

# Magnetic Bead-Based Salivary Peptidome Profiling Analysis for Severe Early Childhood Caries

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## Key Words

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry · Peptidome · Saliva · Severe early childhood caries

## Abstract

**Objective:** To investigate the differential salivary protein expression profiles between children with severe early childhood caries (S-ECC) and caries-free (CF) children at the age of 3 years. **Methods:** We used matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) combined with weak cation exchange magnetic beads, and peptide mass fingerprints were created by scanning mass spectrometry signals. Salivary samples from 20 children were analyzed (10 for each group). **Results:** Eleven protein peaks were significantly different ( $p < 0.05$ ) between the two groups. Eight of these peaks were higher in the S-ECC group and three were higher in the CF group. To establish a diagnostic model for discrimination between the two groups, we chose three peptides (3,186.2, 3,195.8 and 3,324.8 Da) that exhibited the best fitted curve, by which the two groups were better separated when compared with other combinations. **Conclusions:** The salivary biomarkers identified revealed significant differ-

ences between the CF and the S-ECC group. Our results provide novel insight into the salivary protein profile of preschool-age children with dental caries and may lead to the development of a new strategy for screening high-risk populations.

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Dental caries is a chronic infectious disease affecting 60–90% of schoolchildren and remains a major oral health problem in most industrialized countries [Bian, 2006]. A susceptible host, cariogenic bacteria and cariogenic diets together contribute to the occurrence of dental caries. Severe early childhood caries (S-ECC) is a particular type of dental caries indicated by (a) any sign of smooth-surface caries in children younger than 3 years of age, (b) one or more decayed, missing (because of caries) or filled smooth surfaces in primary maxillary anterior teeth in those aged 3–5 years, or (c) a decayed, missing or filled surface (dmfs) score of  $\geq 4$  at age 3,  $\geq 5$  at age 4 or  $\geq 6$  at age 5 [Drury et al., 1999]. Primary teeth are especially vulnerable to the disease and can become infected by cariogenic bacteria immediately after erupting in the oral cavity during the first year of life. The prevalence of caries increases gradually

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with age, becoming greater in 3-year-olds and reaching a peak in 6- to 8-year-olds. According to the third national oral health survey, the caries prevalence rate is 66% among 5-year-olds in China [Qi, 2008]. Early diagnosis and early prophylaxis are important for disease control.

Of the identified host factors, saliva has received particular attention. Human whole saliva is a complex biological fluid that originates primarily from three major salivary glands: the parotid, submandibular and sublingual glands. Salivary constituents [Dusa Vukosavljevic and Siqueira, 2011], flow rate and buffering capacity have been correlated with caries risk. Human saliva, which contains approximately 2,290 proteins [Loo et al., 2010], protects the integrity of oral tissues and can provide diagnostic clues concerning local and systemic diseases and conditions [Llena-Puy, 2006; Dusa Vukosavljevic and Siqueira, 2011]. In recent years, with the development of mass spectrometry (MS), salivary proteins have been applied to the monitoring of general health and to the early diagnosis of disease [Hu et al., 2007].

To date, most studies examining the correlation between dental caries and salivary proteins/peptides have focused on only one or a few proteins/peptides, and studies of the proteome/peptidome of saliva have identified and isolated several salivary proteins/peptides and determined their specific roles in the oral cavity [Vitorino et al., 2005, 2006; Preza et al., 2009; Zehetbauer et al., 2009; Hart et al., 2011]. Protein profiling methods such as two-dimensional gel electrophoresis, high-performance liquid chromatography and MS have been applied to characterize differences in the protein composition of saliva between patients with and without dental caries [Al-Tarawneh et al., 2011]. Nevertheless, comparisons among these studies are difficult owing to differences in samples and disease severity, nonstandard sample collection and handling processes, insufficient numbers of samples or differences in technologies and analytical methods. Thus, the relationship between dental caries and human salivary proteins is not well defined, and biomarker information remains unclear. Moreover, no studies have addressed saliva proteome differences in early childhood dental caries [Al-Tarawneh et al., 2011; Martins et al., 2012]. To understand the possible relationship between salivary peptides and dental caries, further investigation of specific proteins is essential.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), a sensitive MS-based proteomic technique, has been used to detect peptides over a large mass range. The mass spectra generated by this technique are easy to interpret [Al-Tarawneh

et al., 2011], and this method has been used to analyze proteins in many diseases and disorders, including Sjögren's syndrome [Ryu et al., 2006] and breast cancer [Streckfus et al., 2006].

In the present study, MALDI-TOF MS was used to analyze salivary peptides in the range of 1,000–10,000 Da, which were selected with weak cation exchange (WCX) magnetic beads. The effectiveness of this combination of techniques has been confirmed in many serum/saliva-based peptide profile identification studies, including those in nasopharyngeal carcinoma [Tao et al., 2012] and gastric cancer [Wu et al., 2009]. We compared the peptide mass profiles of saliva samples from 3-year-old children with and without S-ECC to determine whether we could evaluate the risk of a child developing caries by the detection of salivary profiles.

## Subjects and Methods

### *Ethics Statement*

This study was approved by the Peking University Biomedical Ethics Committee. The parents of the pediatric subjects signed an informed consent form before the start of the study.

### *Subjects*

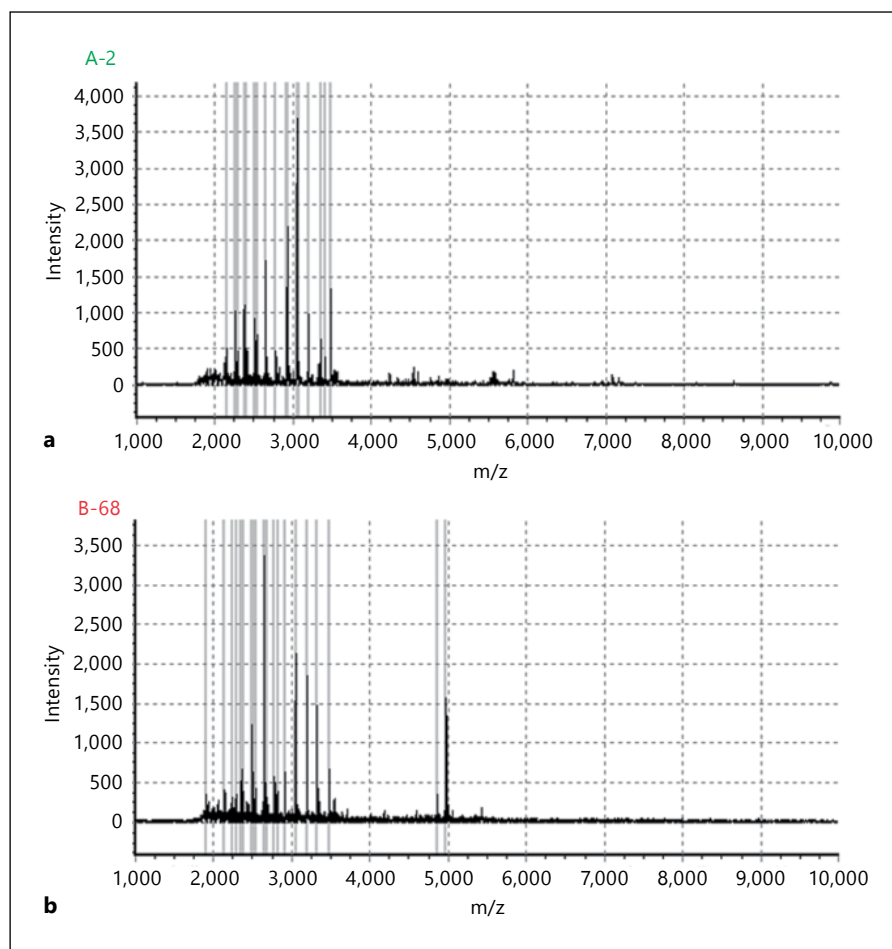
A cohort of 3-year-old children who had just entered Wangjing Xincheng kindergarten in Chaoyang District (Beijing, China) was recruited in September 2012. All study subjects were systemically healthy. Those with influenza or an upper respiratory tract infection and those who had received antibiotic therapy within 1 month or fluoride prophylaxis within 1 year of the study were excluded. The experimental group comprised 10 children who had been diagnosed with S-ECC (S-ECC group; dmfs score in primary maxillary anterior teeth  $\geq 4$ ). Ten age- and sex-matched children who were completely free of carious lesions served as the caries-free control group (CF group). A detailed caries status was determined for all 20 children. The sex distribution in the S-ECC group was 3:7 (male:female) compared to 4:6 in the CF group.

### *Saliva Collection and Processing*

All individuals were instructed to rinse orally with water after breakfast and then rest for 10 min before saliva collection at 8:30 a.m. Stimulated whole saliva samples were collected for 5 min. The 1.5-ml stimulated whole saliva samples were immediately placed on ice. Insoluble material, cells and debris were removed by centrifugation at 10,000 g for 10 min at 4°C. The supernatants were collected, and 1 mM ethylene diamine tetraacetic acid (Sigma, St. Louis, Mo., USA) and 1 mM phenylmethyl sulfonyl fluoride (Sigma) were added to inhibit protease activity. Protein concentrations were measured by the Lowry method and the ELx808 Protein Assay (BioTek, Hercules, Calif., USA). Supernatants were kept at -80°C until further analysis.

### *Reagents and Instruments*

The WCX magnetic bead kit (SPE-C) was from Bioyong Tech (Beijing, China). Alpha-cyano-4-hydroxycinnamic acid (CHCA) was freshly dissolved in 100% ethanol (chromatographic grade)



**Fig. 1.** Complete mass spectra of proteins in the range of 1,000–7,000 Da show the peptide fingerprints of saliva samples from representative patients without caries (**a**) and with caries (**b**).

and 100% acetone (chromatographic grade) to prepare the sample matrix for MALDI-TOF MS (Bruker Bio-Sciences, Bremen, Germany).

#### WCX Fractionation and MALDI-TOF MS

The suspension provided in the WCX magnetic bead kit was mixed with the saliva samples by shaking. The bound peptides were eluted from the magnetic beads and transferred to a 0.5-ml sample tube. Five microliters of CHCA substrate solution (0.4 g/l, dissolved in acetone and ethanol) and 0.8–1.2  $\mu$ l of eluted peptides was mixed, and 0.8–1.2  $\mu$ l of this mixture were applied to a metal target plate and dried at room temperature. The prepared samples were analyzed by MALDI-TOF MS. Peptides with molecular weights ranging from 1,000 to 10,000 Da were detected, and 400 shots of laser energy were used. To obtain peptide mass fingerprints, 50 individual MS signal scans were accumulated.

#### Statistical Analysis

An analysis of variance was used to identify differences in protein levels among saliva samples from the two groups. The *t* and *w* tests were used for comparisons among groups. The data were analyzed using the BioExplorer statistical package (Bioyong Tech). *p* values <0.05 were considered to indicate significance.

## Results

To investigate the differences between the S-ECC and CF saliva samples, the entire mass spectra of the peptides from the extracted saliva samples from 20 subjects (*n* = 10 per group) were obtained by MALDI-TOF MS (fig. 1). Saliva peptidome fingerprint peaks from each patient were characterized based on the maximum intensity within a particular mass-to-charge ratio (*m/z*) range. The molecular weights of the majority of the peptides were 1,000–7,000 Da. The mass peaks were quantified and compared.

An average of 91 protein mass peaks was detected when the two groups were compared. The peak intensities differed significantly for 11 peptides (2,004.8, 2,014.0, 2,255.0, 2,609.1, 2,926.2, 3,186.2, 3,195.8, 3,324.8, 5,554.7, 5,570.6 and 5,812.6 Da) (table 1). The S-ECC group showed higher mass peaks for peptides of 2,255.0, 5,554.7, 5,570.6, 2,926.2, 2,004.8, 5,812.6, 2,609.1 and 2,014.0 Da,

**Table 1.** Significant ( $p < 0.05$ )  $m/z$  values discriminating samples from the two groups, and the sequences of the differentially expressed peptides

Mean $m/z$ value	p value	Tendency <sup>1</sup>	Peptide sequence	Potential proteins
3,186.2	0.015	↓	R.EYVNWTPADPVYVQYYNLSYTSKLGSK.D	uncharacterized protein AF_2048 ( <i>A. fulgidus</i> )
3,195.8	0.033	↓	K.SARLLAFLALIMIGSVFAYMLSGGSAEHR.E	
3,324.8	0.025	↓	R.GISQVMIVDFGETVPLYFVDAGMSKIYFAK.E	
2,255.0	0.006	↑	K.EAALTSEEVGADLEQVEVLQK.K	spectrin alpha chain (SPTAN1)
2,926.2	0.027	↑	Q.GLLPVLESFKVSFLSALEEYTKKLT.Q	apolipoprotein A-I precursor (apoA1)
2,004.8	0.042	↑	unknown	unknown
2,014.0	0.049	↑	unknown	unknown
2,609.1	0.043	↑	unknown	unknown
5,554.7	0.014	↑	unknown	unknown
5,570.6	0.020	↑	unknown	unknown
5,812.6	0.042	↑	unknown	unknown

<sup>1</sup> Tendency refers to the trend in the intensity of  $m/z$  values between the two groups. ↑ = Higher intensity in the group with caries than in the group without caries; ↓ = lower intensity in the group with caries than in the group without caries.

whereas the mass peaks for the peptides of 3,186.2, 3,324.8 and 3,195.8 Da were higher in the CF group (fig. 2, 3).

Compared with the other peptides, the 3,186.2, 3,195.8 and 3,324.8 Da peptides tended to be downregulated in the S-ECC group and formed a nearly straight line, indicating that they might have originated from the same potential protein. Thus, we chose the peaks described above as candidate biomarkers. Based on a search using Mascot ([http://www.matrixscience.com/cgi/protein\\_view.pl?file=..%2Fdata%2F20130130%2FFtEcIfSam.dat&hit=1](http://www.matrixscience.com/cgi/protein_view.pl?file=..%2Fdata%2F20130130%2FFtEcIfSam.dat&hit=1)), these three peptides were predicted to be the uncharacterized protein AF\_2048, which originates from *Archaeoglobus fulgidus*. Additionally, the 2,255.0 and 2,926.2 Da peptides were predicted to be spectrin alpha chain (SPTAN1) and apolipoprotein A-I precursor (apoA1), respectively.

We selected the 3,186.2, 3,195.8 and 3,324.8 Da peptides to establish a diagnostic model of S-ECC (fig. 4). Samples from the two groups were well separated based on these three peptides, indicating that the fitted results were satisfactory. However, four CF samples (green symbols in fig. 4) were found among the S-ECC samples; this implies that these children may be at high risk for caries.

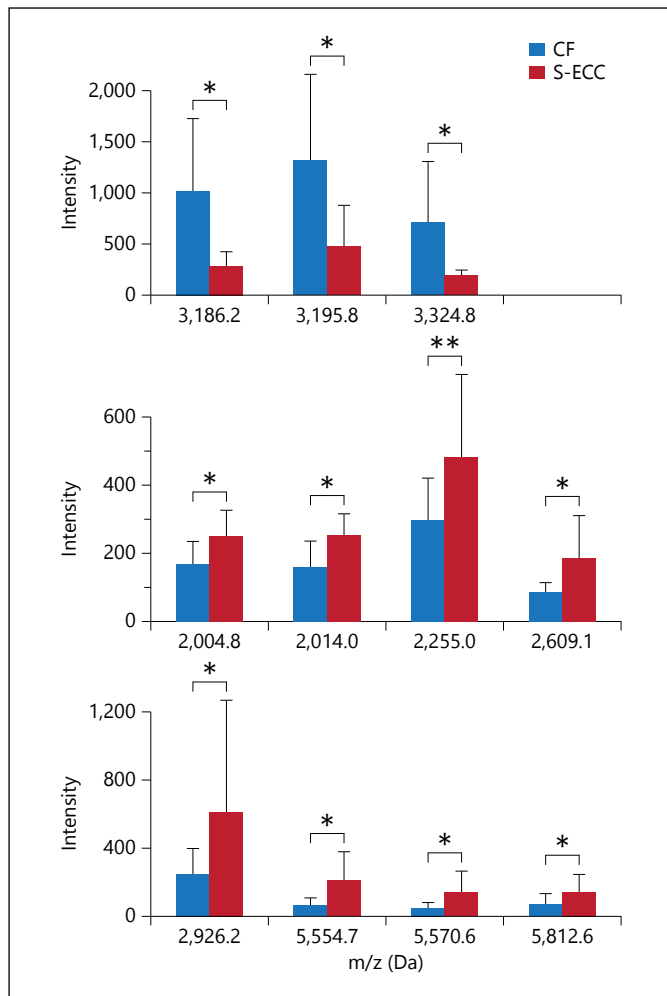
## Discussion

Primary caries can lead to mastication problems and thus affect nutrition, growth and mental well-being, in addition to causing dysplasia of future permanent teeth.

Thus, early diagnosis and prophylaxis are vital to control caries formation. Currently, dental caries is diagnosed mainly by clinical examination, usually after irreversible damage to the tooth has begun. The present study highlights a new tool for diagnosing dental caries and identifying susceptible subjects before demineralization of the tooth structure begins.

The components of saliva can provide a large range of health information, and saliva collection is non-invasive, simple and inexpensive. Thus, interest in the use of saliva for the detection of oral and systemic conditions has increased. Proteomics/peptidomics and related techniques have advanced significantly over the last two decades, and a saliva-based proteomic approach enables the exploration of diagnostic biomarkers.

To examine a possible correlation between salivary peptide composition and dental caries, Vitorino et al. [2005] analyzed saliva samples from CF and caries-susceptible (CS) groups and reported a strong correlation between large amounts of phosphopeptides (PRP1/3, histatin 1 and statherin) and the absence of dental caries. This emphasizes the importance of these peptides in the maintenance of tooth integrity. A subsequent study by Vitorino et al. [2006] used 2-DE combined with MALDI-TOF MS to evaluate differential protein expression patterns in the whole saliva of 16 CF and 16 CS subjects aged 18–29 years. The study found significantly higher quantities of acidic proline-rich proteins, lipocalin, cystatin SN and cystatin S in samples from CF subjects, whereas the levels of amylase,



**Fig. 2.** Column view of mass spectra from both groups. It showed higher peak intensities at peptides of 2,004.8, 2,014.0, 2,255.0, 2,609.1, 2,926.2, 5,554.7, 5,570.6 and 5,812.6 Da, and lower intensities at peptides of 3,186.2, 3,195.8 and 3,324.8 Da for the S-ECC group compared with the CF group. \*  $p < 0.05$ ; \*\*  $p < 0.01$ .

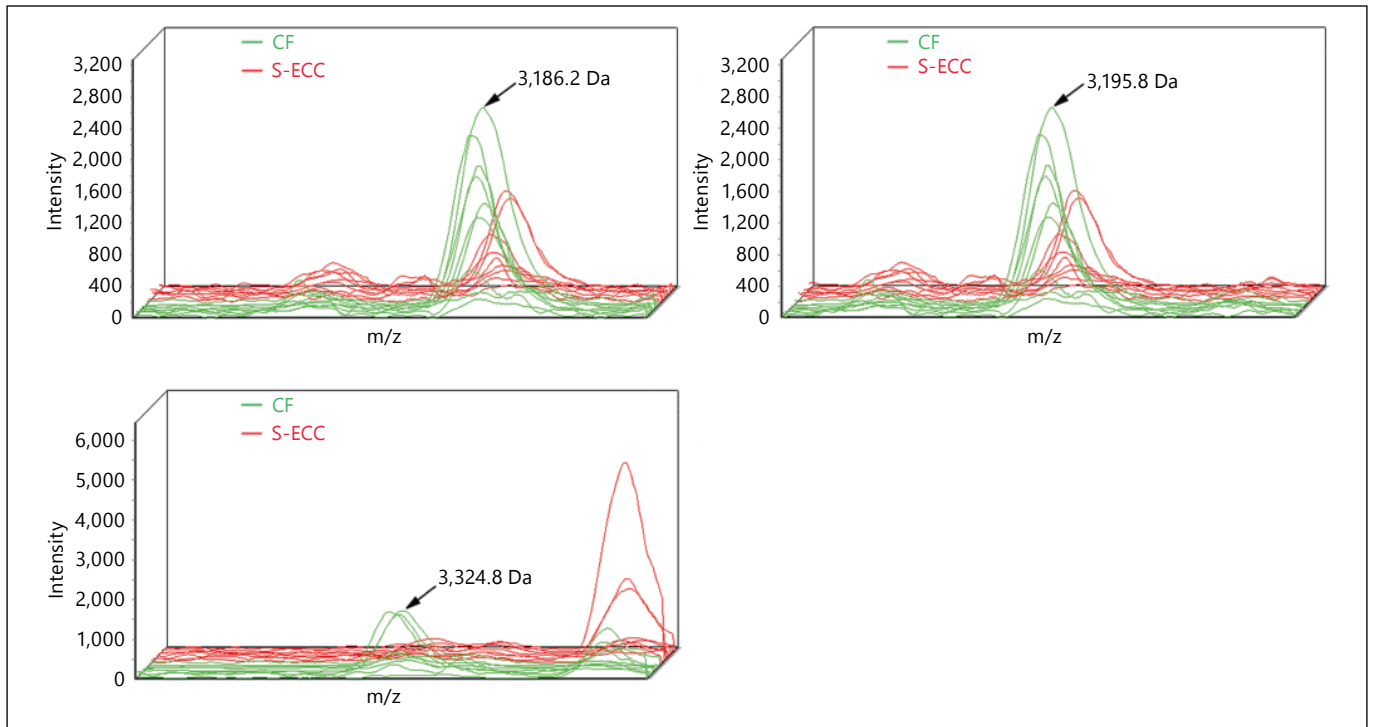
immunoglobulin A and lactoferrin appeared to be higher in samples from subjects with a high DMFT index. These phosphopeptides, including acidic proline-rich proteins, histatin, statherin and cystatin, which were shown to maintain calcium saturation around teeth, may promote the process of remineralization and inhibit demineralization [Schenkels et al., 1995; Van Nieuw Amerongen et al., 2004]. Hart et al. [2011] employed high-throughput methodologies, including multiplexed microbial arrays and surface-enhanced laser desorption/ionization time-of-flight MS profiling, to characterize the oral flora and salivary proteomes of 204 children aged 1–8 years in Brazil; their study demonstrated that the combination of proteomic and mi-

crobial profiling was beneficial for classification accuracy and that the combined data improved predictive models for CS and CF individuals. The best predictive model had a 6% test error, >92% sensitivity and >95% specificity. However, the association between dental caries and human salivary proteins is unclear, and biomarkers have not yet been identified by previous studies.

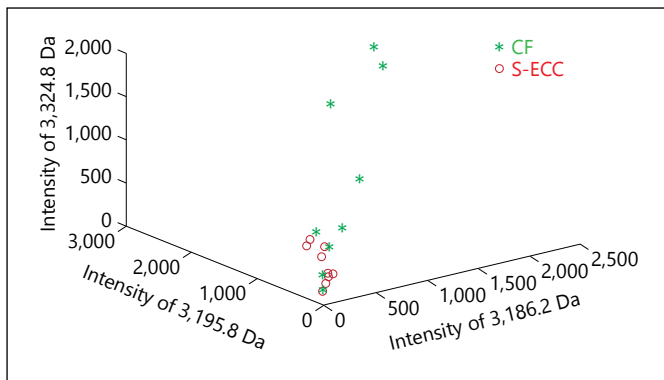
In the present study, we used a MALDI-TOF MS-based proteomic method to analyze peptides selected with WCX magnetic beads from saliva samples of 10 children with S-ECC and 10 CF children. Eleven peak intensities (table 1) differed significantly between the S-ECC and CF groups, indicating that our method can be used to analyze the peptide profiles of children with and without dental caries. Peptides of 3,186.2, 3,195.8 and 3,324.8 Da were lower in the S-ECC group and formed nearly a straight line in the fitted curve analysis (fig. 4), suggesting that they may originate from the same protein. Samples from the two groups were well separated based on these three peptides; therefore they were chosen as potential biomarkers. As mentioned above, some phosphopeptides were lower in the CS group in previous studies [Vitorino et al., 2005, 2006], suggesting that they play a role in protecting tooth integrity. In this case, the three peptides might come from the uncharacterized protein, AF\_2048, which we assumed acts as a positive factor in oral health. Four samples fell among the S-ECC samples, indicating that these individuals may be at risk for caries.

A Mascot search matched the 3,186.2, 3,324.8 and 3,195.8 Da peptides to the uncharacterized protein AF\_2048 from *A. fulgidus*, which belongs to the Archaea. Archaea are prokaryotic microorganisms that were initially thought to comprise only extreme thermophiles (growing at 80°C or above), extreme halophiles (growing in 2 M to saturated NaCl) and strictly anaerobic methanogens. However, they have since been found in most ecosystems [Cavicchioli et al., 2003]. Hulcr et al. [2012] analyzed bacteria and Archaea from the belly buttons of humans and found three phylotypes of Archaea that had not been previously reported in human skin. It is not yet clear whether these prokaryotes are beneficial or pathogenic to human life. As these three peptides were determined to be highly expressed in the CF group in the present study, we boldly presumed that a group of supragingival Archaea, from which the three peptides might have originated, might be responsible for oral health benefits.

However, it must be kept in mind that a peptide sequence usually does not exclusively define a single protein. Ultimately, the aim of our investigation was to determine the protein or gene from which the peptides were



**Fig. 3.** Three-dimensional  $m/z$  ratio intensity maps show significantly different intensities for proteins at 3,186.2, 3,195.8 and 3,324.8 Da.



**Fig. 4.** Scatter plots of the two groups were generated by combining the 3,186.2, 3,195.8 and 3,324.8 Da proteins. The scatter plots show a well-fitted curve.

derived, and this is not an easy task. Several peptide constituents showed marked differences between the two groups, suggesting that they can serve as biomarkers to effectively diagnose dental caries. The early determination of a high risk for caries would enable preventive measures designed to stop infection at the very beginning of a lesion. Unfortunately, identifying all of the peaks in the present

study was quite challenging. Some of the peptides might have been secreted by oral microbes whose polypeptide fingerprint spectra have not been well established.

There are some limitations to this technique. First, the sample spots used in MALDI-TOF MS often suffer from heterogeneity due to a different matrix, which can lead to high variability in signal intensities and coefficients of variation that make quantitative analysis by this ionization technique difficult. Second, laser power is not fixed from time to time, making the acquired data less consistent and accurate. Moreover, to avoid interference by macromolecular proteins, we adopted WCX magnetic beads to select peptides in the range of 1,000–10,000 Da, which might miss some important biomarkers of larger molecular weight. Lastly, it is difficult to carry out secondary MS. Therefore, with a small sample size, the specificity of our data was not completely satisfactory.

In subsequent work, we intend to apply the potential biomarkers identified here to a larger population in order to establish a concise diagnostic model for dental caries. The analysis of saliva is inherently difficult because it contains a large number of proteins that are present in a wide concentration range and may be post-translationally modified. Nevertheless, it is likely that applications for

saliva analysis will continue to expand, providing new and promising tools for investigating physiological and pathophysiological states.

## Conclusions

Our study demonstrated salivary peptide profile differences between S-ECC and healthy children, as determined by the analysis of magnetic bead-selected peptides using MALDI-TOF MS. This method is suitable for preliminary biomarker discovery studies and provides a new tool for revealing significant differences between CF and S-ECC salivary profiles, and may ultimately help identify high-risk individuals so that measures can be taken to stop infections and prevent lesions. Expanding the dataset of carious patients and identifying more biomarkers will help to establish a concise model for the early diagnosis of dental caries.

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## Acknowledgements

The authors gratefully acknowledge the assistance of Yan Li of Bioyong Tech for her expertise in MS. The study was partly supported by the Foundation of National Health and Family Planning Commission of the PRC (No. 201002017). The funders had no role in the study design, data collection and analysis, decision to publish or preparation of the manuscript.

## Author Contributions

S. Zheng and F. Chen conceived the study and experimental design; Y. Si, S. Zheng and S. Ao performed the clinical examination; F. Chen and S. Ao performed the experiments; W. Wang and S. Ao analyzed the data; Y. Si and S. Ao wrote the paper.

## Disclosure Statement

The authors declare no conflicts of interest.

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