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The stability of children's salivary peptidome profiles in response to short-term beverage consumption

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ABSTRACT

Background: Salivary peptidome profiling analysis has advantages of simplicity and non-invasiveness and great potentiality for screening, monitoring or primary diagnosis of diseases, but may be subjected to change against interferences like diet.
Methods: We conducted a 5-day study to investigate the influence of 3 kinds of beverages (orange juice, sugar-free tea, and sugar-free liquid yoghurt; water as control) on children's salivary peptidome using mass spectrometry techniques.
Results: All the groups shared a relatively stable pattern in heatmaps during the experimental days. Principal component analysis plot presented slight shifts in all the intervention groups between the baseline and intervention period while samples were not distinctly separated by date. The numbers of significantly changed peptides after short-term orange juice and tea intervention were four and three, respectively, while no changes occurred in the yoghurt group and control. Four of these peptides were identified as histatin-3, collagen alpha-1(IV) chain, zinc finger protein 805, and quinolinate synthase A.
Conclusions: Salivary peptidome has its own stability against beverage intervention, confirming the feasibility and validity of using it as a potential reference for the healthy state of the body, with diet habits recorded and considered as a confounder if necessary.

1. Introduction

Saliva contains a huge number of protein species, including numerous low-molecular-weight peptides, which could reflect the biological processes taking place in oral cavity [1] and indicate the physiological and pathological state of the whole body [2]. With the advantages of simple and non-invasive collection methods, and uncomplicated and comparatively inexpensive processing procedures, salivary peptidome profiling analysis was considered to have great potentiality in screening, monitoring or primary diagnosis of oral and systemic diseases, such as dental caries, periodontal diseases, oral cancer, Sjögren's syndrome, cardiovascular disease and chronic kidney disease [3–8].

As an emerging technique in recent years, the matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI-TOF-MS), which owns advantages of high sensitivity and easy operating procedures, has been widely used for peptidome analysis and biomarker characterization [9]. Given that eligible biomarkers for diseases should maintain strong stability and be slightly affected by some common confounding and interference factors such as dietary intake [10], whether salivary biomarkers could keep this characteristic become quite important since saliva experiences daily disturbances such as food and beverage intake frequently. Several previous studies found that certain types of salivary proteins might be partly affected by diet [11,12] or react with some composition of food [13], but the effects of diet intake on the profiles of salivary peptides (potential biomarkers)

Abbreviations: MALDI-TOF MS, Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; WCX MB, weak cation exchange magnetic bead; CB, MB-WCX binding solution; CW, MB washing solution; CE, MB elution solution; CHCA, α-cyano-4-hydroxycinnamic acid; S/N, signal-to-noise; FDR, False discovery rate; PCA, Principal component analysis; nano-LC/ESI–MS/MS, nano-liquid chromatography-electrospray ionization-tandem mass spectrometry; EGCG, Epigallocatechin gallate; GCG, gallocatechin gallate; PRPs, Salivary proline-rich proteins; HOMD, Human Oral Microbiome Database

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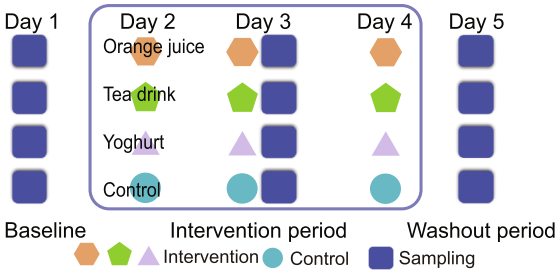

Fig. 1. The schematic diagram of the sampling and study procedures.

remains unclear, particularly for that of beverages, which are much popular worldwide and could serve as representatives for daily interferent on the oral peptidome [14,15].

Children, a population with relatively poor oral hygiene skills but rarely affected by periodontitis and most non-communicable diseases [16,17], may be an appropriate option as subjects for studies related to interventions on oral microenvironment. Therefore, we conducted a short-term longitudinal study to investigate the influence of 3 kinds of popular beverages (orange juice, sugar-free tea, sugar-free liquid yoghurt, as representatives of the effects of sugar, tea polyphenols, exogenous bacteria / milk proteins, respectively) and water (as control) on children's salivary peptidome using the techniques of MALDI-TOF MS and Nano-LC/ESI-MS/MS, to further explore the characteristics of salivary peptidome in response to beverage interventions (Fig. 1).

2. Methods

2.1. Study participants

60 study participants were recruited from 190 children (aged 3–5 years) in one kindergarten at Chaoyang District of Beijing, according to the inclusion criteria as follows: (i) no history of systemic diseases, oral diseases and dental treatment; (ii) no antibiotic use within the past month; (iii) no upper respiratory infections within the past fortnight [18]. In total 39 participants (Supplementary Table 1) completed the whole study course, while the other 21 were dropped because of absence, poor compliance, or contamination of saliva sample (e.g. bloody saliva). All these children had been living at the kindergarten under a unified schedule for at least 7 months before the study commenced, with informed consents signed by their guardians. Ethics approval was obtained from the Ethics Committee of Peking University School and Hospital of Stomatology (issue number: PKUSSIRB-201413035).

2.2. Sample collection

Children were grouped into Group J, T, Y, and W (for Juice, Tea, Yoghurt and Water) with gender and age matched as far as possible (Supplementary Table 2). The sampling procedures lasted for five consecutive days in April 2014 (Monday to Friday), with the first and last day set as the baseline and washout period, respectively. Weekdays were selected to perform the sampling of saliva to reduce the impact of different family diet habits during weekends, as these children had undergone the same schedules on their weekdays at the kindergarten. Beverage intervention were implemented from day 2 to day 4 ("experimental days"), during which period approximately 80 mL of the corresponding beverage (Supplementary Table 3) was given to each participant at 4 separate time points (9:30, 10:00, 11:00 and 14:00). Besides the designated beverages, children were instructed to drink water only during the 5 days.

Due to potentially poor compliance of the 3–5 year-olds, stimulated method of tooth brushing by professionals [19] was more frequently used to collect the adequate amount of saliva. Stimulated whole saliva (~2 mL) was collected at 14:30 every day. With the assistance of passive tooth brushing by trained dentists from Peking University School and Hospital of Stomatology, children kept their heads bent slightly forward to make saliva drooling from the mouth floor to the funnel. Both the intake of beverages at 4 time points and the collection of saliva were recorded by research fellows. 9 children missed intervention for at least once because of absence, while 2 children had taken extra milk during the intervention period. For the sake of strict quality control, samples collected from these participants were all excluded from the present study. The saliva samples were collected in sterile, ice-chilled tubes and immediately placed in liquid nitrogen for temporary storage to avoid dramatic changes in the components of saliva in vitro. At few ($\leq$2) hours later on the same day, saliva samples were centrifuged at 10,000 × g for 10 min at 4℃, and for each sample a volume of 1 mL supernatants was sent for mass spectrometry as soon as possible, whereas the rest part was stored in −80℃ refrigerator until further use.

2.3. WCX enrichment and MALDI-TOF MS

All saliva samples were fractionated using a weak cation exchange magnetic bead (WCX MB) kit (Bioyong Tech, Beijing, China). Samples were purified and isolated with the steps (Supplementary Fig. 1) below (following previous studies by our research group [5,20]): (i) 20 μL of beads, 150 μL of MB-WCX binding solution (CB), and 20 μL of salivary sample were mixed carefully and incubated for 5 min. (ii) The tubes were placed on the MB separation device (Bioyong Tech) and the beads were allowed to collect on the tube wall for 1 min. (iii) The supernatant was removed by washing and mixed thoroughly with 80 μL of MB washing solution (CW). (iv) Another 10 μL of MB elution solution (CE) was added, and the beads were allowed to gather on the tube wall in the separation device for 2 min. (v) Clear supernatant was transferred into a fresh tube, and its peptide composition was analyzed directly by the ClinTOF-II device (Bioyong Tech, Beijing, China) or stored at −20 ˚C.

Anchor chip spotting and MALDI-TOF MS profiling were performed with the following steps: Initially, the matrix solution, 5 mg/mL CHCA (α-cyano-4-hydroxycinnamic acid) in 50% acetone / 0.1% TFA solution was prepared; Then, 1 μL of purified peptide solution was spotted onto a MALDI-TOF MS target by the ClinTOF-II; After drying at room temperature, 1 μL of matrix solution was spotted onto the sample, and dried again before analysis; MALDI-TOF MS were performed using the ClinTOF-II device. Before analyzing, a three-peptide mixture (monoisotopic molecular weights of 1532.8582, 2464.1989, and 5729.6087 Da, Product Numbers P2613, A8346, and I6279, respectively; Sigma) was used for calibration. Profile spectra were acquired from an average of 400 laser shots per sample. Data with the mass range of 1,000–10,000 were collected. Each sample of saliva was analyzed for 3 times.

2.4. Data processing and statistical analysis

All the spectra obtained from the samples were analyzed using BioExplorer 1.0 (Bioyong Tech) software. The mean relative peak intensities were acquired with the chemical and electrical noises subtracted. Then, the spectra were normalized using total ion current, with intensities and peak mass-to-charge ratio (m/z) values determined in the range of 1,000–10,000. A signal-to-noise (S/N) ratio $>$ 5 was required. To align the spectra, the mass shift was set as no $>$ 0.1%. The peak area was analyzed for quantitative standardization.

Data were analyzed using BioExplorer 1.0 statistical package and SPSS 20.0 software (IBM, Armonk, NY). Differences in peptide levels among the 3 experimental days were evaluated using repeated-measures ANOVA by Mauchly's Test of Sphericity and the tests of effects within subjects: If P (Mauchly's Test of Sphericity) $\geq$ 0.05, the P value of sphericity assumed was adopted; then, if P (Mauchly's Test of Sphericity) $<$ 0.05, and epsilon (ε) $<$ 0.75, the P value of Greenhouse-Geisser was adopted; otherwise, if P (Mauchly's Test of Sphericity) $<$ 0.05, and epsilon (ε) $>$ 0.75, the P value of Huynh-

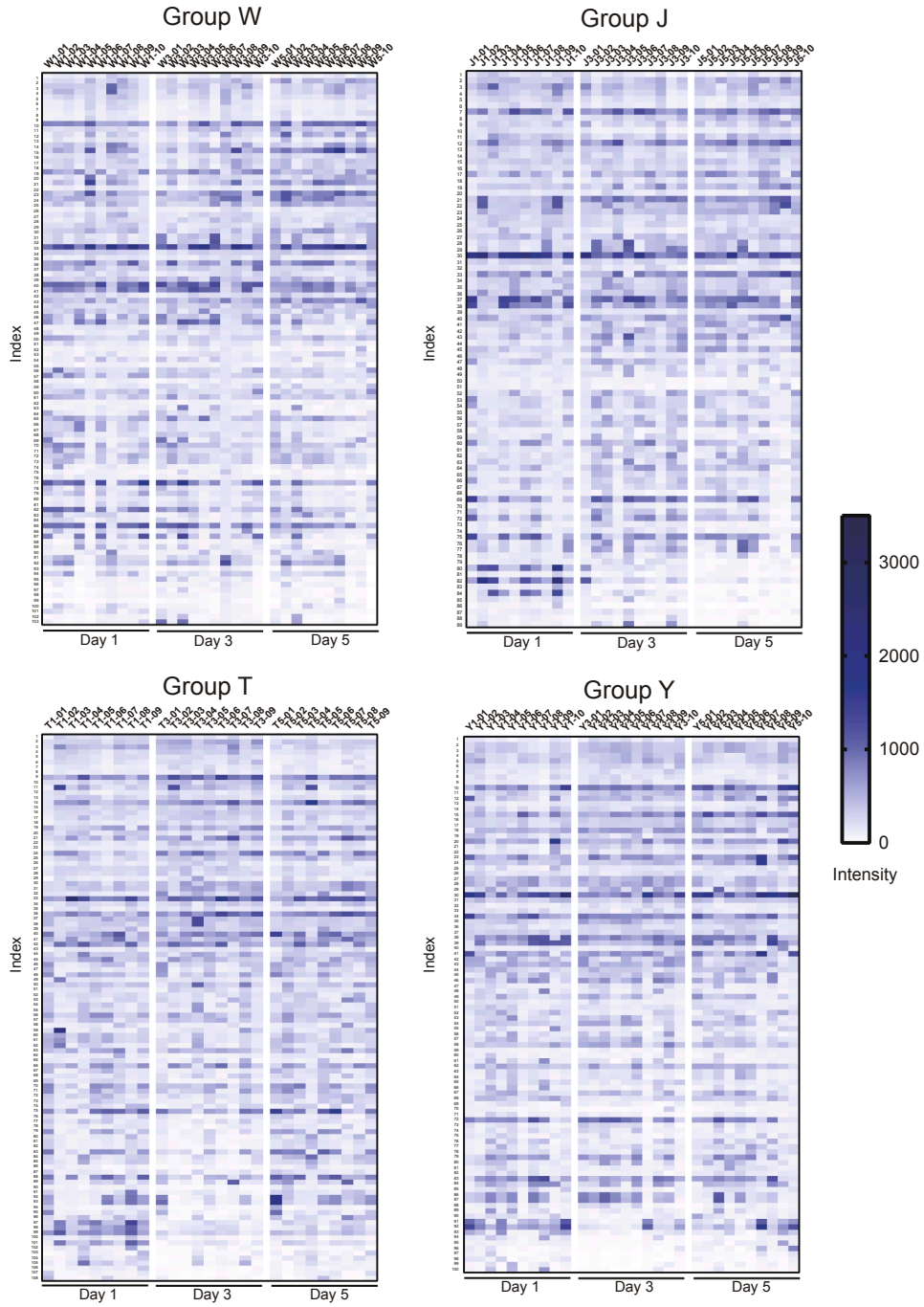

Fig. 2. Heatmaps of all the peptide peaks in the 4 groups (W, J, T, and Y) on day 1, 3, and 5. The *m/z* values (y-axis) is listed in Supplementary Table 4 with the same order.

Feldt was adopted. For all other tests except Mauchly's Test of Sphericity, the threshold of statistical significance was set at $P < 0.01$. False discovery rate (FDR) was used to control for multiple testing.

2.5. Principal component analysis

STAMP 2.1.3 software [21] was used to perform the Principal Component Analysis (PCA) on peptidome profiles in different groups.

2.6. Peptide sequences

The sequences of significantly changed peptides in the model were identified using a nano-liquid chromatography-electrospray ionization-tandem mass spectrometry (nano-LC/ESI–MS/MS) system consisting of an ACQUITY UPLC system (Waters, Milford, MA) and an LTQ Obitrap XL mass spectrometer (Thermo Fisher Scientific, Rockford, IL) equipped with a nano-ESI source. The MS/MS experimental protocol was as follows: The supernatant was loaded onto a C18 trap column (Symmetry nano-ACQUITY; Waters) (180 μm × 20 mm × 5 μm) with a flow rate of 15 μL/min. Then, the desalted peptides were enriched on a C18 analytical column (Symmetry nano-ACQUITY) (75 μm × 150 mm × 3.5 μm) at a flow rate of 400 μL/min. Third, the mobile phases A (5% acetonitrile and 0.1% formic acid) and B (95% acetonitrile and 0.1% formic acid) were used on the analytical columns. The profile of solvent B was at 5% during elution, was ramped to 30% after 5 min and remained for 45 min, then increased to 90% for 8 min, and decreased to 5% for 5 min. The

mass spectrometry device was operated in data-dependent mode. The *m/z* range of full scan was 400–2000 with a mass resolution of 100,000 (*m/z* 400). The eight monoisotope ions with the highest intensities were the precursors for collision-induced dissociation. MS/MS spectra were limited to two consecutive scans per precursor ion followed by 60 s for dynamic exclusion. Acetylation (N-Term) and oxidation (M) were used as post-translational modifications. The initial screening was carried out after inputting the MS/MS figure to the Proteome Discoverer 1.4 software (Thermo Fisher Scientific).

The parameters used were listed below: the minimum number of peaks in MS/MS figures was set as 10, the range of molecular weight of the parent ion was 350–5,000, and the threshold of S/N ratio was 1.5. After initial screening, the mass spectra were searched using Mascot software (version: 2.3.2) based on variable and fixed modifications. Database included the Homo sapiens data (human, 20,255 sequences) of SwissProt 2012_03 (535,248 sequences; 189,901,164 residues) and the bacteria data (eubacteria, 331,887 sequences) of SwissProt 2015_01 (547,357 sequences; 194,874,700 residues). The parameters for generating the peak lists were as follows: FDR ≤ 1%, accuracy of peptide tol: 50 ppm, accuracy of MS/MS tol: 0.3D.

3. Results

3.1. The stable profiles of salivary peptide

In the 4 groups, in total 39 healthy children (average age of 56.3 ± 6.3 months; 18 males and 21 females; details in Supplementary Table 1) completed the full course of the present study. No statistical significance was found between the 4 groups in age and gender ($p > 0.05$). A total of 117 saliva samples were collected for the 3 experimental days.

The entire mass spectra of peptides from the saliva samples were analyzed and compared using MALDI-TOF MS. The maximum intensity of a saliva peptidome peak appearing between two adjacent *m/z* values were regarded to belong to its most proximal *m/z* value; most peaks were in the range of 1000 ± 4,000 (Supplementary Fig. 2). An average of 100 peaks were detected in each group after alignment (Fig. 2).

3.2. The response of salivary peptides to beverages

All the groups shared a similarly stable pattern on day 1, 3, and 5 for peaks with *m/z* values < 3,000, whereas the patterns of peaks with *m/z* values > 3,000 appeared to be diverse and uneven in Group J, T and Y (Fig. 2). The result of Principal Component Analysis figured out changes in salivary peptides of the 4 groups, showing that individuals had an indiscrete distribution in most areas in Group Y while some samples on day 1 kept separated from those on day 3 and 5 in Group J and T (Fig. 3A-3D).

3.3. Differentially expressed peptide peaks

The masses were quantified and compared to select out differentially expressed peptides (Fig. 4). In Group W and Y, there were no significantly changed peptides. In Group J, a total of four peptides exhibited significant differences, with *m/z* values of 1531.6, 3365.1, 3436.4 and 3483.6 (FDR < 0.17). The intensity of the peptide with *m/z* 1531.6 declined on day 3 and returned to the baseline level on day 5, while the other three peptides kept the declining trend on day 5. In Group T, a total of three peptides were found to be significantly changed, including those with *m/z* values of 3367.7, 3441.1, and 3482.8 (FDR < 0.2). All of them had a declining tendency on day 3 but appeared to recover on day 5.

Nano-LC/ESI-MS/MS was carried out to identify possible proteins which these peptides might derive from. It was regarded as the same peptide if the difference between experimental and theoretical *m/z* values was < 5. Finally, four of the six differentially expressed peptides (experimental *m/z* values: 1531.6, 1851.4, 1886.5, and 2331.0) were successfully identified (Table 1), with three of them from human and the other one from bacteria.

4. Discussion

With the technique of mass spectrometry, the present study explored the variation of salivary peptidome against interventions by three kinds of popular beverages, which was an important issue of concern in salivary biomarker studies that daily activities such as diet intake might affect the compositions of saliva.

Most salivary peptides kept stable after beverage intake in the present study. Previously, it was reported that the secretion of salivary proteins could be influenced by circadian rhythm, taste, and masticatory movement [22–24]. Studies also found that the total amount and composition of salivary proteins, as well as the salivary flow rate, could be influenced by taste stimulation [25,26]. However, in another study, most influences of sodium-chloride and sucrose solutions on salivary protein composition were observed in 1 min, with almost no effects observed after 20 min [27], which was even less than the interval between beverage drinking and sampling procedures in our study (30 min or so). Based on the above, the most probable explanation is that beverages might result in temporary influences on salivary proteins, but have little persistent effects. As these endogenous salivary peptides were believed to be originated from large proteins via the counterbalancing regulation by their inhibitors [1,28] and proteolysis by proteases which might be related to some pathological conditions sometimes [28], it was speculated that the nature of barely-affected activity of proteases was playing a more important role than the interaction been proteins and beverage ingredients in maintaining the stable state of salivary peptidome in response to beverage intake.

In Group J, four significantly changed peptides were detected after drinking orange juice, which beverage was rich in carbohydrates and health-promoting bioactive compounds such as flavanones hesperidin and naringenin [29]. As previously reported, the intake of naringenin was able to reduce the salivary flow rate in rats [30], while 50 mM sucrose could elevate the concentration of α-amylase in saliva [31]. In the present study, histatin-3, a member of the histatin family with antimicrobial functions [32], was the only one successfully identified in all significantly changed peptides in Group J, with no previous reports regarding its relationship with the orange juice so far. In Group T, three peptides exhibited a significant change in response to green tea, which mainly consists of polyphenols (~90%, a kind of tannin) and catechins such as epigallocatechin gallate (EGCG) and gallocatechin gallate (GCG) [33]. Some salivary proteins, including salivary proline-rich proteins [34], salivary histatins [35], α-amylase, and salivary mucins [36,37], were demonstrated to have the ability of binding polyphenols, but the three significantly changed peptides observed in our study did not belong to any of them. Furthermore, by virtue of the antibacterial effects of EGCG [38], the microbiome structure in saliva might be subjected to a change. Although one significantly changed peptide was identified to be derived from a bacterial protein, namely quinolinate synthase A of *Dehalococcoides mccartyi*, it remained doubtful as this microbe was not listed in the Human Oral Microbiome Database (HOMD) project (version 15.2). However, since quinolinate synthase exists widely in prokaryotes [39], while some genera (e.g. Chloroflexi) from the same phylum with Dehalococcoides were already found in the oral cavity [40], the limited capacity of the database might be a potentially possible reason for this questionable finding. Furthermore, with the involvement of yoghurt in the present study, we intended to discover the influence of probiotics and milk proteins on salivary peptidome, as the consumption of probiotics-containing dairy products could increase the pH value of saliva and positively affect the microbial ecology of the oral biofilm to some extent [41], but our results suggested that probiotics in yoghurt seemed not to alter the salivary peptidome profile.

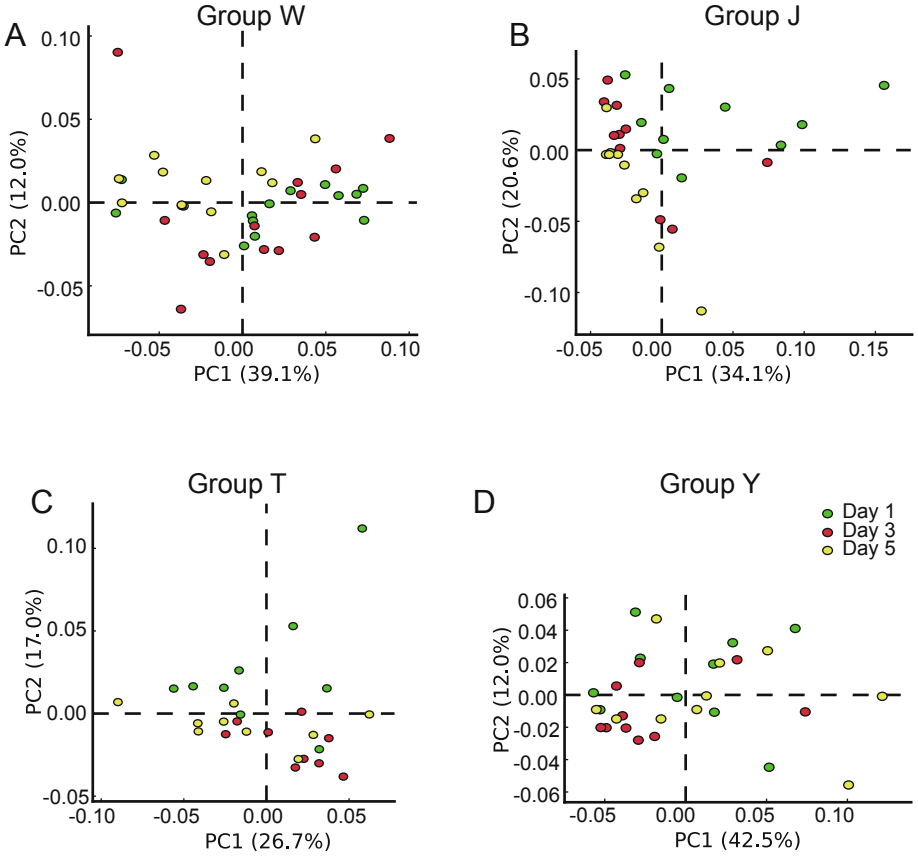

Fig. 3. The Principal Component Analysis (PCA) of Group W**(A)**, J**(B)**, T**(C)**, and Y**(D)** on day 1, 3, and 5.

By comparing the significantly changed peaks with previous studies on salivary peptide biomarkers [4–8,20–24], one peptide with *m/z* value of 3436.4 was reported as potential biomarkers for periodontal diseases [5]. Moreover, histatin-3, which was identified both in the present study (in Group J) and in a previous biomarker study for chronic kidney disease [8], was proved to be particularly sensitive to proteolytic degradation [42]. This remind us that some peptides, including but not limited to the above two, may need more attention when extrapolating the findings related to them in some salivary biomarker studies. It would be better if diet habits (including beverage-drinking habits) are recorded before sampling and considered as a potential confounder if necessary in future studies.

Some limitations of the present study should be carefully noted. First, the beverage intervention lasted for a short term of 3 consecutive days in this study. Since the association between proteolysis and diet habits was found [11], while some people might drink certain beverages for months or years, long-term investigations of the influence of beverage intake on oral microenvironment are still needed. Second, the long spatial distance between the kindergarten and our laboratory caused inevitable freezing and thawing of saliva samples, though the time for transport of samples had been shortened as far as possible. It is necessary to take into consideration this point to optimize the study procedures and diminish its impact in future studies. Third, among the three beverages adopted in our study, orange juice and yoghurt could have much complex composition. Hence, which component of these beverages might impact more on the salivary peptidome still needs further exploration in subsequent studies. Finally, the peptide-identifying methodologies and procedures would surely benefit from the

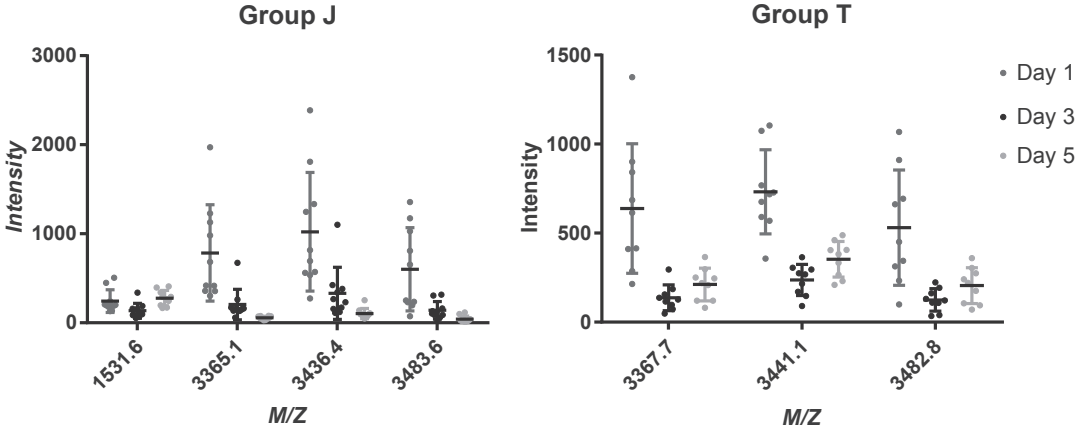

Fig. 4. Significantly changed peptides in Group J and T. ($P < 0.01$) Dot graphs present values of mean ± S.D.

Table 1
Identification of significantly changed peptides.

Group	m/z	Description	Species	Mr(expt)	Mr(calc)	ppm	Score	Expect	Rank	Unique	Sequence
J	1532	Histatin-3	Homo sapiens	1529.7706	1529.7348	23.4	17	1.7e + 002	1	U	F.HEKHHSHRGYRS.N
J	3365	Unknown	–	–	–						–
J	3436	Unknown	–	–	–						–
J	3484	Unknown	–	–	–						–
T	3368	Collagen alpha-1(IV) chain	Homo sapiens	3364.7166	3364.726	−2.90	1	1.1e + 004	4	U	D.KGLPGLDGIPGVKGEAGLPGTPGPTGPAGQKGEPGSD.G
T	3441	Zinc finger protein 805	Homo sapiens	3446.5296	3446.6922	−47.19	16	1.5e + 002	1	U	D.QDGFSEMQGERLRPGLDSQKEKLPGKMSPK.H
T	3483	Quinolinate synthase A	Dehalococcoides mccartyi	3485.8168	3485.699	33.7	2	2.7e + 004	8	U	A.RYPGLPVVCYVNSTAEVKAESDICCTSANAVKV.V

expansion and extension of databases and the development of relevant techniques.

5. Conclusions

In conclusion, salivary peptidome has its own stability in response to beverage consumption, whereas short-term intervention by two representative beverages (orange juice and tea) could change the profile of salivary peptidome slightly. This confirms the feasibility and validity of taking account of the salivary peptidome profile as a potential reference for the healthy state of the body with diet habits recorded and considered as a confounder if necessary, providing a fundamental basis for future studies to discover biomarkers in saliva.

CRediT authorship contribution statement

Fangqiao Wei: Investigation, Data curation, Visualization, Writing - original draft. **Xiangyu Sun:** Writing - review & editing. **Peiyuan Tong:** Resources. **Yufeng Gao:** Resources. **Ce Zhu:** Visualization. **Feng Chen:** Project administration. **Shuguo Zheng:** Conceptualization, Methodology.

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Declaration of interests.

The authors declare no competing financial interests.

Appendix A. Supplementary material

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