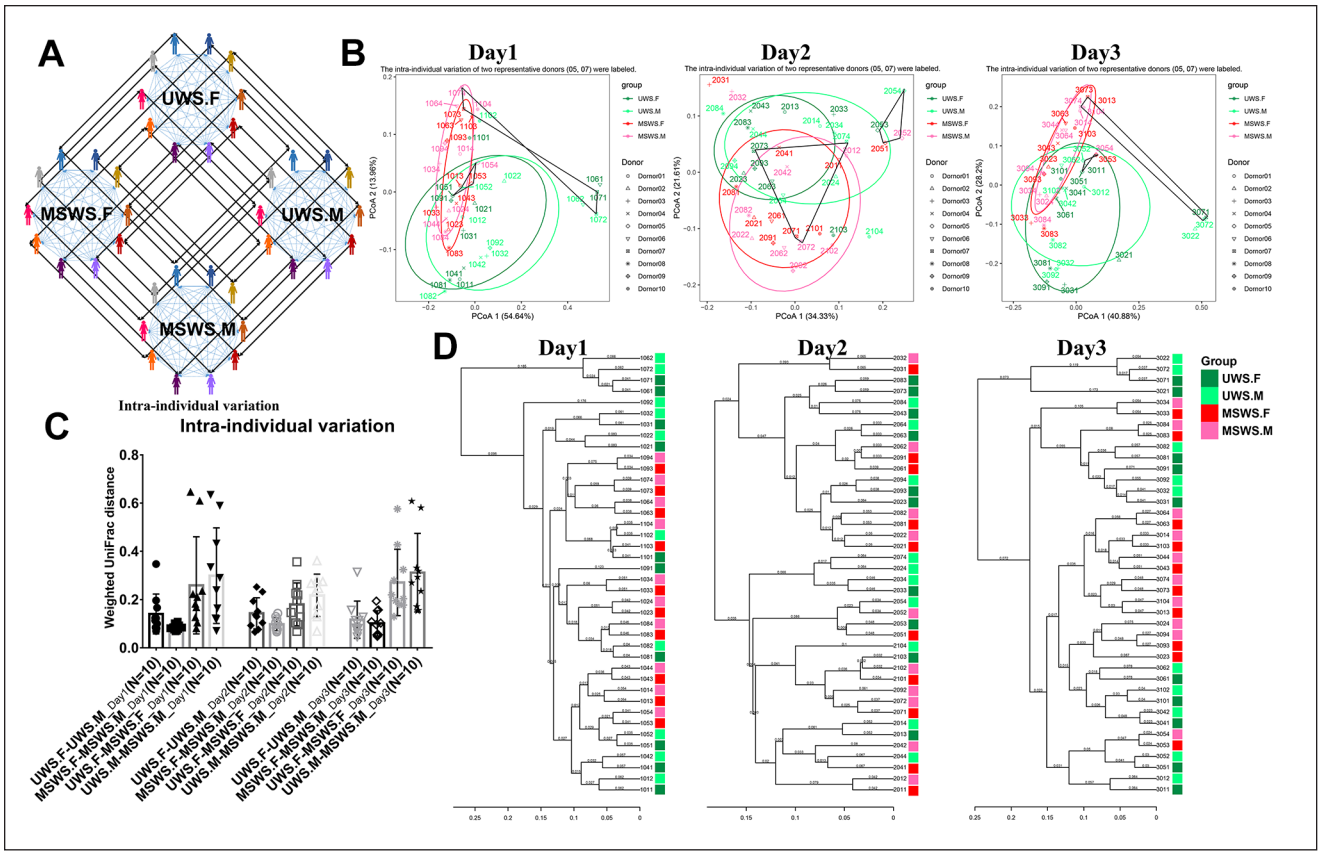

Figure 3. The intraindividual variation introduced by the change of collection method demonstrated via the distance algorithm. (**A**) The schema diagram showing the intraindividual variation of salivary microbiota between determined collection methods. (**B**) The principal coordinate analysis based on weighted UniFrac distance showing the intraindividual variation on each sampling day, with 2 representative donors (donor 5, donor 7) labeled. (**C**) The corresponding intraindividual weighted UniFrac distances of the 4 determined comparisons on each sampling day. (**D**) The unweighted pair group method with arithmetic mean (UPGMA) hierarchical clustering analysis based on weighted UniFrac distance according to collection methods on each sampling day.

to the salivary microbiota structure was observed in our study, which is consistent with previous reports (Lazarevic et al. 2010; Dzunkova et al. 2018).

The unstimulated saliva is undoubtedly the easiest way, while for practical reasons, stimulated saliva is often preferred on account of its fast sampling procedure with better standardization of saliva flow, advantages for certain groups of people, and lower viscosity (Navazesh 1993; Humphrey and Williamson 2001; Dodds et al. 2005; Dawes 2008). With a focus on the microbial research field, a preliminary experiment based on 2 individuals revealed higher bacterial diversity in unstimulated saliva samples compared with stimulated saliva samples (Simon-Soro et al. 2013). This seemed to be consistent with the idea that stimulated saliva dilutes the salivary analytes. However, the present study indicated that MSWS samples exhibited higher bacterial diversity than UWS samples (Appendix Fig. 6). This bias might be due to limited reads (2,700 per sample) being available, and the sequencing depth was insufficient to reflect the diversity information (Simon-Soro et al. 2013). Two previous studies also verified stimulated saliva was superior to unstimulated saliva for specific bacterial identification through culture-dependent technology (Asikainen et al. 1991; Dasanayake et al. 1995). From the above, we can infer that chewing in the oral cavity during stimulated saliva collection does increase the microbial diversity in saliva samples, not dilute it. However, the potential effects of sampling methods on salivary proteome or other salivaomics remain underexplored since different components get into saliva in different ways (Helmerhorst and Oppenheim 2007; Wong 2012).

Based on the literature review of the effects of unstimulated and stimulated saliva on the overall microbiota profiles, contradictory conclusions were observed. Belstrøm et al. (2016) verified that stimulated saliva is an adequate surrogate of unstimulated saliva for microbiome-related studies. Oppositely, Gomar-Vercher et al. (2018) demonstrated that stimulated and unstimulated saliva samples have significantly different bacterial profiles, which is also supported by the findings in our study. As the throughput of detection methods is quite limited (the mean Shannon index is around 2.75) in the former research (Belstrom et al. 2016), the different characteristics between saliva samples collected with and without stimulation are more

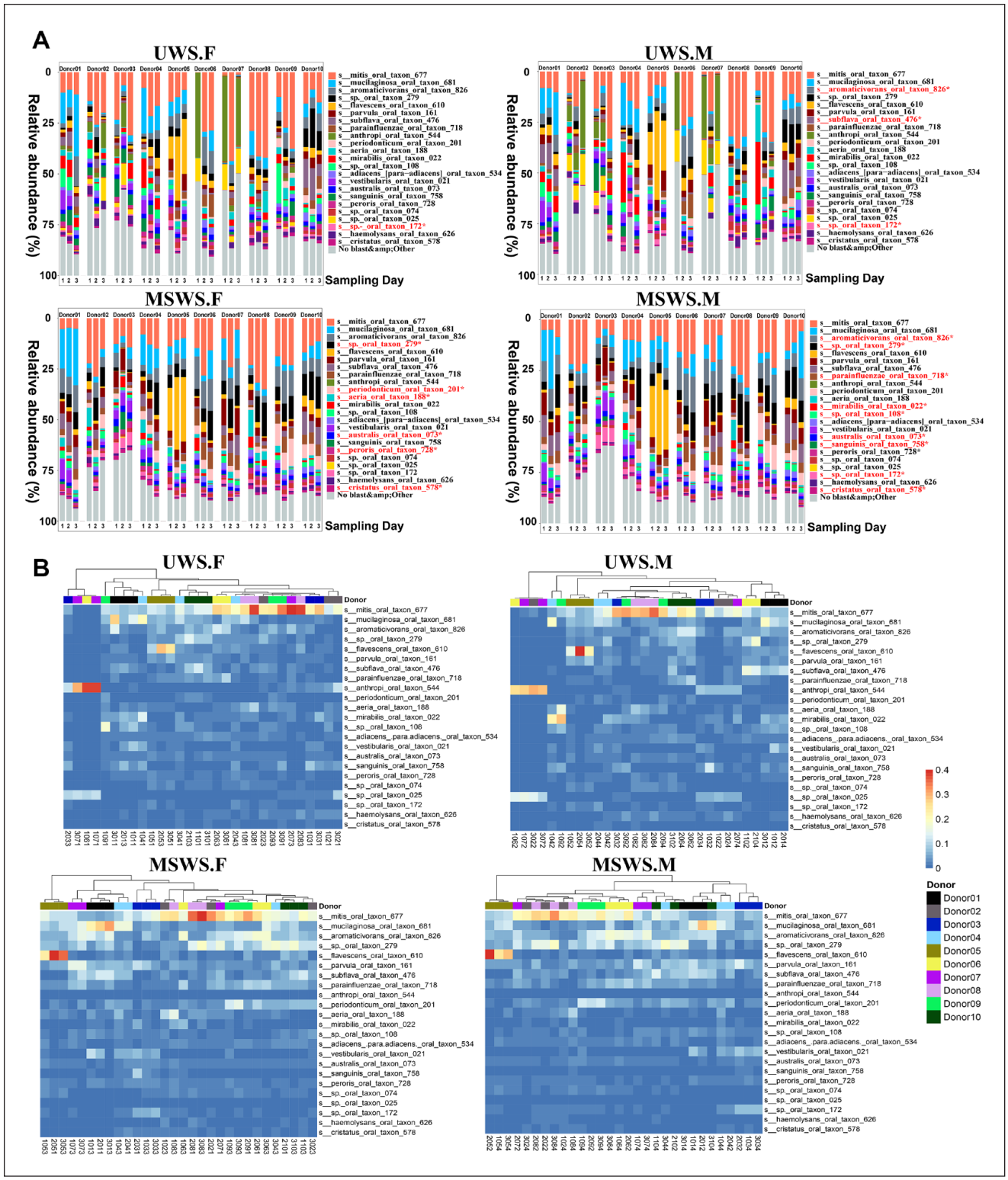

Figure 4. The intraindividual temporal dynamics introduced by the change of sampling time point across a short-term time scale demonstrated via the dominant species (mean relative abundance >1%). (**A**) The histogram demonstrates the community structure via the relative abundance of the dominant species according to sampling order within each collection method ($N = 30$). The temporal dynamics of dominant species were compared and the statistically different species are noted with red font and asterisks. The P values were obtained by the repeated-measures analysis of variance as detailed above. *$P < 0.05$. (**B**) The unweighted pair group method with arithmetic mean (UPGMA) clustering analysis based on the relative abundance of the dominant species according to donor ID within each collection method.

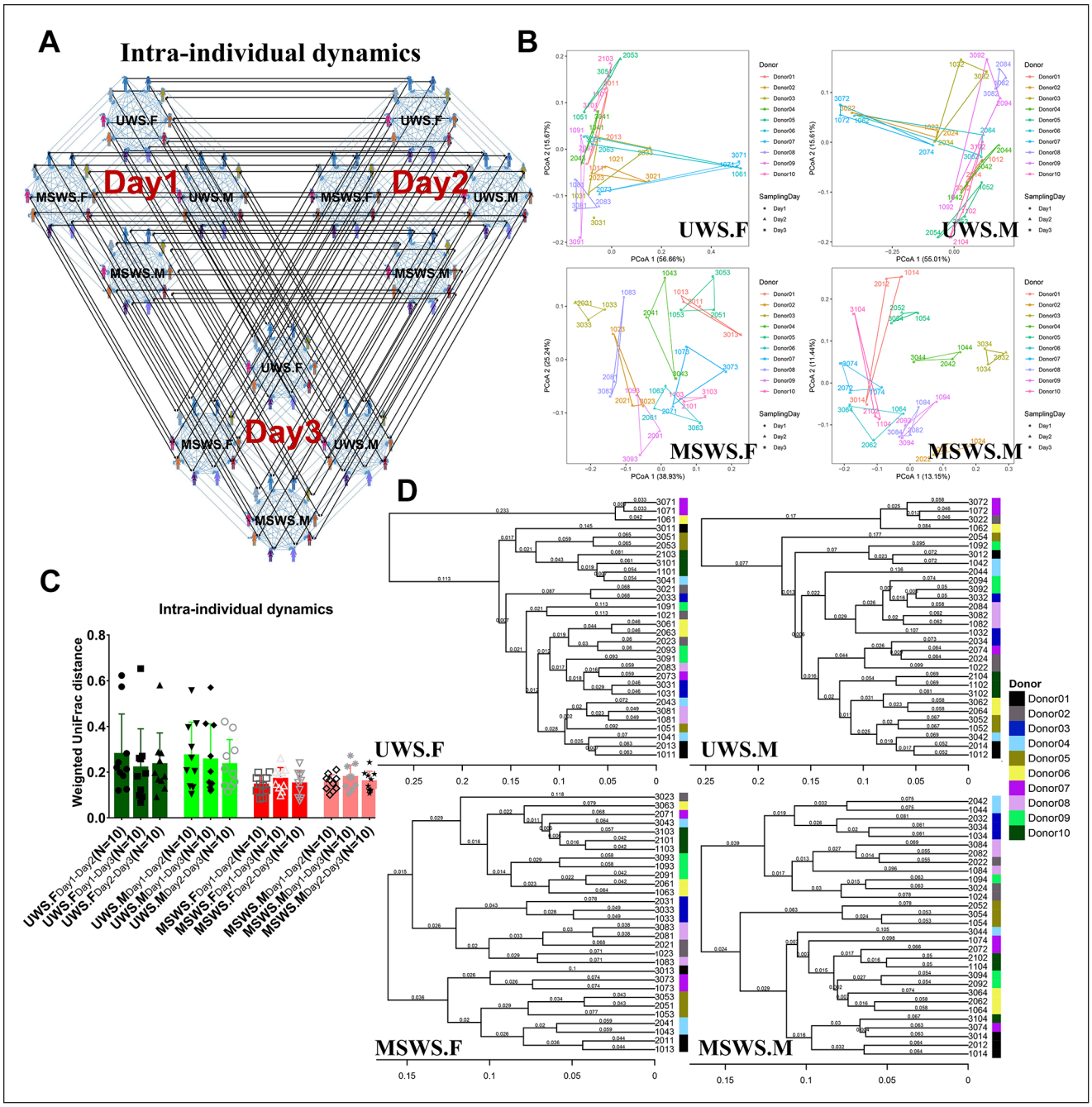

Figure 5. The intraindividual temporal dynamics introduced by the change of sampling time point across a short-term time scale demonstrated via the distance algorithm. (**A**) The schema diagram showing the intraindividual temporal dynamics of salivary microbiota between any 2 sampling days using the same collection method. (**B**) The principal coordinate analysis based on weighted UniFrac distance showing the intraindividual temporal dynamics within each collection method. (**C**) The corresponding intraindividual weighted UniFrac distances between any 2 sampling days within each collection method. (**D**) The unweighted pair group method with arithmetic mean (UPGMA) hierarchical clustering analysis based on weighted UniFrac distance according to donor ID within each collection method.

believable. In short, the change of unstimulated and stimulated condition could bring intraindividual variation beyond the tolerance of the individual specificity, while the change of midstream and stimulated segment was relatively acceptable comparing to the individual specificity (Figs. 2 and 3).

Our study has reported short-term variability of the high-level taxonomic composition of salivary microbiota in all collection methods. The results pointed out that the change of sampling time point across a short-term time scale might introduce conspicuous interference with the individual specificity

(Figs. 4 and 5). This conspicuous interference means taking a "stable" and "representative" sample is quite challenging. One strategy to deal with this challenge is repeated sampling at multiple time points and then interpreting the complex relationships and causality between microbiome composition and disease without the interference of temporal dynamics (Sugihara et al. 2012). This complex project might have to rely on Bayesian community-wide microbial SourceTracker (Knights et al. 2011) and an artificial neural network approach (Larsen et al. 2012). One recent study suggested that the rate of change of the microbiome may itself be a clinical feature (Gajer et al. 2012). We also observed differences in the temporal variability of salivary microbiota in different individuals (donor 5 showed strong stability, while donor 7 showed high variability), suggesting that characterizing temporal variability may be an important part of characterizing an individual's microbiome.

Limitations within this study and corresponding future research directions should be acknowledged. First, although this is the first supervised study with a longitudinal design aiming to elucidate the perturbation of collection method and temporal dynamics on salivary microbiota, there are only 10 systematically and orally healthy participants involved, making the extrapolation of the findings limited. Nevertheless, the evidence could be strengthened by future studies by increasing the complexity of sample constitution and expanded sample size. Moreover, it is worth investigating the variability of single collections of saliva without standardization of confounding factors. Second, the sequencing analysis used in this study was based on the *16S rDNA* double V3 to V4 region amplicon sequencing library and Illumina MiSeq PE300 platform, which had difficulty in providing absolute taxa abundance. It is expected that further studies could use high-throughput absolute abundance quantification techniques and provide absolute taxa abundance for the salivary microbiome at the species level (Tourlousse et al. 2017; Bender et al. 2018).

In summary, salivary microbiota showed individual specificity from individuals with similar oral and general health status. The intraindividual variation brought by the change of collection condition or sampling time point might introduce conspicuous interference with the individual specificity. We recommend keeping culture conditions consistent and fixing the sampling process within a study to eliminate any potential effect brought by the sampling. The strategy of repeated sampling at multiple time points as representative samples, as well as interpreting the complex relationships and causality between microbiome composition and disease thoroughly without the interference of temporal dynamics, is optimal for the research exploring the relationship between the human microbiome and disease.

Author Contributions

C. Zhu, X.Y. Sun, S.G. Zheng, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; C. Yuan, F.Q. Wei, contributed to conception, design, and data interpretation, drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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