CHAPTER

6

Effect of experimental gingivitis induction and erythritol on the salivary MALDI-TOF MS peptide profiles of systemically healthy young adults

Andrei Prodan, Sultan Imangaliyev, Henk S. Brand,
Evgeni Levin, Martijn N.A. Rosema, Ad de Jong,
Armand Paauw, Wim Crielaard, Bart J.F. Keijser,
and Enno C.I. Veerman
Chapter 6

Abstract

Introduction Understanding the changes occurring in the oral ecosystem during development of gingivitis could help to improve prevention and treatment strategies for oral health. Erythritol is a non-caloric polyol proposed to have beneficial effects on oral health.

Objectives To examine the effect of experimental gingivitis induction and the effect of erythritol on the salivary peptide profiles of healthy adults.

Methods In a two-week experimental gingivitis challenge intervention study, MALDI-TOF-MS profiling was performed on saliva samples from 61 healthy adults, collected at seven time-points. The effect of erythritol was studied in a randomized, controlled trial setting. Effects were analyzed using mixed-design ANOVA models.

Results 83 salivary peptide peaks in the mass range 2 – 15 kDa were quantified. There were no significant effects of the experimental gingivitis or of erythritol on any of the peaks analyzed (all effect $p$-values > 0.05, before adjusting for multiple comparisons).

Conclusions No significant effects of experimental gingivitis induction and/or of a daily dose of erythritol on the MALDI-TOF salivary peptide profiles of healthy adults were observed.
Introduction

Oral health is integral part of the general health [189]. Gingivitis is an oral health condition, a local inflammatory response caused by an oral bacterial biofilm [190]. Gingivitis is regarded as a transitional stage between oral health and oral disease, as it does not affect the supporting structures of the teeth and is generally reversible with improved oral hygiene [191]. Despite this, gingivitis is a potential risk factor in the development of periodontitis, one of the major dental diseases [190, 192]. Biomarkers that can give information on the state of the oral cavity with regard to gingivitis could be useful tools for understanding the changes occurring in the oral ecosystem during gingivitis development, potentially leading to improved oral health prevention strategies. Saliva is a biological fluid that can be collected easily and non-invasively and has been explored as a source of biomarkers for oral as well as systemic diseases [193, 194].

Erythritol is a non-caloric polyol used as a sweetener. Its safety has been well documented: it is classified as GRAS (Generally Recognized As Safe) by the U.S. Food and Drug Administration and is considered safe for use as a food additive (E968) by the European Food Safety Authority [144]. Erythritol is non-cariogenic and has been shown to lower plaque levels and caries rates in a prospective clinical trial [145, 146]. The positive effect of erythritol with regard to caries may be due it potential stabilizing effect on the oral microflora: in-vitro studies have found that erythritol inhibits the growth of Streptococcus mutans strains [148]. Erythritol may therefore have a protective effect on the oral homeostasis.

A previous study examining variation in salivary MALDI-TOF peptide profiles found that individuals could be clustered based on their peptide profiles into four subgroups [195]. Salivary P-C peptide and its fragmentation pattern had an important role in the clustering. Individuals from the four subgroups showed significant differences in their underlying salivary biochemistry with regard to the activity of lysozyme and chitinase, two enzymes relevant for oral health which are involved in the salivary innate defense system [195]. Therefore, it is possible that changes in salivary peptide profiles during an induced shift in the oral ecosystem could yield information on the underlying mechanisms contributing to the stability and resilience of that system, and thus ultimately to oral health.

The aim of this study was to examine the changes in the salivary peptide profiles of young healthy adults acquired using MALDI-TOF MS during a two-week experimental gingivitis challenge intervention, and to assess the effect of a daily intake of erythritol on these changes during a controlled, randomized clinical study.
Materials and methods

Study design

This study was part of the TI Food and Nutrition project ‘An exploratory study on the dynamic (microbial, biochemical and immunological) interactions of the oral ecosystem during induction of mild gingival inflammation. Dynamics of a healthy oral ecosystem’.

Whole unstimulated saliva was collected in a single-center, challenge intervention, randomized study at the Academic Centre for Dentistry Amsterdam (ACTA). The study population was a convenience sample of systemically healthy adults. The study design and inclusion/exclusion criteria have been previously described in detail [196]. In brief, the challenge intervention was based on a full-mouth modified experimental gingivitis protocol [149]. The first stage was a two-week baseline period in which the treatment group started the use of erythritol (Day -14 to Day 0). All participants were then requested to refrain from any form of oral hygiene for two weeks (Day 1 to Day 14) resulting in plaque accumulation and induction of (mild) inflammation. The two weeks of plaque accumulation were followed by a one week resolution phase. At the onset of the resolution phase all participants were provided with a standardized manual toothbrush, fluoride toothpaste, and specific instructions to re-initiate tooth brushing.

One group of participants (the treatment / erythritol group) was requested to take six doses of erythritol per day during the entire duration of the study (five weeks), amounting of 12.0 g/day of erythritol. The erythritol group (n = 20) and the control group (n = 41) were balanced with regard to age and sex.

Unstimulated saliva samples were collected from all participants at seven time-points across the challenge intervention period: at Day -14, Day 0, Day 2, Day 5, Day 9, Day 14 and Day 21.

The study was conducted in accordance with the Declaration of Helsinki (2013) of the World Medical Association and approximating Good Clinical Practice guidelines [151]. The study protocol was reviewed and approved by the Medical Ethics Committee of the Free University of Amsterdam Medical Centre (2014_505) and was registered at the Dutch Trial Register (NL51111.029.14). All participants signed an informed consent form.

Sample collection and MALDI-TOF-MS analysis

Unstimulated saliva samples were collected and processed as previously described [100]. In brief, participants were instructed to spit for 5 min at 30 s intervals into ice-chilled 30-ml polypropylene tubes (Sterilin, Newport, U.K.). After vortexing, samples were clarified by centrifugation for 10 min at 4°C and 10,000 g. The resulting clarified saliva was diluted with a NaCl solution to a final concentration of 250 mM NaCl and stored at -80°C.
Samples were desalted prior to MALDI-TOF MS analysis using C18 ZipTips (Merck Millipore, Darmstadt, Germany). The purified peptides were spotted directly onto a MALDI target plate with 1 µL of matrix solution (10 mg α-cyano-4-hydroxy cinnamic acid in 1 ml of ACN/water 1:1 (v/v) with 2.5% trifluoroacetic acid). Spectra were recorded in linear mode at a mass range of 2 – 15 kDa with a 200 Hz laser at 355 nm on an Autoflex III MALDI-TOF mass spectrometer (Bruker, Bremen, Germany). Details of the MALDI-TOF protocol have been previously described [195]. Samples were analyzed in duplicate. Visual quality control was performed for all raw spectra. Samples were re-analyzed if either one of the duplicate spectra showed excessive noise or large baseline drift, or if the duplicate spectra exhibited dissimilarities. Spectra of a reference saliva sample were acquired in quadruplicate at each session in order to aid the subsequent sample spectral alignment and to assess the reproducibility of the assay. The mean coefficient of variation of the assay was 18%.

Raw MALDI-TOF MS spectra were processed in Matlab R2012b using the Mathworks bioinformatics tool box (MathWorks, Natick, MA, U.S.A). The workflow consisted of spectra resampling followed by baseline subtraction, smoothing, peak detection and finally peak binning (peak coalescing) using a hierarchical clustering algorithm.

**Statistical analysis**

The effects of the challenge intervention, of the erythritol treatment, as well as potential interaction effects between the two were examined with mixed-design ANOVA models implemented in Scientific Python using the “pyvttbl” package, available for download at https://code.google.com/archive/p/pyvttbl/ [154]. Peaks were excluded from the analysis if they were present in <2% of the total number of samples. P-values were calculated for the within-subject effect (the challenge intervention), the between-subject effect (erythritol), and the interaction between the main effects, respectively. A Huyhn-Feldt correction was used to compensate for any deviation from sphericity in the data [155]. The significance level (alpha) was 0.05. The Benjamini-Hochberg False Discovery Rate (FDR) procedure was used to correct for multiple comparisons [73]. The FDR was set at 0.05.
Chapter 6

Results

Of the 63 enrolled volunteers, 61 participants completed the entire study and were included in the final analysis. Sample volumes from one participant from the control group were insufficient. Therefore, MALDI-TOF MS data from a total number of 60 participants were included in the final analysis (40 from the control group; 20 from the erythritol group). The age of the participants was 25.2 ± 6.2 (mean ± SD). A number of 83 MALDI-TOF MS peaks were present in more than 2% of samples (i.e. in more than 8 out of 420 samples) and were therefore included in the mixed-design ANOVA analysis. The complete dataset is provided as Supplementary Information 1.

There was a large overlap between the peaks found in this study and the peaks found in the saliva of young healthy adults in a previous, cross-sectional study, using an identical MALDI-TOF protocol [195]. The cross-sectional study found 80 peaks, 69 of which were also found in the present study (83% overlap). The frequency of the 69 overlapping peaks (the proportion of samples in which the respective peaks were found) in the two studies were similar (Supplementary Information 2).

Most of the 14 peaks that did not overlap (i.e. 12 out of 14) had very low frequencies, being present in <5% of samples. The degree of concordance between the peaks detected in the two studies of salivary MALDI-TOF is illustrated in Fig. 1.

Figure 1. Venn diagram illustrating the overlap (yellow) between the peaks found in a previous cross-sectional study (red circle on the left, 80 peaks) and the present study (green circle on the right, 83 peaks).
The mixed-design ANOVA analysis found no significant effects of either the experimental gingivitis challenge intervention or of erythritol on any of the 83 peaks included in the analysis (all $p$-values $> 0.05$, before adjusting for multiple comparisons). The detailed results of the mixed-design ANOVA are provided as Supplementary Information 3.
Discussion

The present study found no significant changes in the salivary peptide profiles of healthy adults during the two weeks period of gingivitis induction or after the one week resolution phase. Also, there was no significant effect of erythritol on the salivary peptide profiles. There was good agreement between the list of peaks detected in the present study and the peaks detected using an identical analytical protocol in a previous cross-sectional study of salivary peptide profiles, performed on a different study population (Fig. 1). The majority of none-overlapping peaks (found in one study, but not the other) were present at low levels, close to the detection limit of the assay, in <5% of the samples analyzed in the respective studies. The present assay therefore appears to be able to consistently detect and measure approximately 70 salivary peptide peaks.

While a previous prospective clinical trial found significant decreases in dental plaque and in dental caries rates, the duration of erythritol intake in that study was three years, much longer than the total of five weeks in the present study [145, 146]. Salivary erythritol concentrations during the present study indicate good levels of compliance with regard to erythritol intake. Mean salivary concentrations in the erythritol group during the experimental gingivitis induction phase (between Day 0 and Day 14) were approximately two orders of magnitude higher than in the control group [196]. However, it may be that any potential effect of erythritol on the oral ecosystem requires a much longer exposure duration than the one used in this study. Moreover, the design of the present study was aimed at observing changes during the induction of gingivitis, not caries progression as in the case of the aforementioned prospective clinical trial.

There could be several reasons for the non-significant effect of the experimental gingivitis induction on the salivary peptide profiles. Firstly, it may be that none of the peptides quantified was influenced by the induction or by the resolution of the 2-week experimental gingivitis challenge. One of the limitations of MALDI-TOF MS is the limited mass range at which it is effective (in this study, between 2 – 15 kDa), therefore giving only a partial coverage of the collection of peptides present in saliva. However, an antibody- and enzymatic activity-based analysis of the same samples set looking at eight larger salivary proteins with known functional relevance for oral health also found no significant changes during the induction of experimental gingivitis [196]. Secondly, it may be that the effect, if it did in fact occur, was too small to detect with the present study sample size (N = 61).

In conclusion, this study found no significant effect of experimental gingivitis induction or of erythritol on the MALDI-TOF salivary peptide profiles of healthy adults.
References


