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ORIGINAL ARTICLE

Characterising salivary peptidome across diurnal dynamics and variations induced by sampling procedures

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Abstract

Objectives This study aimed to characterise diurnal dynamics of salivary peptidome and variations induced by sampling procedures.

Materials and methods A supervised short-term longitudinal study was conducted amongst ten healthy participants. Saliva samples were collected by different procedures (stimulated/unstimulated conditions, forepart/midstream segments) on three consecutive days. The peptidome compositions of saliva samples were analysed using matrix-assisted laser desorption/ionisation-time of flight-mass spectrometry (MALDI-TOF MS).

Results The salivary peptidome exhibited a stable trend generally, even though some diurnal dynamics happened in aspects of both overall structure and certain single peptides. The results indicated saliva samples collected under unstimulated and stimulated conditions have significantly different structures of peptidome, whilst the peptidome profile of stimulated saliva was more abundant than that of unstimulated saliva. It was also indicated that the midstream segment effect might exist in the segmented process of saliva sampling.

Conclusions In summary, salivary peptidome was able to maintain stability though some dynamic changes might happen within a short-term period. Stimulated and unstimulated saliva samples had significantly different peptidome profiles, whilst the stimulated whole saliva was a larger pool of low molecular weight peptides.

Clinical relevance The stability of the salivary peptidome highlights the reliability of salivary peptidome as a source of diagnostic biomarker. We recommend keeping one collection condition (stimulated/unstimulated) consistently within one study on salivary peptidome. Stimulated whole saliva would be preferred if more abundant peptidome profile is needed.

Keywords Salivary peptidome · Diurnal dynamics · Sampling procedure · Midstream segment effect · Longitudinal study

Introduction

Human saliva is a biological fluid with emerging potential for early disease detection and risk assessment. In the past decades, growing evidence had unveiled that diverse salivary molecular constituents (DNA, mRNA, microRNA, proteins, metabolites, and microbiota) could serve as discriminatory biomarkers for both oral and systemic diseases [1–3]. The peptidome, namely the complete set of peptides with a low molecular weight (commonly below 10 kDa) which contained protein fragments, shed proteins and endogenous bioactive peptides, was recognised as a treasure chest of candidate biomarkers [4]. Salivary peptidome was expected to carry physiologic and pathologic information from all the perfused tissues [5, 6] and had certain advantages in terms of the simple and non-invasive sampling procedures of saliva, providing promising

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application values in disease diagnosis, prognosis, and therapy development [7–9].

Saliva has an inherent characteristic of dynamic changes since it is interacting with the body's internal physiologic procedure and external environment all the time, resulting in a question if the saliva sample could maintain a stable state generally. The diurnal dynamics of saliva hence became an important factor in determining the relationship between salivary analytes and the body state of health or disease [10].

The mixed whole saliva was regarded as the optimal option for saliva collection since it offered a feasible and simple process [11–13], which could be collected under unstimulated or stimulated conditions. The unstimulated condition was the easiest way for saliva sampling, whereas the stimulated condition was used under certain circumstances on account of its faster-salivating flow [13]. Previous studies found that stimulated sampling of saliva would not only change the salivary flow rate but also alter the main source of glands for saliva secretion [14], which brought a great methodological concern related to saliva sampling. Meanwhile, different segments of body fluid samples were already investigated and compared in urine test, which confirmed that the midstream urine sample was much cleaner and more stable than the forepart (which was so-called "midstream segment effect") and ought to be used as the golden standard for most sampling procedures in urinalysis [15], but it was still questionable whether saliva had similar properties. In consideration of the stability and cleanliness of saliva samples, some researchers had already preferred to retain midstream saliva for subsequent analysis by discarding or swallowing the forepart [16–18] without satisfactory evidence. So far, the influences of different collection conditions (stimulated/unstimulated) and segments (forepart/midstream) on compositions of salivary peptidome were still one scientific question left to be resolved.

To that end, we designed the present study to characterise the diurnal dynamics of salivary peptidome and variations induced by sampling procedures. Since salivary components could be influenced by the environment [19], general and oral health state [20], diet [21], physical exercise [22], gender [22], medication [23], and oral hygiene behaviours [24], it is indispensable to control these confounding factors before further explorations of salivary analytes. Hence, we conducted a supervised short-term longitudinal study on salivary peptidome in systemically and orally healthy participants with some confounding factors (gender, oral and general health state, medication history, physical exercise, diet, and oral hygiene behaviours) strictly controlled to analyse salivary peptidome profiles using the technique of matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS).

Materials and methods

Study participants

Participants (saliva donors) were recruited from the Peking University School and Hospital of Stomatology from May to June in 2018. After systemic medical history review and oral clinical examination, ten subjects (5 males and 5 females) were finally enrolled into the present study, who conformed to the following inclusion criteria: (1) age greater than 18 years old; (2) no systematic diseases; (3) no history of antibiotic exposure in the previous 3 months; (4) currently not pregnant or lactating; (5) no smoking or alcohol-drinking habit; (6) no presence of untreated dental caries, periodontal diseases, or other oral diseases, and not using oral appliances (e.g., orthodontic appliances). The flow diagram of this study was shown in Supplementary Fig. 1.

Control of confounding factors

Before the sampling procedures started, a washout period was designed to homogenise the confounding factors. The washout period for oral hygiene behaviours last for 2 weeks, during which period all participants used the unified suite of commercial oral care products including toothpaste, toothbrush, and dental floss with written instructions. Before the sampling began, all the participants also shared the same planned diet and beverage for 1 week for the homogenisation of diet. All the donors were instructed to avoid strenuous physical exercises for 3 days before sampling. These control measures of oral hygiene behaviours, diet, and physical exercises were also maintained during the 3-day sampling period.

Sampling of saliva

To minimize the risk of contamination or disturbance, participants were requested not to eat, brush teeth, drink, exercise, or chew gum for at least 2 h before the sampling started [25]. The participants were instructed to rinse their mouth with deionised water and then rest for 10 min before the collection of saliva started at 9:00–10:00 a.m. The graduated polypropylene tubes (Eppendorf, Hamburg, Germany) used for saliva collection were all pre-marked with sample IDs and had marker lines highlighting the 1.5-ml scale. The sample ID consisted of four digits, with the first digit indicating the number of sampling days (1, 2, 3), the second and third digits indicating the saliva donor number (01, 02, 03, 04, 05, 06, 07, 08, 09, 10), and the fourth digit indicating the sampling order (1, 2, 3, 4).

Saliva samples in the UWS (unstimulated whole saliva) group were collected by passive drooling naturally with their head bent slightly forward and the tongue placed on the palate. Samples in the MSWS (mechanically stimulated the whole saliva) group were collected by chewing a paraffin gum once per second to stimulate saliva production, then saliva was drooling from the floor of mouth with participants' heads bent slightly forward. A funnel (Zhenqi, Shanghai, China) was used to assist saliva flowing into the tube.

On sampling Day 1 and Day 3, whole-saliva samples were collected in terms of the following sequence: Initially, 1.5 mL unstimulated forepart whole saliva was collected by passive drooling, then 1.5 mL unstimulated midstream whole saliva was collected straight after the forepart segment. After a 15-min interval for rest, 1.5 mL stimulated forepart whole saliva was collected under the stimulation of chewing a paraffin gum, followed immediately by another 1.5 mL stimulated midstream whole saliva collected under the same stimulation. On sampling Day 2, the sampling order was changed to collect stimulated whole saliva first to refrain from the influence brought about by the order of collection (Supplementary Fig. 1). These four types of whole saliva samples ($N = 120$) categorised by collection procedures were described 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), respectively. Salivary flow rate was calculated by dividing the volume by the time taken to collect the sample.

Processing of saliva samples

The saliva samples were placed on ice immediately and transported to the laboratory within 2 h after collection for processing procedures. All the samples were centrifuged at 10,000 × g for 10 min at 4 ℃ to separate the pellet and supernatant. Then, the supernatants were divided into 150 µL aliquots, which received treatment of proteome stabilisation by adding 30 µL absolute ethanol (stabiliser for proteins) [26] and were stored at − 80 °C until further analysis.

WCX fractionation and MALDI-TOF MS

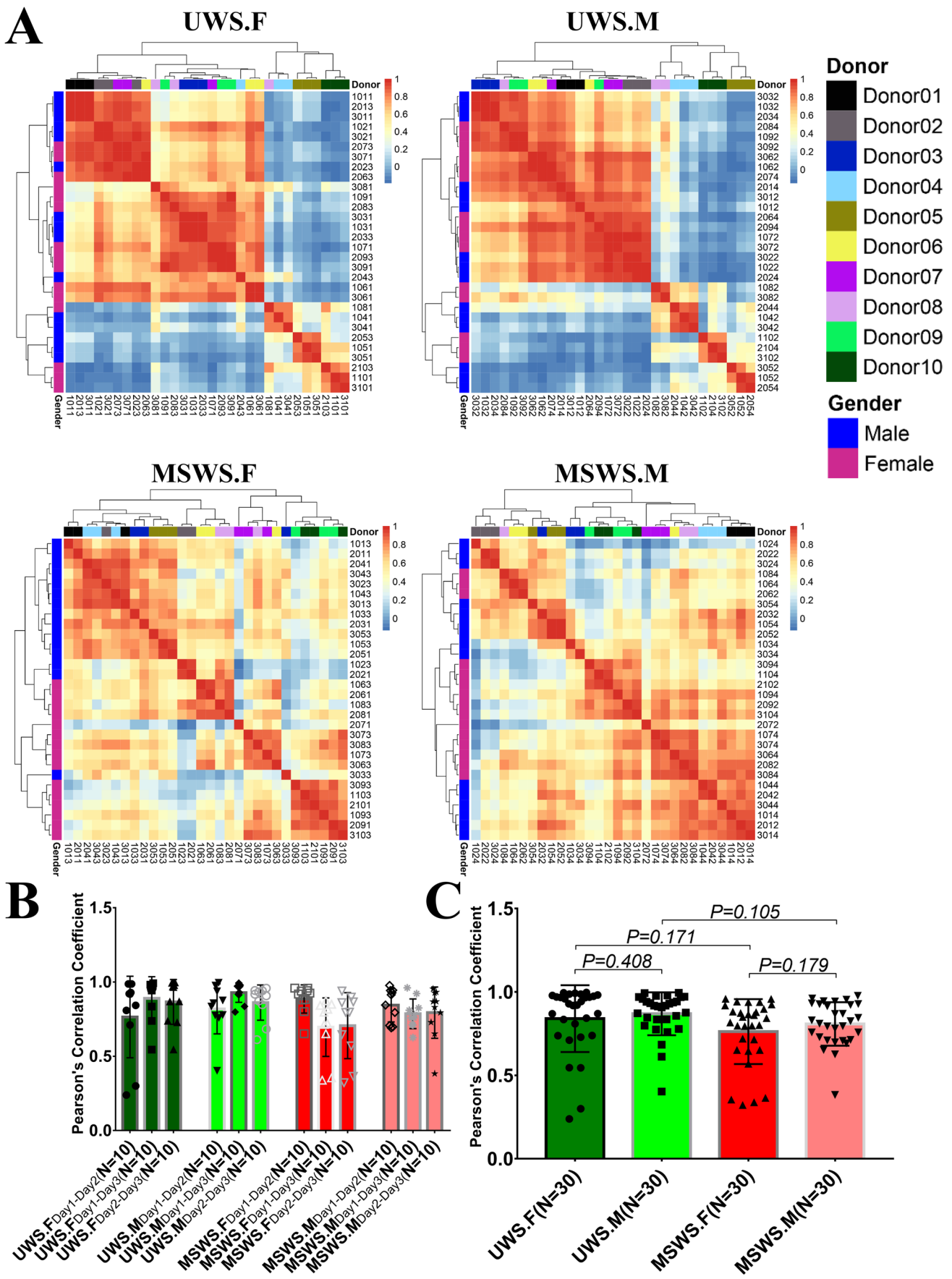
At this stage, all the samples were fractioned and purified using a weak-cation exchange magnetic-bead (WCX MB) kit (Bioyong Tech, Beijing, China) with the following steps: (1) 150 µL of WCX MB binding solution, 20 µL of beads, and 10 µL of the sample were mixed carefully and incubated for 5 min at room temperature (~ 20℃); (2) the tubes were placed on the WCX MB separation device for 1 min to collect the beads onto the tube wall, and then the supernatant was removed; (3) the beads were washed by 150 µL washing solution for 2 min, then the tubes were placed on the separation device for 1 min; (4) Step 3 was repeated, and all the supernatant was removed; (5) 10 µL of WCX MB elution solution was added, and the beads were allowed to gather on the tube wall in the separation device for 2 min; (6) the clear supernatant was transferred to a new tube, and the peptides were analysed immediately on a MALDI-TOF MS instrument (Bioyong Tech, Beijing, China).

One microlitre of the purified peptide solution was spotted onto a MALDI-TOF MS target and dried at room temperature. Then, 1 µL of the matrix solution was spotted to cover the sample and dried again. The matrix solution was 8 mg/mL CHCA in 50% acetonitrile/0.1% TFA/49.9% deionised water. The samples were loaded according to the order of the sample ID. A three-peptide mixture (monoisotopic molecular weight of 1533.8582, 2465.1989, and 5730.6087 Da; Sigma product numbers P2613, A8346, and I6279, respectively) was employed to calibrate the mass spectrometer. Profile spectra were obtained from an average of 400 laser shots per sample. The mass-to-charge ratio (m/z) values in the range of 1000–10,000 were collected. Each sample was analysed in triplicate in the MALDI procedures, whilst the subsequent analyses were based on the average spectra for each sample obtained by three-time repeated measurements using a merge calculator program (Bioyong Tech). All the experiments were conducted by the same technician without changing reagent lots, standards, or control materials to minimize analytical variations.

Data processing and statistical analysis

All the spectra obtained from the samples were analysed by BioExplorer 1.0 (Bioyong Tech, Beijing, China). The mean intensity of technical repetitions was determined, and chemical/electrical noises were subtracted. Then, the spectra were processed using wavelet smooth method and normalized by applying the total ion current. The peak m/z values and intensities were determined in the mass range of 1000–10,000. The signal-to-noise ratio was required to be more than 5. To align the spectra, a mass shift of no more than 0.1% was determined. The above methodology has been used and validated successfully in a series of previous studies by our research group [27–30].

The visualized analysis was conducted using the peak intensity profile and the Bray–Curtis distance algorithm. The Bray–Curtis distance was a frequently used abundance-based and non-phylogeny-based ecological/biodiversity distance algorithm [31, 32], which ranged from 0 to 1: "0" indicated that the composition of the


A
UWS.F
UWS.M
MSWS.F
MSWS.M
Donor
Donor01
Donor02
Donor03
Donor04
Donor05
Donor06
Donor07
Donor08
Donor09
Donor10
Gender
Male
Female
B
Pearson's Correlation Coefficient
1.5
1.0
0.5
0.0
UWS.FDay1-Day2(N=10)
UWS.FDay1-Day3(N=10)
UWS.FDay2-Day3(N=10)
UWS.MDay1-Day2(N=10)
UWS.MDay1-Day3(N=10)
UWS.MDay2-Day3(N=10)
MSWS.FDay1-Day2(N=10)
MSWS.FDay1-Day3(N=10)
MSWS.FDay2-Day3(N=10)
MSWS.MDay1-Day2(N=10)
MSWS.MDay1-Day3(N=10)
MSWS.MDay2-Day3(N=10)
C
Pearson's Correlation Coefficient
P=0.105
P=0.171
P=0.408
P=0.179
UWS.F(N=30)
UWS.M(N=30)
MSWS.F(N=30)
MSWS.M(N=30)

◂**Fig. 1** Diurnal dynamics of the salivary peptidome compositions accessed by the peak intensity profile. **A** The correlation heatmap based on Pearson's correlation coefficient of peak intensity profile within each collection procedure. The horizontal annotation was clustered according to the Donor ID, and the vertical annotation was clustered according to gender. **B** The intra-individual Pearson's correlation coefficients between any two sampling days within each collection procedure. **C** Comparisons of intra-individual Pearson's correlation coefficients represented for diurnal dynamics amongst collection procedures using repeated measures analysis of variance (ANOVA)

two samples was completely the same, whilst "1" represented that the composition of the two samples was completely dissimilar. Log intensities rather than raw intensities were adopted in the comparison since they provided an intuitive interpretation of differences in terms of fold changes, as commonly used in mass spectrometric profiling approaches. The peak intensity profile was conducted by principal component analysis (PCA) and visualized via STAMP [33]. Venn diagram was generated to visualize the shared and unique peaks amongst groups based on the occurrence of peaks across groups regardless of their intensity. The peak intensity profile was also conducted by the correlation heatmap with the unweighted pair-group method with arithmetic means (UPGMA) hierarchical clustering analysis. Pearson's correlation coefficient matrix was calculated, and the intra-individual Pearson's correlation coefficients were extracted. The Bray–Curtis distance matrix was calculated, and the intra-individual Bray–Curtis distances were extracted. The principal coordinate analysis (PCoA) and UPGMA hierarchical clustering analysis were performed based on the Bray–Curtis distance matrix. The significance of differentiation of peptidome compositions amongst groups was assessed by analysis of similarities (Anosim) using the R package "vegan." The intra-individual Pearson's correlation coefficient and Bray–Curtis distance were displayed using GraphPad Prism 7 (GraphPad, San Diego, CA, USA).

Data analysis was performed using SPSS 23.0 software (IBM, Armonk, NY, USA). The comparison of salivary flow rate, intra-individual Pearson's correlation coefficient, intra-individual Bray–Curtis distance, and peak intensity were evaluated using repeated-measures analysis of variance (ANOVA) with a post hoc Bonferroni correction used for multiple comparisons. If $P_{\text{Mauchly's test of sphericity}} \geq 0.05$, the P values of sphericity assumed were adopted; if $P_{\text{Mauchly's test of sphericity}} < 0.05$ and the epsilon $(\varepsilon) < 0.75$, P values of Greenhouse–Geisser were adopted; if $P_{\text{Mauchly's test of sphericity}} < 0.05$ and the epsilon $(\varepsilon) > 0.75$, P values of Huynh–Feldt were adopted. Results of comparisons (UWS.F vs UWS.M, MSWS.F vs MSWS.M, UWS.F vs MSWS.F, and UWS.M vs MSWS.M) were reported. $P < 0.05$ was regarded as the threshold for statistical significance (two-sided).

Results

Comparison of salivary flow rates

The socio-demographic background and oral health status of the ten donors enrolled were shown in Supplementary Table 1, whilst the comparisons of salivary flow rates were shown in Supplementary Fig. 2. The diurnal variations of salivary flow rates within each collection procedure exhibited a steady trend across the three sampling days, except for UWS.M (Supplementary Fig. 2A). As expected, there were significant differences in the flow rates between unstimulated and stimulated saliva for the same segment (forepart/midstream); salivary flow rates varied by different segments collected under stimulated condition, but no significant differences were found for that of unstimulated condition (Supplementary Fig. 2B).

Diurnal dynamics of salivary peptidome profiles

The peptide fingerprints characterised the diurnal dynamics within each collection procedure horizontally and the variations of sampling procedures vertically (Supplementary Fig. 3). The virtual gel electrophoresis (left column) and the peak intensity profile after normalisation (right column) were shown in Supplementary Fig. 4. The number of peptide peaks after normalisation was 42 for UWS.F, 55 for UWS.M, 71 for MSWS.F, and 70 for MSWS.M, respectively.

The intra-individual diurnal dynamics of the salivary peptidome compositions were accessed by the peak intensity profile (Fig. 1) and verified via the Bray–Curtis distance algorithm (Figs. 2 and 3). The correlation heatmap based on Pearson's correlation coefficient of peak intensity profile within each collection procedure was shown in Fig. 1A. Two or three coloured patches from the same donor were closely clustered together, indicating the stability of the salivary peptidome compositions. The intra-individual Pearson's correlation coefficients between any two sampling days within each collection procedure were extracted (Fig. 1B). The mean magnitude of the coefficients was around 0.85, suggesting that the salivary peptidome compositions were stable across that time scale. Interestingly, the vertical annotation in the MSWS.F showed a significant trend of gender aggregation, which was significantly reduced in MSWS.M (Fig. 1A), but statistical analysis results showed that there was no significant difference represented for the midstream segment effect (Fig. 1C). As for the PCoA and UPGMA hierarchical clustering analysis based on the Bray–Curtis distance within each collection procedure (Figs. 2A and 3), the phenomenon of gender aggregation in MSWS.F and reduction

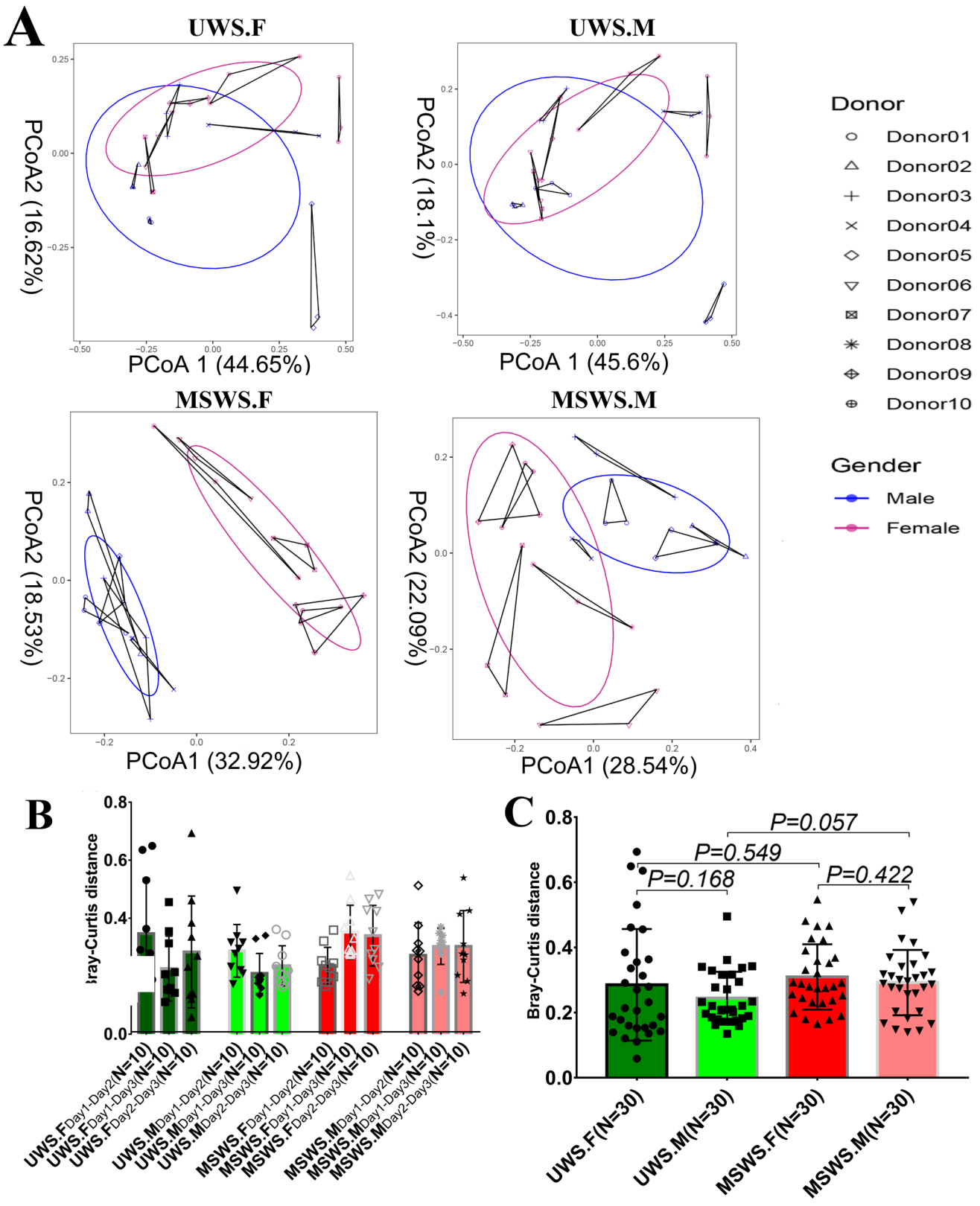


Fig. 2 Diurnal dynamics of the salivary peptidome compositions verified via the Bray–Curtis distance algorithm. **A** The principal coordinate analysis (PCoA) based on the Bray–Curtis distance showing the diurnal dynamics within each collection procedure. **B** The corresponding intra-individual Bray–Curtis distances between any two sampling days within each collection procedure. **C** Comparisons of intra-individual Bray–Curtis distances represented for diurnal dynamics amongst collection procedures using repeated measures ANOVA

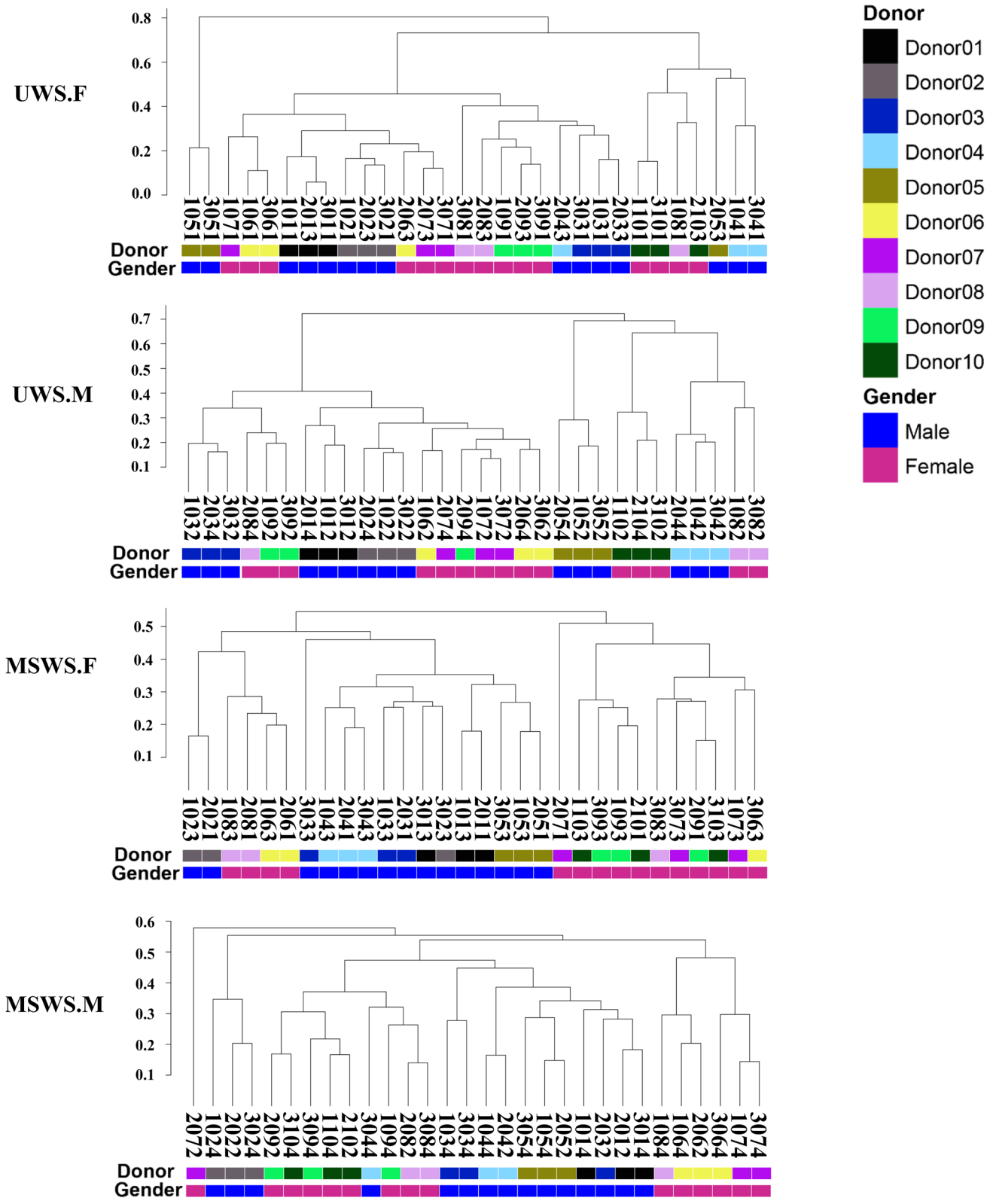


Fig. 3 The unweighted pair-group method with arithmetic means (UPGMA) hierarchical clustering analysis based on the Bray–Curtis distance within each collection procedure. The upper annotation was clustered according to the Donor ID, and the lower annotation was clustered according to gender

of aggregation in MSWS.M were also observed. The corresponding intra-individual Bray–Curtis distances between any two sampling days within each collection procedure were exhibited in Fig. 2B. The trend of intra-individual Bray–Curtis distances of the midstream segment seemed closer than its corresponding forepart; however, no significant differences were observed when Bray–Curtis distance was compared (Fig. 2C).

The diurnal dynamics of each single peptide peak intensity (log_2 transformed) within each collection procedure across the three sampling days were also performed (Supplementary Figs. 5–8). The numbers of significantly dynamic peptide peaks were as follows: 1/42 (UWS.F), 0/55 (UWS.M), 12/71 (MSWS.F), and 1/70 (MSWS.M), respectively. The results indicated that the abundance of peptides in saliva samples collected by different procedures was shown to maintain a stable trend, whilst only a small part of peptides exhibited highly dynamic characteristics. The results also implied that the midstream segment had fewer significantly dynamic peptides, which was more stable in consideration of diurnal dynamics.

Variations of salivary peptidome profiles induced by sampling procedures

The distributions of peptide peaks in saliva samples collected by different collection procedures were shown in Fig. 4A. A total of 92 peptide peaks were detected, with 31 of them shared by all the four types of collection procedures. The Venn diagram illustrated comparisons between different collection procedures (Fig. 4B), demonstrating that a group of specific peptide peaks existed in both comparisons between different collection conditions and between segments. Figure 4C showed comparisons of the intensities of the 31 shared peaks between different collection procedures and segments (the full histogram was divided into 4 separate histograms to show the results more clearly), indicating that one-half of the shared peptide peaks exhibited statistically different intensities between unstimulated and stimulated conditions, whilst a small part of shared peptides had statistical significance in comparisons between the forepart and midstream segments.

The results of PCA based on peak intensity profile on each sampling day revealed a distinct separation trend between unstimulated and stimulated conditions in both segments, whereas a relatively inconspicuous separation trend between the forepart and midstream segments was observed under both conditions (Fig. 5A). This finding was verified in the results of cluster analysis based on Pearson's correlation coefficient of peak intensity on each sampling day (Fig. 5B). The horizontal annotation (referring to collection procedure) indicated unstimulated and stimulated saliva have distinct peptidome compositions, whilst the vertical annotation (referring to Donor ID) showed that most of the forepart and midstream saliva from the same donor were clustered closely, suggesting that the forepart and midstream saliva had similar peptidome compositions.

The UPGMA hierarchical clustering analysis and PCoA based on the Bray–Curtis distance on each sampling day were also conducted (Figs. 6 and 7A), which demonstrated the same finding that a distinct separation trend existed between unstimulated and stimulated conditions in both collection segments whilst a relatively inconspicuous separation trend was found between the forepart and midstream segments under both collection conditions. The corresponding intra-individual Bray–Curtis distances of statistical comparisons between different collection procedures on each sampling day were shown in Fig. 7B, C, which verified that the variations introduced by change of collection conditions had a much higher level of Bray–Curtis distances than that introduced by change of collection segments.

Discussion

The verification of diurnal dynamics and determination of sampling procedures were considered as a prerequisite concern to be resolved in salivary peptidome research [34]. In our short-term longitudinal study, we characterised the diurnal dynamics of salivary peptidome and variations of sampling procedures using MALDI-TOF MS profiling method with strict control of certain confounding factors during the whole study process, which result was a full view of the peptide diversity but not limited to one or more specific peptide(s) that potentially served as the biomarker for a disease [35]. These findings at the *m*/*z* value level would be of great scientific significance to reveal the stability of salivary peptidome and the influences of diurnal dynamics and different collection procedures, as well as to provide reliable information and valuable research directions for future research on salivary peptidome.

As we know, human beings could always interact with the environment, and their own physiologic procedure kept changing all the time, giving rise to the dynamic variations in certain salivary components. However, the findings of the diurnal dynamics of the salivary peptidome profiles in the present study indicated that the overall compositions and intensities of most peptide peaks could keep a consistent tendency over time to maintain the stability of saliva. Nevertheless, the standardised collection, storage, and preparation procedures of saliva samples, as well as precise detection process and data interpretation analysis, were all necessary in the discovery process of salivary biomarkers to overcome the challenges brought about by dynamic changes and obtain reproducible and credible results as far as possible.

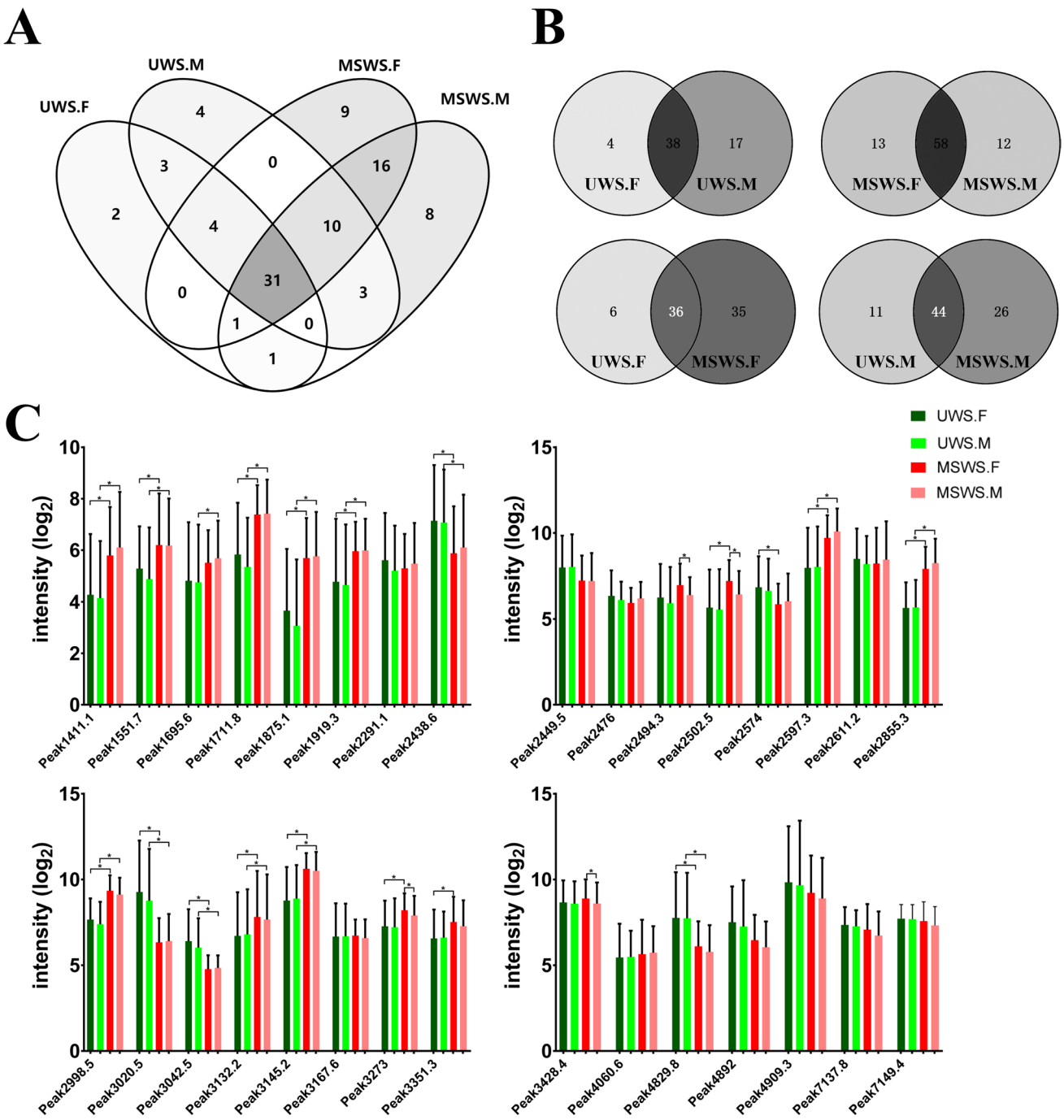


Fig. 4 **A** The Venn diagrams depicted distributions of peptide peaks in saliva samples collected by different collection procedures. **B** The Venn diagrams depicted comparisons between different collection states. **C** Comparisons of the intensities of the 31 shared peaks between different collection procedures and segments ($\log_2$ transformed). The *P* values were obtained by the repeated measures ANOVA as detailed above, * indicated $P < 0.05$.

We found that saliva samples collected by different collection conditions (unstimulated/stimulated) did have significantly different peptidome compositions, which exhibited an inconsistent finding with one previous proteome study [36] as the target regions of mass spectrometry was quite different: the present study focused on peptides within the *m/z* range of 1000–10,000, whereas Golatowski's study [36] focused on the whole proteome. For the sake of comparability, it was recommended to keep the same collection condition (unstimulated/stimulated) consistently throughout one study on salivary peptidome. Our results also demonstrated that the peptidome profile in MSWS was more abundant

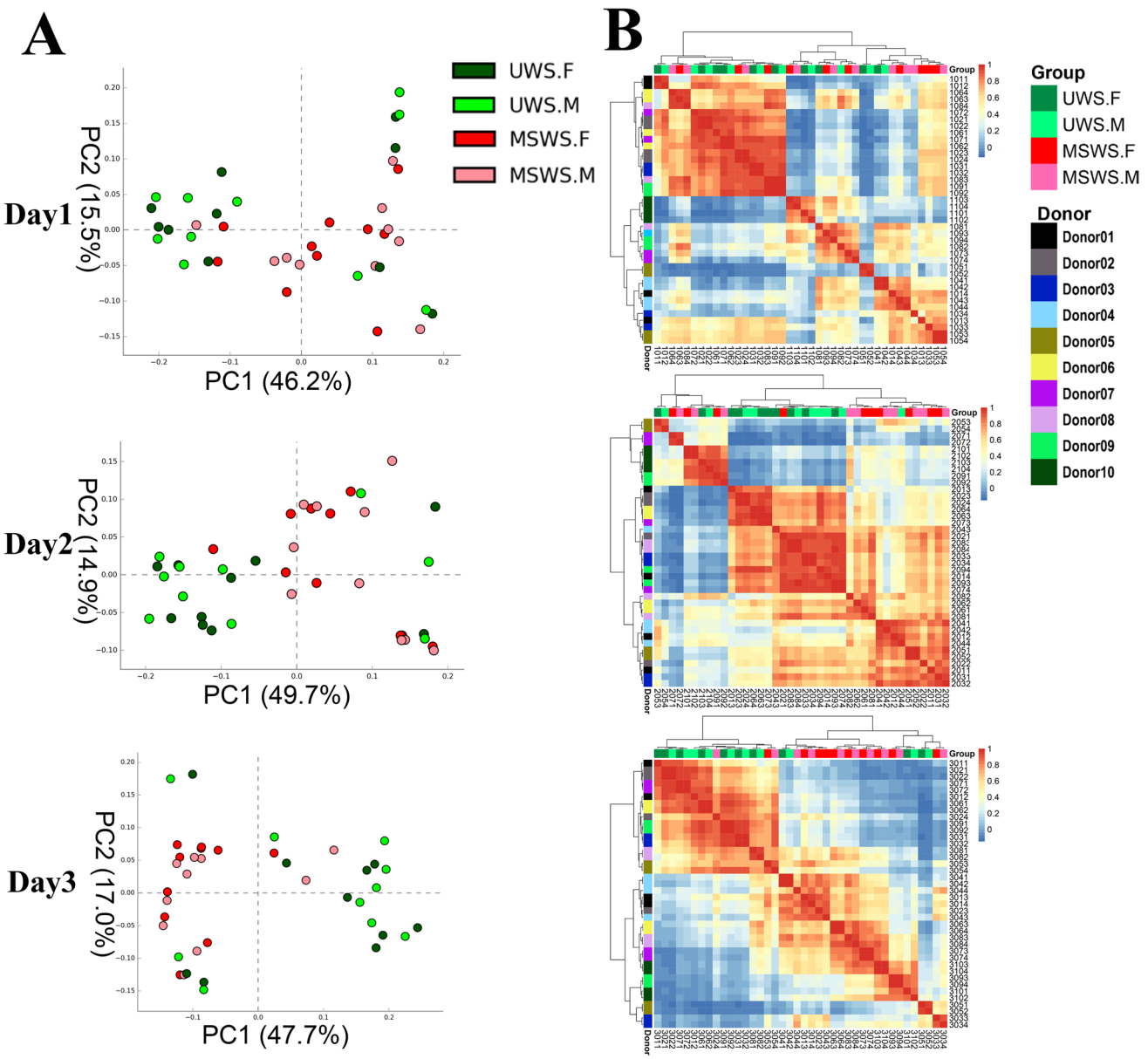


Fig. 5 The variations of peptidome profiles induced by sampling procedures for saliva samples. **A** The principal component analysis (PCA) based on peak intensity profile showing the variations introduced by the change of collection procedure on each sampling day. **B** The correlation heatmap based on Pearson's correlation coefficient of the peak intensity profile. The horizontal annotation was clustered according to the collection procedure, and the vertical annotation was clustered according to the Donor ID

than UWS, indicating that stimulated saliva might be a larger pool of low molecular weight peptides than the unstimulated counterparts. One explanation of this phenomenon was that the concentration of mucins in UWS was much higher than that in MSWS [37], which might result in a reduction of proteolysis activity and peptide diversity due to the aggregation between mucins and other salivary proteins [38]. Therefore, the collection of stimulated saliva samples might be a better choice if one salivary peptidome study aimed to investigate a wider range of peptides.

The "midstream segment effect" might be an unneglectable factor in studies on salivary peptidome. Based on our findings, MSWS.F exhibited an evident gender aggregation trend compared with MSWS.M, whilst the midstream segment was more stable in diurnal dynamics with fewer significantly dynamic peptides, suggesting that discarding or swallowing the forepart segment of saliva [16–18] seemed to be practical in salivary peptidome research. However, it was worth noting that results of statistical analysis showed no significant differences represented for the midstream

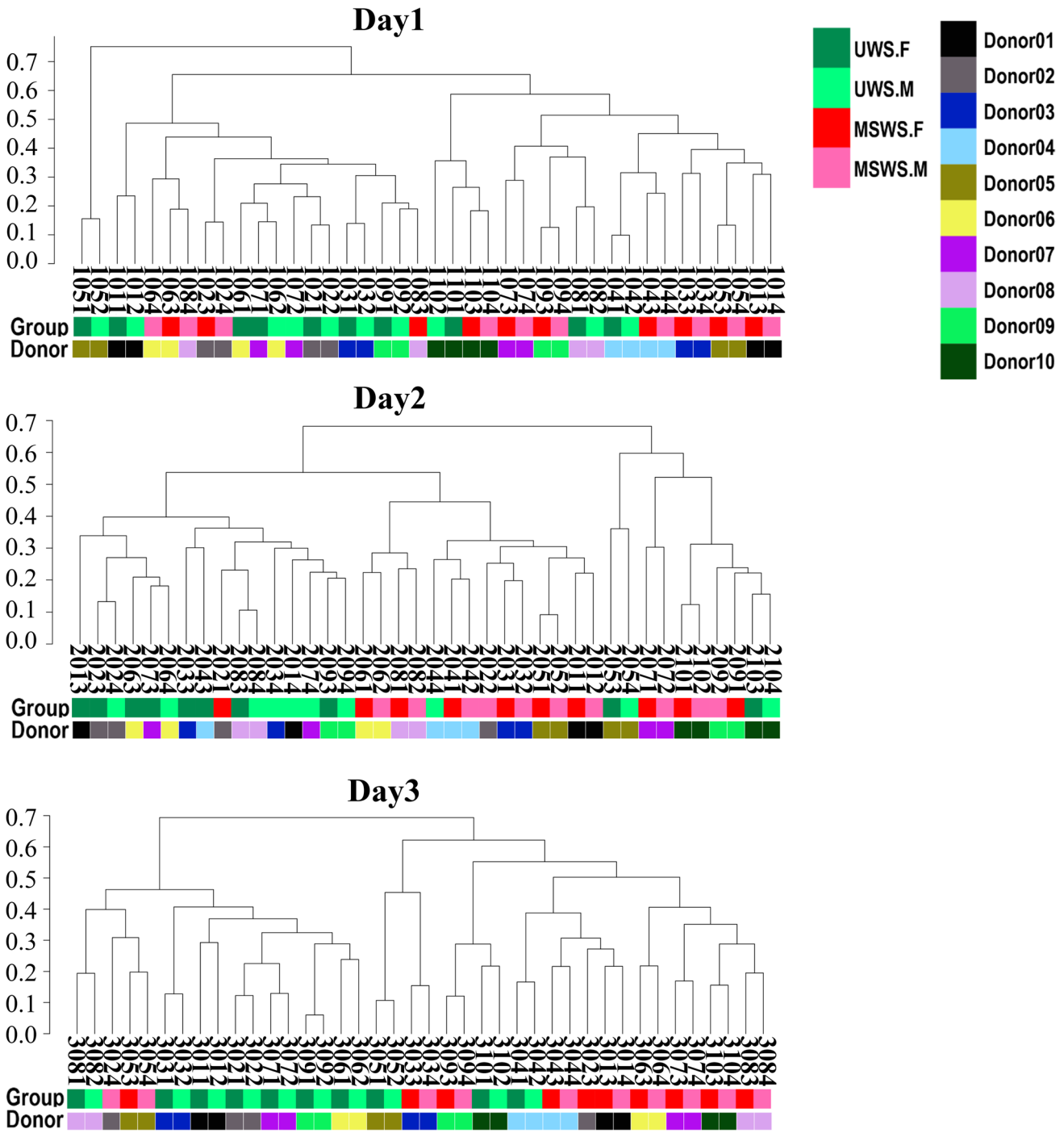


Fig. 6 The variations of peptidome profiles induced by sampling procedures for saliva samples using the unweighted pair-group method with arithmetic means (UPGMA) hierarchical clustering analysis based on the Bray–Curtis distance on each sampling day. The upper annotation was clustered according to the collection procedure, and the lower annotation was clustered according to the Donor ID

segment effect (Fig. 1C). Since the present findings were completely based on setting a cutoff volume as 1.5 mL between the forepart and midstream segments, future studies on investigation of the midstream segment effect with attempts of other cutoff volumes would contribute to the current knowledge on this issue.

The sample size of peptidome study was also an important issue worth discussing. In recent years, mass spectrometric profiling approaches such as MALDI-TOF MS were emerging to be utilized in exploration for disease biomarkers such as peptides. However, most studies in this field lacked a specific calculation process for

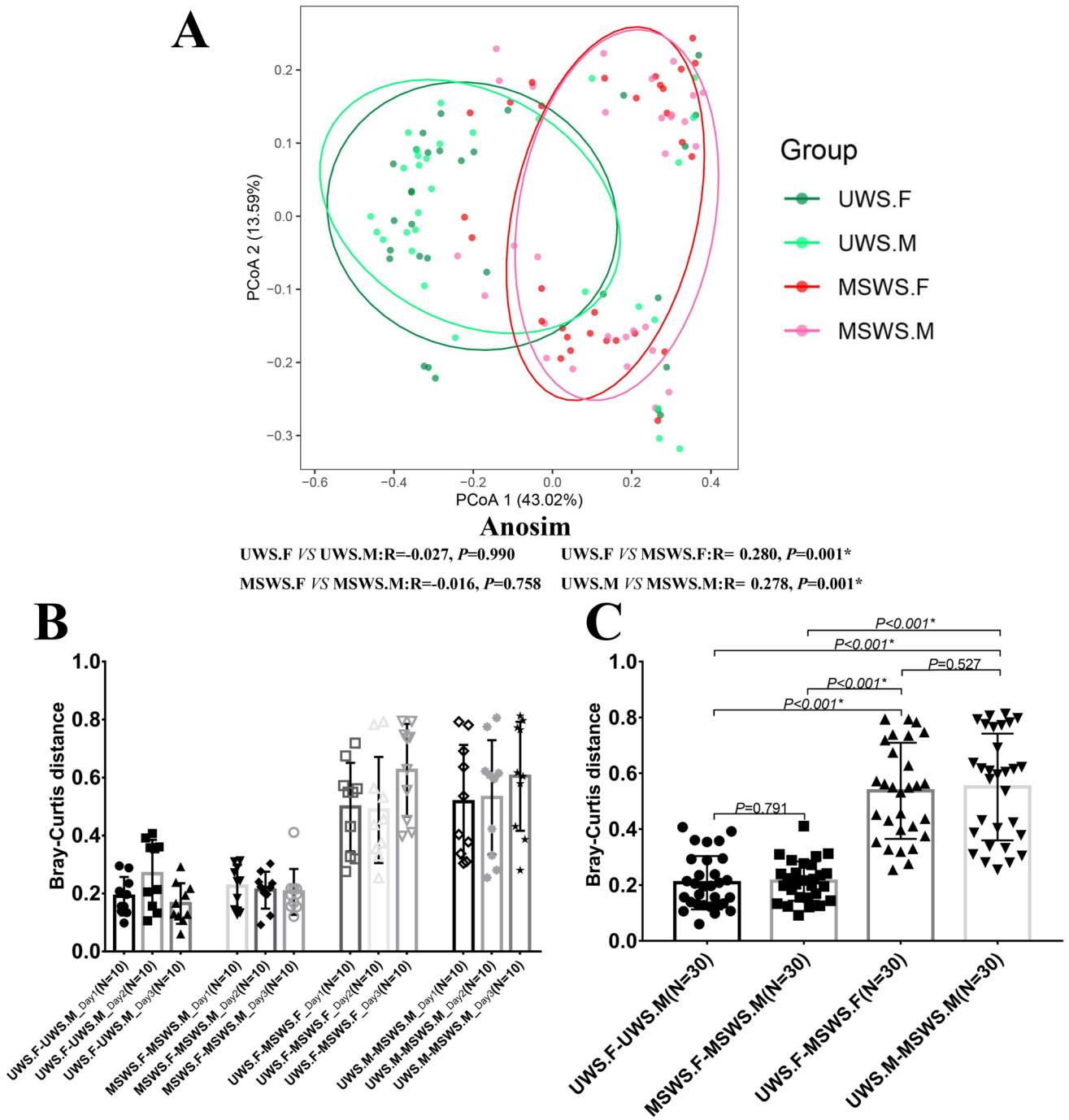


Fig. 7 Demonstration of the variations of peptidome profiles induced by sampling procedures for saliva samples using the distance algorithm. **A** The principal coordinate analysis (PCoA) based on the Bray–Curtis distance showing the variations introduced by the change of collection procedure on each sampling day with the Anosim test. **B** and **C** The corresponding intra-individual Bray–Curtis distances of statistical comparisons between different collection procedures on each sampling day. * indicated $P < 0.05$

sample size determination in the stage of study design, because it was quite difficult to carry out a pilot experiment for the construction of a calculation model for sample size. However, we attempted to conduct the "Back Testing" to evaluate the sample size based on a simple linear mixed model [39], which allowed the inclusion of estimates of biological and technical variation inherent in the experiment using the data obtained from the results of this study. The number of biologically distinct samples N in each group was given by: $N = 2\left(\frac{Z_{\alpha/2}+Z_{\beta}}{\Delta}\right)^2 [\frac{\tau_p^2}{m} + 6_p^2]$. We adopted a two-sided 5% significance-level test (α) with 95% power (β), and the Δ to detect a difference of

magnitude was set as $\log_2(2)$. The technical replicates (m) were 3, and the components of variance for biological variation (σ_p^2) and technical variation (τ_p^2) were calculated from $\log_2$ transformed intensities. The estimated required sample size equation was $= 2\left(\frac{1.96+1.645}{\log 2(2)}\right)^2\left(\frac{0.62}{3}+0.24\right) \approx 12$. This post-mortem validation indicated that the sample size used in the present study was acceptable, particularly in consideration of its longitudinal design.

Findings in the present study must be interpreted in the context of limitations to enlighten future research directions. First, there were only ten systematically and orally healthy participants involved, making the extrapolation of the findings a bit limited, though we have conducted the "Back Testing" to validate its adequacy to some extent. Also, the evidence could be strengthened by future studies on a larger population with higher complexity, e.g., patients with specific diseases. Second, competing ionisations between peptide moieties that lead to ion suppressions could mask actual abundance changes in measurements of intensities by MALDI-TOF MS, bringing about the most evident limitation on MALDI-TOF MS profiling in clinical proteomics based on the complexity of biological fluid. Hence, the reproducibility of the MALDI-TOF MS workflow should be considered within clinical MALDI-TOF MS profiling. Third, the available information was limited by the lack of peptide identification in our MALDI-TOF profiling method. Despite this, we believe the comparisons of overall *m/z* profiles within this study were of clinical significance since the panel of *m*/*z* value profiles based on MALDI-TOF MS profiling could be fundamental to the peptidome composition and also serve as an indicator of peptidome diversity [35].

Conclusions

In summary, salivary peptidome was able to maintain stability though some dynamic changes might happen within a short-term period. Stimulated and unstimulated saliva samples have significantly different peptidome profiles, whilst the stimulated whole saliva would be a larger pool of low molecular weight peptides.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00784-022-04722-4.

Author contribution All authors have made substantial contributions to the conception and design of the study. All authors have been involved in data collection, and Z.C. has been involved in data analysis. All authors have been involved in data interpretation, and Z.C. has been involved in drafting of the manuscript. X.Y.S. and S.G.Z have been involved in revising it critically, and all authors have given the final approval of the version to be published.

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Data availability Data sets used for analyses in the present study are available upon request.

Declaration

Ethics approval This study was approved by the Peking University School and Hospital of Stomatology Ethics Committee (PKUSSIRB-201944061 and PKUSSIRB-202274053). All the procedures were performed in accordance with the Declaration of Helsinki and the STROBE guidelines.

Consent to participate Informed consent was obtained from all participants before the study began.

Conflict of interest The authors declare no competing interests.
The following four comparisons: UWS.F vs UWS.M, MSWS.F vs MSWS.M, UWS.F vs MSWS.F, UWS.M vs MSWS.M were reported. UWS.F, the forepart segment of unstimulated whole saliva. UWS.M, the midstream segment of unstimulated whole saliva. MSWS.F, the forepart segment of mechanically stimulated whole saliva. MSWS.M, the midstream segment of mechanically stimulated whole saliva (similarly hereinafter).

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