CHAPTER

5

Effect of experimental gingivitis induction and erythritol on the salivary metabolome and functional biochemistry of systemically healthy young adults

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Chapter 5

Abstract

Introduction Understanding the changes occurring in the oral ecosystem during development of gingivitis could help 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 and the effect of erythritol on the salivary metabolome and salivary functional biochemistry.

Methods In a two-week experimental gingivitis challenge intervention study, non-targeted, mass spectrometry-based metabolomic profiling was performed on saliva samples from 61 healthy adults, collected at five time-points. The effect of erythritol was studied in a randomized, controlled trial setting. Fourteen salivary biochemistry variables were measured with antibody- or enzymatic activity-based assays.

Results Bacterial amino acid catabolites (cadaverine, N-acetylcadaverine, and α-hydroxyisovalerate) and end-products of bacterial alkali-producing pathways (N-α-acetylmorhithine and γ-aminobutyrate) increased significantly during the experimental gingivitis. Significant changes were found in a set of 13 salivary metabolite ratios composed of host cell membrane lipids involved in cell signaling, host responses to bacteria, and defense against free radicals. An increase in mevalonate was also observed. There were no significant effects of erythritol. No significant changes were found in functional salivary biochemistry.

Conclusions The findings underline a dynamic interaction between the host and the oral microbial biofilm during an experimental induction of gingivitis.
Introduction

Oral health is of paramount importance for functions like chewing, tasting, speech, and social interaction [1]. Oral diseases such as caries, gingivitis, and periodontitis have high prevalence rates, reduce quality of life, and can have a profound negative effect on general health [2, 3]. The oral cavity is a specialized ecological niche with thousands of microbial species, and the site of complex interactions between oral microbiota, salivary components, and host immune factors [4]. The commensal oral microflora has adapted to the human host during a long evolutionary relationship. Several studies have uncovered considerable cross-talk between oral microflora and host, mediated by collaborative cell sensing, immunomodulation, and discriminatory systems [5-7]. However, an inadequate level of oral hygiene, increased consumption of carbohydrates, and excessive host inflammatory responses - among other factors - can alter the composition and the functional profile of the oral microflora and shift this ecosystem away from homeostasis and towards a state of disease [4, 8]. Understanding these changes in the oral ecosystem is an essential step in the development of prevention strategies and improved treatments for oral health.

Metabolomics is the study the small molecules (<2000 Da) found in living cells or environments [9]. Metabolomics is complementary to transcriptomics and proteomics, with the advantage of being further ‘downstream’ of gene expression and thus more closely reflecting the activity of the biological system at a functional level [10].

Studies of the changes in metabolomic profiles caused by various life-style, pharmaceuticals, and diseases (e.g. diabetes, cancer) have offered valuable biological insights and uncovered biomarkers for diagnostic purposes [9, 11]. Metabolomics is also increasingly used in oral sciences. The salivary metabolite profile of healthy individuals has already been catalogued [12]. Several studies have found a periodontitis-specific metabolomic fingerprint in the saliva and gingival crevicular fluid of periodontitis patients, characterized by increased concentrations of degradation products of macromolecules and intensified host-bacterial interactions [13-15]. However, no mass-spectrometry-based studies of salivary metabolome changes occurring in healthy individuals during the development of gingivitis have yet been published. The changes in the salivary metabolome during different stages of oral plaque overgrowth and oral biofilm maturation, and during the corresponding reaction of the host defense systems to these challenges, could yield important clues to the biological mechanisms that maintain a healthy mouth.

Erythritol ((2R,3S)-butane-1,2,3,4-tetraol) 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 [16]. Erythritol is non-cariogenic, inhibits Streptococcus mutans strains in vitro, and lowers plaque levels,
caries rates, and caries progression in *in vivo* trials [17-20]. Erythritol may therefore exert a stabilizing effect on the oral homeostasis.

The primary aim of this study was to examine the changes in the salivary metabolome of young healthy adults during a two-week experimental gingivitis challenge intervention, and to explore the potential effect of a daily intake of erythritol on these changes during a controlled, randomized clinical study. Non-targeted, multi-platform, mass spectrometry (MS)-based metabolic profiling was performed on saliva samples from the study participants. The secondary aim was to look at potential changes in host salivary biochemistry. An array of targeted functional biochemistry analyses (antibody-based ELISAs and enzymatic activity assays) were used to measure salivary proteins with known relevance for oral health: mucin MUC5B, albumin, secretory-IgA, lactoferrin, amylase, lysozyme, chitinase, and proteases, alongside salivary pH, buffered pH, flow rate, and total protein content.
Materials and Methods

Study design, study population and inclusion/exclusion criteria

The study was carried out within the framework 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 CONSORT flow diagram of the study is shown in Fig. 1.

The challenge intervention was based on a full-mouth modified experimental gingivitis protocol [21]. 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 (the challenge intervention) 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. The duration of the resolution phase was one week (Day 14 to Day 21). The study design is illustrated in Fig. 2.

One group of participants was requested to take six doses of erythritol per day during the entire duration of the study (five weeks): two weeks prior, two weeks during, and one week after the challenge intervention (Day -14 through Day 21). The erythritol was administered orally, in the form of tablets containing approximately 90% erythritol and 10% maltodextrin, 0.5% magnesium stearate and 0.1% menthol. Each tablet contained 2.0 g of erythritol, amounting to a daily dose of 12.0 g. The No-Observed-Adverse-Effect Level (NOAEL) for laxative effects for erythritol is 0.71 g/kg/bw/day, much higher than the dose administered in this study [16]. Participants were instructed to take one tablet after breakfast, lunch, and dinner, and additionally during the mid-morning, mid-afternoon, and mid-evening. Participants allocated to the erythritol group were specifically instructed not to chew the tablets, but to keep them as long as possible in their mouth, for an optimal exposure of the oral cavity to erythritol. The planned treatment (erythritol) group / control group sample size ratio was 1.00 / 2.00.

Unstimulated saliva samples were collected from all participants at seven timepoints across the challenge intervention period: at Day-14, Day 0 (beginning of the challenge intervention), Day 2, Day 5, Day 9, Day 14 (end of the challenge intervention and beginning of the resolution phase), and Day 21 (end of the resolution phase). The team
collecting and analyzing the samples was blinded to the erythritol group or control group status of the participants or samples. Saliva samples were split into separate aliquots for metabolome analysis and for each targeted salivary biochemistry assay. Volunteers were included in the study if they were aged between 18 and 55 yr, systemically healthy (as assessed by a medical questionnaire), had a minimum of 20 natural teeth (with first and second molars present), and had visited a dentist for a regular check-up within the last year. The exclusion criteria were: a dental pocket probing depth of $\geq 5$ mm with bleeding on probing and attachment loss $\geq 2$ mm (Dutch Periodontal Screening Index score $3+/4$) [22], $>40\%$ bleeding on Probing (BOP), dental neglect (such as overt dental caries), smokers (more than one cigarette per day for a least one year), removable partial dentures or night guard, oral/peri-oral piercings, apparent oral lesions, abuse of drugs/alcohol, dental student or dental professional, pregnancy, use of antibiotics during the previous three months, prescribed medication (excepting contraceptives), and evidence of systemic disease.

The study was conducted in accordance with the Declaration of Helsinki (2013) of the World Medical Association and approximating Good Clinical Practice guidelines [23]. 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.

**Saliva sample collection and metabolomics**

Unstimulated saliva was collected between 09:00 and 12:00. Participants were instructed to allow saliva to accumulate on the floor of the mouth and to spit at 30 s intervals into pre-weighed 30 ml polypropylene tubes (Sterilin, Newport, UK), which were kept on ice. The collection period was 5 min. Participants were instructed not to eat or drink two hours prior to the appointment. Samples were immediately frozen and stored at -80 °C.

Non-targeted metabolite profiling of saliva samples was performed on a fee-for-service basis at Metabolon® (Durham, NC, USA). Samples were extracted and prepared for analysis using Metabolon’s standard solvent extraction method [24]. Samples were prepared using the automated MicroLab STAR® system (Hamilton Company). To remove protein, dissociate small molecules bound to protein or trapped in the precipitated protein matrix, and to recover chemically diverse metabolites, proteins were precipitated with methanol under vigorous shaking for 2 min (Glen Mills GenoGrinder 2000) followed by centrifugation. The resulting extract was divided into five aliquots: two for analysis by two separate reverse phase (RP)/UPLC-MS/MS methods with positive ion mode electrospray ionization (ESI), one for analysis by RP/UPLC-MS/MS with negative ion mode ESI, one for analysis by HILIC/UPLC-MS/MS with negative ion mode ESI, and one sample reserved for backup. Samples were placed briefly on a TurboVap® (Zymark) to remove the organic solvent. The sample extracts were stored overnight under nitrogen before
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preparation for analysis. All methods utilized a Waters ACQUITY ultra-performance liquid chromatography (UPLC) and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution. The sample extract was dried then reconstituted in solvents compatible to each of the four methods. Each reconstitution solvent contained a series of standards at fixed concentrations to ensure injection and chromatographic consistency. One aliquot was analyzed using acidic positive ion conditions, chromatographically optimized for more hydrophilic compounds. In this method, the extract was gradient eluted from a C18 column (Waters UPLC BEH C18 - 2.1 x 100 mm, 1.7 μm) using water and methanol, containing 0.05% perfluoropentanoic acid (PFPA) and 0.1% formic acid (FA). Another aliquot was also analyzed using acidic positive ion conditions, chromatographically optimized for more hydrophobic compounds. In this method, the extract was gradient eluted from the same aforementioned C18 column using methanol, acetonitrile, water, 0.05% PFPA and 0.01% FA and was operated at an overall higher organic content. Another aliquot was analyzed using basic negative ion optimized conditions using a separate dedicated C18 column. The basic extracts were gradient eluted from the column using methanol and water, with 6.5 mM ammonium bicarbonate at pH 8. The fourth aliquot was analyzed via negative ionization following elution from a HILIC column (Waters UPLC BEH Amide 2.1x150 mm, 1.7 μm) using a gradient consisting of water and acetonitrile with 10 mM ammonium formate, pH 10.8. The MS analysis alternated between MS and data-dependent MS² scans using dynamic exclusion. The scan range varied slightly between methods but covered 70-1000 m/z.

Raw data were extracted, peak-identified and quality controlled using Metabolon’s hardware and software. Compounds were identified by comparison with Metabolon’s reference library (retention times, MS and MS/MS spectra) [25]. A data normalization step was performed to correct variation resulting from instrument inter-day tuning differences. Each compound was corrected in run-day blocks by registering the medians to equal one (1.00) and normalizing each data point proportionately. Overall process variability was determined by calculating the median Relative Standard Deviation (RSD) for all endogenous metabolites present in 100% of technical replicates of a pooled sample. The RSD was 10%. Missing values were assumed to be caused by the compound in question being below the detection limit in the respective samples and were imputed with the minimum detected value for that compound across all samples (after the normalization step). Due to differences in ionization potentials between different compounds, normalized peak areas were scaled by the median value for each compound (i.e. each value for a particular compound in a sample was expressed as a multiple of the median value for that compound across all samples in which it was detected) [25]. Metabolome data was acquired for the five time-points corresponding to the experimental gingivitis challenge (Day 0, Day 2, Day 5, Day 9, and Day 14). Due to practical limitations, no metabolome data was
obtained for Day -14 and Day 21, and therefore these two time-points were excluded in the salivary metabolome analysis.

**Targeted functional salivary biochemistry assays**

Salivary pH, buffered pH, flow rate, total protein content, mucin MUC5B, albumin, secretory-IgA, lactoferrin, amylase, lysozyme, chitinase, and proteases were measured as described in a previous study [26]. In brief, total protein content was assayed using a Pierce BCA Protein Assay Kit (Thermo Scientific). MUC5B, albumin, secretory-IgA, and lactoferrin were analyzed using enzyme-linked immunosorbent assays (ELISAs). Amylase, lysozyme, chitinase, and proteases were quantified with enzyme activity assays. Reference samples of pooled saliva were analyzed on each ELISA / enzymatic activity plate to assess assay reproducibility. The mean RSD for the array of targeted salivary biochemistry assays was 8.1% (range 4.2% – 11.8%). Functional salivary biochemistry data were obtained from all seven time-points in the study (Day -14 to Day 21).

**Statistical analysis**

Exploratory data analysis was performed with Principal Component Analysis (PCA) in SPSS version 21. 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/ [27]. Metabolites absent in more than 33% of samples were excluded from the mixed-design ANOVA analysis. The primary output of the mixed-design ANOVA models were p-values for the within-subject effect, the between-subject effect, and the interaction between the main effects, respectively. The within-subject (repeated-measures) factor was time (i.e. five time-points for metabolites / seven time-points for the salivary functional biochemistry variables). The between-subject factor was erythritol (erythritol vs. control). A Huynh-Feldt correction was used to compensate for any deviation from sphericity in the data [28].

Metabolite data were log10-transformed (examination of histograms showed that the data were approximately log-normal distributed). Mixed-design ANOVA was performed on each individual metabolite present in more than two thirds of the 305 samples from which metabolome data were acquired, as well as on all metabolite ratios constructed from these metabolites. Ratio data were also log10-transformed to insure that ratio values were independent of the metabolite order and to half the multiple comparison burden, as log(A/B) = -log(B/A) [29, 30]. Multiple comparison adjustment was performed using the Bonferroni correction. The p-value significance threshold for individual metabolites was calculated at 1.2 x 10^{-4}. The p-value significance threshold for metabolite ratios was 6.0 x 10^{-7}. Additionally, for each ratio a p-gain statistic was calculated to assess whether the
additional information gained from the ratio was significant compared to the information from the respective individual metabolites [30]. The $p$-gain was defined as the change in the $p$-value obtained when using the metabolite ratios compared to the smallest of the two $p$-values obtained when analyzing the two respective metabolites separately [29, 30]. Metabolite ratios were considered significant if they had a $p$-value < $1.2 \times 10^{-7}$ as well as a $p$-gain > 408. Significant effects were examined with post-hoc Šidák tests.

Mixed-design ANOVA was also performed on data from each of the 12 targeted salivary biochemistry variables measured, with the addition of two extra time-points (Day - 14 and Day 21) compared to the metabolome data. The $p$-value significance threshold for the functional salivary biochemistry variables (using the Bonferroni correction) was calculated at $4.2 \times 10^{-3}$.

The statistical significance level (alpha) was 0.05. Standardized effect sizes are reported as partial eta-square ($\eta^2$) as defined by Cohen: partial $\eta^2 = \frac{SS_{between}}{SS_{between} + SS_{error}}$ [31]. Partial $\eta^2$ for a specific effect expresses the proportion the variance explained by that effect out of the variance remaining after excluding variance explained by other effects [32]. Plotted 95% confidence intervals were calculated according to Loftus and Mason’s procedure for within-subject and mixed-design studies [33].
Results

Study work flow and demographics

Of the 63 volunteers enrolled in the study, 61 participants completed the entire study and were included in the final analysis (Fig. 1). The study design is illustrated in Fig. 2. The erythritol group (N = 20) and the control group (N = 41) were balanced with regard to age and sex (Table 1). The final treatment (erythritol) group / control group sample size ratio was 1.00 / 2.05.

Figure 1. CONSORT flow diagram of the study - Dutch Trial Register (NL51111029.14).
Figure. Study design.

Table 1. Age and gender distribution of participants.

<table>
<thead>
<tr>
<th></th>
<th>Males (N = 21)</th>
<th>Females (N = 40)</th>
<th>All (N = 61)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Min</td>
<td>Max</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>(N = 41)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>45</td>
<td>26.0 ± 6.9</td>
</tr>
<tr>
<td>15 males,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>26 females</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Treatment</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(erythritol)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>(N = 20)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>19</td>
<td>23</td>
<td>21.2 ± 1.5</td>
</tr>
<tr>
<td>6 males,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14 females</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>All</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(N = 61)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>47</td>
<td>25.2 ± 6.2</td>
</tr>
<tr>
<td>21 males,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40 females</td>
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</tbody>
</table>
Salivary metabolites

The analysis identified a total of 497 metabolites (mass range 75 - 862 Da) in the 305 saliva samples obtained from the 61 participants at five time-points during the experimental gingivitis challenge. The mean number of metabolites identified per sample was 424.1 (SD = 28.4). The complete salivary metabolite dataset is given in Online Resource 1. A number of 446 metabolites out of the 497 total were found in more than 50% of samples, while 291 metabolites were found in more than 95 percent of samples. Only 186 metabolites were detected in 100% of samples. An overview of the distribution of metabolites between the main classes is shown in Table 2. The best represented classes of metabolites were amino acids and amino acid derivatives (29.6% of total) and lipids (28.4%). Among the 141 lipids, the most common sub-classes were phospholipids (34) and sphingolipids (24).

Table 2. Overview of metabolites organized by super-pathway.

<table>
<thead>
<tr>
<th>Super-pathway</th>
<th>Total metabolites detected from super-pathway</th>
<th>Mean number of metabolites per sample from super-pathway ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino Acids</td>
<td>147</td>
<td>135.8 ± 6.5</td>
</tr>
<tr>
<td>Lipids</td>
<td>141</td>
<td>130.1 ± 6.4</td>
</tr>
<tr>
<td>Xenobiotics</td>
<td>60</td>
<td>34.2 ± 4.6</td>
</tr>
<tr>
<td>Nucleotides</td>
<td>50</td>
<td>42.5 ± 4.8</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>34</td>
<td>26.8 ± 3.2</td>
</tr>
<tr>
<td>Peptides</td>
<td>34</td>
<td>27.2 ± 5.3</td>
</tr>
<tr>
<td>Cofactors and Vitamins</td>
<td>22</td>
<td>18.6 ± 2.7</td>
</tr>
<tr>
<td>Energy metabolism</td>
<td>9</td>
<td>9.0 ± 0.2</td>
</tr>
</tbody>
</table>

To indicate the compliance in the treatment group and the efficacy of the erythritol treatment delivery, Fig. 3 shows the erythritol concentration at the different time-points in the control and treatment groups, respectively. The mean salivary erythritol concentration was approximately two orders of magnitude higher in the treatment group compared to the control.

PCA analysis was performed to explore the metabolomic data and to assess whether any patterns were discernable based on either within-subject (challenge intervention) or between-subject (erythritol) factors. The PCA scores plots are shown in Fig. 4. No grouping was apparent for either of the two factors.
Effect of experimental gingivitis on the salivary metabolome

**Figure 3.** Mean erythritol concentrations in the erythritol and control groups, respectively, at each of the five time-points measured during the 14-day experimental gingivitis challenge. Erythritol concentrations are expressed as multiples of the median concentration of erythritol (across all samples in which it was detected). Error bars show 95% confidence intervals of the mean.

**Figure 4.** PCA scores plots based on data from all 497 metabolites detected. a) Samples labeled according to control and treatment (erythritol) groups; b) Samples labeled according to the time-point in the experimental gingivitis challenge intervention. PC1 and PC2 accounted for 40.3% and 6.6% of variance, respectively (46.9% cumulative).
Data from each of the 408 salivary metabolites detected in more than two thirds of the samples was modeled in a mixed-design ANOVA. The full list of mixed-design ANOVA analyses results for both within-subject (challenge intervention) and between-subject (erythritol) effects are given in Online Resource 2. Table 3 lists the ten salivary metabolites for which the effect of the challenge intervention was significant after adjusting for multiple comparisons using the Bonferroni correction. There was no significant effect of erythritol on any of the metabolites after multiple comparison adjustment.
Table 3. List of salivary metabolites found to change significantly between time-points during the experimental gingivitis challenge intervention (after Bonferroni correction for multiple comparisons). \(p\)-values of the within-subject effect of the mixed-design ANOVA (\(N = 61\)), relative changes in median values compared to Day 0, and effect sizes are listed for each ratio.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Super-pathway / Pathway</th>
<th>(p)-value (^a)</th>
<th>Max relative change in median value (compared to Day 0)</th>
<th>Effect size (partial (\eta^2)) (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-(\alpha)-acetylornithine</td>
<td>Amino acid / Urea cycle; Arginine and proline metabolism</td>
<td>5.8 (\times 10^{-10})</td>
<td>+ 75%</td>
<td>0.23</td>
</tr>
<tr>
<td>cadaverine</td>
<td>Amino acid / Lysine metabolism</td>
<td>8.6 (\times 10^{-9})</td>
<td>+ 69%</td>
<td>0.16</td>
</tr>
<tr>
<td>N-acetyl cadaverine</td>
<td>Amino acid / Lysine metabolism</td>
<td>1.5 (\times 10^{-8})</td>
<td>+ 58%</td>
<td>0.16</td>
</tr>
<tr>
<td>(\gamma)-aminobutyrate</td>
<td>Amino acid / Glutamate metabolism</td>
<td>2.0 (\times 10^{-8})</td>
<td>+ 33%</td>
<td>0.19</td>
</tr>
<tr>
<td>mevalonate</td>
<td>Lipid / Mevalonate metabolism</td>
<td>3.3 (\times 10^{-8})</td>
<td>+ 51%</td>
<td>0.15</td>
</tr>
<tr>
<td>cytosine</td>
<td>Nucleotide / Pyrimidine metabolism</td>
<td>1.6 (\times 10^{-7})</td>
<td>+ 47%</td>
<td>0.18</td>
</tr>
<tr>
<td>octanoylcarnitine</td>
<td>Lipid / Fatty acid metabolism (acyl carnitine)</td>
<td>5.3 (\times 10^{-7})</td>
<td>- 32%</td>
<td>0.14</td>
</tr>
<tr>
<td>(\alpha)-hydroxyisovalerate</td>
<td>Amino acid / Leucine, isoleucine and valine metabolism</td>
<td>3.1 (\times 10^{-5})</td>
<td>+ 42%</td>
<td>0.12</td>
</tr>
<tr>
<td>hexanoylcarnitine</td>
<td>Lipid / Fatty acid metabolism (acyl carnitine)</td>
<td>4.5 (\times 10^{-5})</td>
<td>- 22%</td>
<td>0.11</td>
</tr>
<tr>
<td>pseudouridine</td>
<td>Nucleotide / Pyrimidine metabolism, uracil containing</td>
<td>5.8 (\times 10^{-5})</td>
<td>- 28%</td>
<td>0.13</td>
</tr>
</tbody>
</table>

\(^a\)The Bonferroni \(p\)-value threshold for significance was 1.2 \(\times 10^{-4}\).

\(^b\)Approximate interpretation for \(\eta^2\) effect size: \(\eta^2 \approx 0.02\) — small, \(\eta^2 \approx 0.13\) — medium; \(\eta^2 \approx 0.26\) — large effect [31]
Figure 5. Median values (non-log10-transformed data) per time-point for metabolites found to change significantly during the challenge intervention:

a) N-α-acetylgornithine;

b) cadaverine;

c) N-acetylcadaverine;

d) γ-aminobutyrate;

e) mevalonate;

f) cytosine;

g) octanoylcarnitine;

h) α-hydroxy-isovalerate;

i) hexanoylcarnitine;

j) pseudouridine.

Error bars indicate 95% confidence intervals of the medians (N = 61).
Salivary metabolite ratios

Data from each ratio between the 408 salivary metabolites detected in more than two thirds of samples was modeled in a mixed-design ANOVA (83,028 ratios). For 760 ratios the effect of the challenge intervention had significant P-values after adjusting for multiple comparisons. Out of these, 166 ratios had P-gains >408 and are listed in Online Resource 3. However, of these 166 ratios, 135 ratios included at least one member of the 10 metabolites found to significantly change in the univariate mixed-design ANOVA analyses. Of the remaining 31 ratios, 13 ratios involved only metabolites known to be cell membrane lipids (Table 4).

A total of 62 membrane lipids were detected (excluding fatty-acid-free degradation products): 25 sphingolipids, 20 glycerophosphocholines (GPC), 15 glycerophosphoethanolamines (GPE), one glycerophosphoserine (GPS), and one glycerophosphoglycerol (GPG). Ten of these lipids were plasmalogens, a class of lipid where one of the two fatty acids is attached by an ether rather than an ester bond. Thirteen lipids (eight sphingolipids and five GPEs) were involved in the 13 ratios changing. Of the 5 GPEs involved in ratio changes, four were plasmalogens (the exception being 1,2-dioleoyl-GPE).

None of the metabolite ratios showed a significant effect of erythritol after Bonferroni correction.

Figure 6. Median values (non-log10-transformed data) per time-point for two of the metabolite ratios changing significantly during the challenge intervention: a) Ratio R1 [1-(1-enyl-palmitoyl)-2-arachidonoyl-GPE (P-16:0/20:4) : lactosyl-N-palmitoyl-sphingosine (d18:1/16:0)]; b) Ratio R13 [stearoyl sphingomyelin (d18:1/18:0) : sphingomyelin (d18:1/15:0)]. Error bars indicate 95% confidence intervals of the medians.
Table 4. Membrane lipid ratios found to change significantly between time-points during the experimental gingivitis challenge intervention. \( p \)-values for the within-subject effect of the mixed-design ANOVA, \( p \)-gains, and effect sizes are listed for each ratio.

<table>
<thead>
<tr>
<th>No</th>
<th>Metabolite ( M_1 )</th>
<th>Metabolite ( M_2 )</th>
<th>( p )-value (^a) ( M_1/M_2 )</th>
<th>( p )-gain (^b)</th>
<th>Effect size (partial ( \eta^2 ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>R01</td>
<td>1-(1-enyl-palmitoyl)-2-arachidonoyl-GPE (P-16:0/20:4)</td>
<td>lactosyl-N-palmitoyl-sphingosine (d18:1/16:0)</td>
<td>2.9 ( \times ) 10(^{-9})</td>
<td>1.5 ( \times ) 10(^{-3})</td>
<td>0.20</td>
</tr>
<tr>
<td>R02</td>
<td>1-(1-enyl-palmitoyl)-2-linoleoyl-GPE (P-16:0/18:2)</td>
<td>lactosyl-N-palmitoyl-sphingosine (d18:1/16:0)</td>
<td>1.8 ( \times ) 10(^{-7})</td>
<td>2.4 ( \times ) 10(^{-3})</td>
<td>0.20</td>
</tr>
<tr>
<td>R03</td>
<td>1-(1-enyl-palmitoyl)-2-oleoyl-GPE (P-16:0/18:1)</td>
<td>lactosyl-N-palmitoyl-sphingosine (d18:1/16:0)</td>
<td>1.2 ( \times ) 10(^{-8})</td>
<td>3.6 ( \times ) 10(^{-4})</td>
<td>0.20</td>
</tr>
<tr>
<td>R04</td>
<td>1-(1-enyl-stearoyl)-2-arachidonoyl-GPE (P-18:0/20:4)</td>
<td>lactosyl-N-palmitoyl-sphingosine (d18:1/16:0)</td>
<td>2.4 ( \times ) 10(^{-7})</td>
<td>1.8 ( \times ) 10(^{-3})</td>
<td>0.16</td>
</tr>
<tr>
<td>R05</td>
<td>1,2-dioleoyl-GPE (18:1/18:1)</td>
<td>lactosyl-N-palmitoyl-sphingosine (d18:1/16:0)</td>
<td>1.0 ( \times ) 10(^{-7})</td>
<td>4.2 ( \times ) 10(^{-4})</td>
<td>0.16</td>
</tr>
<tr>
<td>R06</td>
<td>behenoyl sphingomyelin (d18:1/22:0)</td>
<td>lactosyl-N-palmitoyl-sphingosine (d18:1/16:0)</td>
<td>3.7 ( \times ) 10(^{-7})</td>
<td>1.2 ( \times ) 10(^{-3})</td>
<td>0.17</td>
</tr>
<tr>
<td>R07</td>
<td>N-palmitoyl-sphinganine (d18:0/16:0)</td>
<td>lactosyl-N-palmitoyl-sphingosine (d18:1/16:0)</td>
<td>3.0 ( \times ) 10(^{-9})</td>
<td>1.5 ( \times ) 10(^{-3})</td>
<td>0.20</td>
</tr>
<tr>
<td>R08</td>
<td>N-palmitoyl-sphingosine (d18:1/16:0)</td>
<td>lactosyl-N-palmitoyl-sphingosine (d18:1/16:0)</td>
<td>4.3 ( \times ) 10(^{-9})</td>
<td>1.0 ( \times ) 10(^{-3})</td>
<td>0.21</td>
</tr>
<tr>
<td>R09</td>
<td>palmitoyl dihydro sphingomyelin (d18:0/16:0)</td>
<td>lactosyl-N-palmitoyl-sphingosine (d18:1/16:0)</td>
<td>5.8 ( \times ) 10(^{-8})</td>
<td>7.6 ( \times ) 10(^{-3})</td>
<td>0.20</td>
</tr>
<tr>
<td>R10</td>
<td>palmitoyl sphingomyelin (d18:1/16:0)</td>
<td>lactosyl-N-palmitoyl-sphingosine (d18:1/16:0)</td>
<td>1.9 ( \times ) 10(^{-7})</td>
<td>2.3 ( \times ) 10(^{-3})</td>
<td>0.18</td>
</tr>
</tbody>
</table>
### R11
- **Stearoyl sphingomyelin** (d18:1/18:0) vs. **lactosyl-N-palmitoyl-sphingosine** (d18:1/16:0)
- Test statistic: $9.3 \times 10^{-8}$
- $p$-value: $4.4 \times 10^{-6}$
- $p$-value adjusted: 0.19

### R12
- **Palmitoyldihydrosphingomyelin** (d18:0/16:0) vs. **sphingomyelin** (d18:1/15:0)
- Test statistic: $3.0 \times 10^{-7}$
- $p$-value: $3.0 \times 10^{-6}$
- $p$-value adjusted: 0.18

### R13
- **Stearoyl sphingomyelin** (d18:1/18:0) vs. **sphingomyelin** (d18:1/15:0)
- Test statistic: $1.3 \times 10^{-7}$
- $p$-value: $3.0 \times 10^{-6}$
- $p$-value adjusted: 0.17

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*The Bonferroni $p$-value threshold for significance for metabolite ratios was $6.0 \times 10^{-7}$. Metabolite ratios had to have a $p$-gain $> 408$ in order to be considered significant.*

## Targeted salivary biochemistry assays

Data from each of the 12 functional salivary biochemistry variables measured were modeled in a mixed-design ANOVA. All results from the analyses of functional salivary biochemistry data are listed in Online Resource 4. Of the 12 variables examined (salivary buffered pH, flow rate, total protein content, mucin MUC5B, albumin, secretory-IgA, lactoferrin, amylase, lysozyme, chitinase, and proteases), only lysozyme ($p = 0.02$) had $p$-values <0.05 for the effect of the experimental gingivitis challenge. However, neither the effect of the experimental gingivitis challenge intervention nor the effect of erythritol was significant in any of the functional salivary variables analyzed after adjusting for multiple comparisons.
Discussion

The results of this study show some significant changes in the salivary metabolome of young healthy adults during a two-week experimental gingivitis challenge intervention. These changes were observed in both the control and the treatment groups, with no significant effect of erythritol found on any of the metabolites analyzed.

Of the ten metabolites found to change significantly during the experimental gingivitis challenge (Table 3), seven metabolites showed increasing trends (N-α-acetylnornithine, cadaverine, N-acetylcadaverine, γ-aminobutyrate, mevalonate, cytosine, and α-hydroxyisovalerate), while the other three (octanoylcaritnine, hexanoylcaritnine, and pseudouridine) decreased (Fig. 5).

Cadaverine, N-acetylcadaverine, and α-hydroxyisovalerate are known products of bacterial amino acid catabolism. Oral bacteria can produce cadaverine and N-acetylcadaverine from lysine, while α-hydroxyisovalerate is derived from valine [34, 35]. This apparent increase in amino acid catabolism by oral bacteria could be a consequence of the accumulation of dental plaque after the cessation of oral hygiene measures. As the oral biofilm is left undisturbed and becomes more mature and organized, its metabolic efficiency increases [36]. An accumulation of catabolic products is therefore in line with the expected intensification of plaque metabolism.

The increase in both γ-aminobutyrate and N-α-acetylnornithine may be explained in the context of an adaptive response of oral bacteria to lower pH in the biofilm’s vicinity. As it matures, the oral biofilm may shift towards a more acidogenic functional profile and become more efficient in fermenting sugars and therefore lowers the pH of its surroundings [37]. However, not all bacterial species in dental plaque are acidoduric (e.g. due to lacking acid-resistant ATPases), and in fact some employ specific metabolic pathways to produce alkali in order to resist an increase in acidic stress [38]. One of the main alkali-producing pathways in bacteria is the Arginine Deiminase System (ADS), which plays a major role in oral biofilm pH homeostasis [39, 40]. The ADS pathway, present in common oral bacteria such as Streptococcus gordonii, Streptococcus parasanguis, Streptococcus rattus, Streptococcus sanguis and Lactobacillus fermentum, catabolizes L-arginine (originating either from salivary secretions or dietary sources) to produce ammonia, with ornithine as a by-product [40]. Together with a subsequent acetylation step by bacterial acetyltransferases, the additional ornithine resulting from an up-regulation of ADS could account for the observed increase in N-α-acetylnornithine.

Another metabolic route used by bacteria to increase their environmental pH is glutamate decarboxylation, present in some species of Bacteroides, Fusobacterium, Eubacterium, and Lactobacillus, among other Gram-positive bacteria [41, 42]. In this reaction cycle, extracellular glutamate is imported into the bacterial cell through a glutamate / γ-aminobutyrate antiporter. The imported glutamate is subsequently
Effect of experimental gingivitis on the salivary metabolome

decarboxylated and the resulting non-ionized γ-aminobutyrate is returned to the extracellular environment through the aforementioned antiporter [41]. The net outcome is an increase in bacterial intracellular pH and an extracellular increase in γ-aminobutyrate.

In the present study, N-α-acetylornithine and γ-aminobutyrate show highly similar trends, with considerable increases during the first five days of the experimental gingivitis before leveling off (Fig. 5, a and d). This could indicate that an up-regulation of bacterial acid-adaptation pathways occurred in the first few days of the challenge intervention.

Mevalonate is a precursor in the mevalonate pathway, where mevalonate is converted to sterol terpenoids (e.g. cholesterol) as well as non-sterol terpenoids (e.g. heme-A, isopentenyl tRNA and ubiquinone) [43]. The mevalonate pathway has anti-inflammatory and immunomodulatory effects [43, 44]. It is particularly important for modulating regulatory T-cell proliferation and function, maintaining a tolerogenic rather than proinflammatory phenotype [44, 45]. The >50% increase in median mevalonate levels observed during the first days of the experimental gingivitis challenge (Table 3, Fig. 5 e) may point towards an early anti-inflammatory response by host epithelial tissues to changes in the oral plaque.

Octanoyl- and hexanoyl-carnitine were both found to decrease during the study (Table 3, Fig. 5 g, i). No significant changes were seen in the other nine detected acyl-carnitines. Carnitine and acyl-carnitines are involved in mitochondrial beta-oxidation of fatty acids [46]. However, bacteria may utilize acyl-carnitines as carbon and/or nitrogen sources [47]. It is unclear why these two particular acyl-carnitines decreased, although it may be linked to preferential use by oral bacteria.

Cytosine increased during the experimental gingivitis challenge intervention, showing a marked change between Day 2 and Day 5 (Table 3, Fig. 5 f) while pseudouridine - another nucleotide – decreased (Fig. 5 j). Whereas cytosine is one of the two canonical pyrimidines in DNA, pseudouridine is a modified pyrimidine found in eukaryotic ribosomal and transfer RNA, but not in messenger RNA [48]. Pseudouridine is a stable end-product of RNA catabolism and has been considered a potential marker of cell turnover [48]. However, in the context of the oral environment, the mechanisms behind the increase in cytosine and the decrease in pseudouridine are unclear. No other nucleotides showed significant changes.

Previous studies have discovered metabolite ratio biomarkers associated with smoking, diabetes, and other health-related conditions [30, 49, 50]. The analysis of metabolite ratios is advantageous. It cancels out systematic experimental variation and, perhaps most importantly, when the two members of the ratio are connected by a biochemical pathway, it can yield valuable additional information on the status of respective pathway [51].

A set of 13 metabolites ratios involving membrane lipids were found to change significantly during the study, with all ratios showing the same trend: an increase from Day 0 to Day 2 followed by a continuous decrease from Day 2 through Day 14 (Table 4, Fig. 6).
Of the diversity of membrane lipid classes detected in this study, only sphingolipids (eight sphingomyelins and three ceramides) and GPEs (one GPE and four GPE-plasmalogens) were involved in the changing ratios.

Sphingomyelins are a class of lipids containing an aliphatic amino alcohol connected to a sphingoid backbone and a fatty acid N-linked via an amide. They are the most abundant lipids in human cell membranes, yet absent in most bacteria [52]. Hydrolysis of sphingomyelins yields ceramides (N-acyl-sphingosines) and free choline. However, ceramides can also be converted back into sphingomyelins [53]. Human cells maintain a controlled balance between sphingomyelins and ceramides, as the relative contribution of each will have a significant effect on the properties of the lipid bilayer [53]. Moreover, these membrane lipids also mediate cell signaling processes and may release second messengers (e.g. arachidonic acid)) [53]. Glycosylated sphingolipids, such as lactosyl-N-palmitoyl-sphingosine (involved in 11 of the 13 ratios found to change during the experimental gingivitis challenge intervention), are involved in cell-to-cell communication and are part of cell surface receptors for bacterial toxins [54-56]. Both sphingomyelins and ceramides are implicated in the intracellular activation of signaling pathways and in the release of cytokines, and therefore have an important role in the host response to microbial challenges [57]. Interestingly, another salivary metabolomics study found palmitoyl sphingomyelin to be elevated in saliva of periodontitis patients compared to healthy controls [14].

Four plasmalogens are involved in four of the 13 changing lipid ratios (see Table 4). Plasmalogens are glycerophospholipids containing a fatty alcohol with a vinyl ether bond at the sn-1 position. Plasmalogens represent up to 20% of the total phospholipids in human cell membranes [58]. While several anaerobic bacteria also contain plasmalogens (e.g., members of Clostridia, Maegashpera, and Veillonella), the composition of the four plasmalogens involved in the changing ratios (e.g. arachidonic / linoleic acid components) suggest that they originate from the host [58, 59]. Besides influencing the fluidity of the cell membrane, plasmalogens have been proposed to act as defensive antioxidants on the cell membrane surface. The vinyl ether bond makes plasmalogens more susceptible and they are preferentially oxidized over other membrane lipids when exposed to free radicals [60]. However, plasmalogen-derived oxidative products cannot further propagate lipid oxidation and the damage to the membrane is therefore limited [61]. Indeed, reactive oxygen species (ROS)-producing human cells have plasmalogen-enriched membranes [62].

The set of membrane lipids ratios found to be significantly changing - involving almost exclusively sphingomyelins, ceramides and plasmalogens - may be linked to increased interactions between host tissues and oral bacteria, brought by changes in the functional profile and metabolism of the oral biofilms during the experimental gingivitis challenge intervention. While the complexity and the inter-connected nature of the functions of many of these membrane lipids make a detailed interpretation of individual
ratios difficult, these findings cumulatively point to a dynamic adjustment of the host cells in response to the effects of the changes in the oral biofilm metabolism.

The study found no significant effects of a daily intake of erythritol on the salivary metabolome during the challenge intervention. 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 five weeks in the present study [17, 18]. Perhaps any effect that erythritol may have on the oral ecosystem requires an exposure duration longer 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.

None of the salivary functional biochemistry variables analyzed in this study showed any changes during the challenge intervention, pointing towards a relatively stable long-term profile of salivary secretions which was not significantly influenced by the changes occurring in the mouth during the experimental gingivitis challenge intervention.

One of the main difficulties in salivary metabolomic analysis is that a large number of metabolites may have both human and bacterial origin [4]. While this may complicate interpretation of results, salivary metabolomics remains a powerful tool for gaining biological insights into the mechanisms involved in the maintenance of oral health.

Concluding remarks

This study aimed to examine the changes in the salivary metabolome of healthy young adults during a two-week experimental gingivitis challenge intervention. A number of significant changes in salivary metabolites and metabolite ratios were found. An increase in bacterial amino acid catabolites (cadaverine, N-acetylcadaverine, and α-hydroxyisovalerate) pointed towards an intensified metabolism of the oral biofilm, while increases in end-products of bacterial alkali-producing pathways (N-α-acetylornithine and γ-aminobutyrate) may be a response of less aciduric oral bacteria faced with an increasingly acidogenic oral biofilm. Synchronous changes in a set of 13 salivary metabolite ratios involving sphingomyelins, ceramides and plasmalogens, all host cell membrane lipids known to be involved in cell signaling, host responses to bacteria, and defense against free radicals could be a consequence of host adaptations to changes in the oral biofilms. An increase in mevalonate, a key member in a pathway involved in anti-inflammatory responses, was also observed. Taken together, the findings of this study underline a dynamic interaction between host and oral microbial biofilms during induction of experimental gingivitis.
Chapter 5

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Chapter 5


