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Intra-oral microbial metabolism and association with host taste perception

Alexander Gardner1,3, Po-Wah So2, Guy H. Carpenter*1

1. Salivary Research, Centre for Host-Microbiome Interactions, Faculty of Dental, Oral & Craniofacial Sciences, King’s College London, London, UK

2. Department of Neuroimaging, Institute of Psychiatry, Psychology and Neuroscience, King’s College London, Maurice Wohl Clinical Neuroscience Institute, London, UK

3. Department of Restorative Dentistry, Dental Hospital and School, University of Dundee, Dundee, UK.

GC and PWS should be considered joint senior author

*Corresponding author: Guy Carpenter, guy.carpenter@kcl.ac.uk, +44 20 7188 7460.

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Abstract

Metabolomics has been identified as a means of functionally assessing the net biological activity of a particular microbial community. Considering the oral microbiome, such an approach remains largely underutilised. While the current knowledge of the oral microbiome is constantly expanding, there are several deficits in knowledge particularly relating to their interactions with their host. This work uses $^1$H-NMR spectroscopy to investigate metabolic differences between oral microbial metabolism of endogenous (i.e. salivary protein) and exogenous substrates (i.e. dietary carbohydrates). It also investigated whether microbial generation of different metabolites may be associated with host taste perception. This work found that in the absence of exogenous substrate, oral bacteria readily catabolise salivary protein and generate metabolic profiles similar to those seen in-vivo. Important metabolites such as acetate, butyrate and propionate are generated at relatively high concentrations. Higher concentrations of metabolites were generated by tongue biofilm compared to planktonic salivary bacteria. Thus, as has been postulated, metabolite production in proximity to taste receptors could reach relatively high concentrations. In the presence of 0.25 M exogenous sucrose, a rapid catabolism was observed with increased concentrations of a range of metabolites relating to glycolysis (lactate, pyruvate, succinate). Additional pyruvate-derived molecules such as acetoin and alanine were also increased. Furthermore, there was evidence that individual taste sensitivity to sucrose was related to differences in the metabolic fate of sucrose in the mouth. Sensitive perceivers appeared more inclined towards continual citric acid cycle activity post-sucrose, whereas relatively insensitive perceivers had a more efficient conversion of pyruvate to lactate. This work collectively indicates that the oral microbiome exists in a complex balance with the host, with fluctuating metabolic activity depending on nutrient availability. There is preliminary evidence of an association between host behaviour (sweet taste perception) and oral catabolism of sugar.

Introduction

Microbial sequencing technologies greatly advanced knowledge of the diversity of the human oral microbiome. Complementary technologies such as metabolomic profiling likely represent an equally valuable, yet under-utilised, source of information (Takahashi 2015). Metabolomic profiling of oral fluid, particularly saliva, generally focuses on biomarker discovery. Metabolomic profiling of saliva appears to offer diagnostic promise for oral cancer (Ishikawa et al. 2016; Sugimoto et al. 2010; Wang et al. 2014; Wei et al. 2011), periodontal disease (Aimetti et al. 2012), dental caries (Fidalgo et al. 2013; Pereira et al. 2019), and primary Sjögren’s syndrome (Kageyama et al. 2015; Mikkonen et al. 2013). Saliva has also been implicated as a source of biomarkers for systemic diseases including breast and prostate cancer (Sugimoto et al. 2010), dementia (Figueira et al. 2016; Tsuruoka et al. 2013), and Alzheimer’s disease (Liang et al. 2015; Yilmaz et al. 2017). These studies typically focus on finding differences between disease and control samples. Investigation of why these metabolic differences arise is lacking. An important step in enhancing the knowledge of the oral microbiome is moving from simply determining what microorganisms are present to determining the significance of their net metabolomic activity (Takahashi 2015). Study of links between the human gut microbiome and metabolome has recently unveiled important insights into the symbiotic relationship between the microbiome and their host (Zierer et al. 2018), including disease processes such as Inflammatory bowel disease, allergic disease and obesity (Hirata and Kunisawa 2017; Liu et al. 2017; Santoru et al. 2017).

There remain important gaps in the collective knowledge of the salivary metabolome. Unlike for the gut, the link between the salivary metabolome and microbiome remains sparsely studied. Prominent gut metabolites including acetate, propionate and butyrate, collectively termed short-chain fatty acids, SCFAs, arising from the bacterial fermentation of dietary fibre are known to confer health...
benefits. These include maintenance of epithelial barrier integrity, immune signalling and anti-cancer regulation (Morrison and Preston 2016; Rooks and Garrett 2016). SCFAs are also prominent salivary metabolites. Until recently, a significant question concerned the origin of salivary metabolites and the extent to which they are derived from the host or the microflora. It has recently been demonstrated that glandular saliva is free of SCFAs, aside from trace levels of acetate. Furthermore, the SCFA concentration of whole mouth saliva (WMS) correlates strongly with the oral microbial load (Gardner et al. 2019).

Glandular saliva as measured by nuclear magnetic resonance spectroscopy ($^1$H-NMR) is relatively sparse in metabolite content compared to WMS. Many other metabolites present in WMS appear to be largely of microbial origin including amines (methyl, dimethyl, and trimethylamine) and amino acids such as phenylalanine and glycine. The few high-concentration metabolites present in glandular saliva include citrate, lactate and urea. Salivary urea is consumed by oral bacteria post secretion, and WMS urea concentrations correlate inversely with both microbial load and plaque abundance (Gardner et al. 2019) (Liebsch et al. 2019). Lactate is arguably the most familiar salivary metabolite amongst dental professionals. The role of lactate production from fermentable carbohydrates in demineralising tooth tissue has been known for 80 years (Miller et al. 1940). While other organic acids have also been implicated in the caries process (Silwood et al. 1999), lactate reaches the highest concentrations following oral exposure to fermentable sugars. Nevertheless, under resting conditions without recent (> one hour) exposure to oral carbohydrates, WMS lactate concentrations are not elevated above parotid saliva concentrations, and are less concentrated than circulating blood lactate concentrations (Gardner et al. 2019). These shifts in metabolism upon exposure to exogenous nutrients underpin a major difference between the gut and oral metabolomes. Whereas gut microbes have a continual source of nutrients in the form of dietary fibre, oral microbial communities must endure significant periods of time without exposure to nutrients consumed by the host. Sleep would likely represent the longest time period for oral bacteria to subsist without oral exposure to exogenous nutrients. However, the saliva initially produced on waking has been demonstrated to be richer in many metabolites including amines and SCFAs, relative to saliva collected throughout the day (Wallner-Liebmann et al. 2016). Therefore, oral bacteria must be capable of metabolising additional substrates.

Another emerging from the growing literature on the gut microbiome is that of the microbiome manipulating host behaviours via their metabolic activity. A complex relationship is being unveiled, linking dietary choices with the microbial metabolism of consumed foods by gut bacteria via molecular signalling that influences host satiety response. Such a relationship essentially forms a feedback cycle where the consumption of unhealthy, processed foods, ultimately leads to a desire to consume more of the same foods resulting in adverse metabolic consequences such as obesity and associated conditions (Sandhu et al. 2017). These host-microbiome interactions have led to gut bacteria being described as “microscopic puppetmasters” (Alcock et al. 2014). Action on taste receptors has been identified as a possible mechanism of such microbial manipulation of their host, however the literature directly supporting this is currently limited to animal models (Alcock et al. 2014). More importantly, despite being focused on oral sensory processes, the emphasis of these animal studies is the gut microbiome. It would seem logical that the oral microbiome would be a more appropriate target when investigating microbial impairment of taste function. It has been hypothesised that metabolic activity of tongue biofilms local to taste receptors may be critical in generating metabolites that modulate individual sensory perception (Neyraud and Morzel 2019). The pattern of bacterial substrate utilisation and metabolite output relevant to these processes is unclear, although catabolism of exogenous nutrients is implicated.
The aim of this work is therefore to explore whether the net metabolic activity of oral microflora might influence host taste perception, particularly in the presence and absence of exogenous nutrients. The pattern of metabolites arising from saliva catabolism by tongue biofilm and WMS bacteria was first established in-vitro, modelling a fasted state such as sleep. Subsequently, in-vivo catabolism of exogenous sucrose was analysed with respect to host taste sensitivity to a sucrose challenge, modelling carbohydrate intake.

Materials and methods

Ethical approval

Work was conducted following approval from King’s College London ethics committee (HR-15/16–2508). All volunteers provided written consent.

Investigation of oral microbial metabolism in the absence of exogenous nutrients in-vitro

Sample collection

Parotid saliva (20 ml) was collected from a single volunteer using a sterilised Lashley cup and 1% mass/volume food-grade tartaric acid stimulation (Gardner et al. 2019). Parotid saliva was filtered through a 0.2 µm filter, aliquoted (500 µl) into sterile tubes, and stored at -80 °C for one week prior to use.

Bacterial inoculums were sourced from six healthy adult volunteers, one hour after eating, drinking or oral exposure to exogenous substances. Dietary information was not gathered. Antibiotic use in the preceding six months and active oral disease (based on visual examination by a dentist) were exclusion criteria. Unstimulated whole-mouth saliva (WMS) was collected from each volunteer. Biofilm samples from the anterior and posterior tongue were collected using sterilised, pre-weighed plastic scrapers (VWR, Lutterworth, UK, cat no: 231-0639). The location of sample was based on proximity to circumvallate papillae posteriorly and fungiform papillae anteriorly.

Inoculation and incubation conditions

Parotid saliva aliquots were thawed on ice. Aliquots were inoculated with 20 µl of WMS or 20 mg of tongue biofilm from either tongue site (i.e. 4% by volume/mass, respectively). Control samples were prepared with 20 µl of sterile PBS. Inoculated sample tubes were stored inside Sterilin universal tubes (ThermoFisher Scientific, Waltham, MA, USA) with wet tissue paper in the bottom to minimise evaporative fluid loss. Samples were incubated at 37 °C for 24 hours in an anaerobic cabinet with gas blend 10% H₂, 10% CO₂ and 80% N₂. Tube lids were pierced with sterilised forceps to allow gaseous exchange.

Non-incubated control samples were prepared immediately prior to analysis. One control was parotid saliva mixed with 4% PBS to control for any effects of incubation alone in the absence of bacteria. The second control was parotid saliva mixed with 20 µl of pooled WMS (4%) to control for any compositional changes arising from the baseline metabolites present in the WMS inoculum. The experimental design is summarised in Supplementary Figure 1.

Sample analyses

Bacterial load

Final bacterial load was assessed post-incubation. Samples were vortexed to homogenise the bacterial content and sample was serially diluted ten-fold to 1:10⁵. Samples diluted to 1:10³ and
were plated (20 µl) onto fastidious anaerobe agar with 5% defibrinated horse blood. Plates were incubated under anaerobic conditions for 48 hours, colonies counted and CFU/ml calculated.

Protein quantification

Bacterial cells were removed by centrifugation at 15,000 g for ten minutes at 4 °C. Samples were analysed by SDS-PAGE as previously described (Gardner and Carpenter 2019). Briefly, 12 µl buffered sample was added per lane, electrophoresed and stained with Coomassie Brilliant Blue R250 (Sigma, Gillingham, UK). Samples of the unincubated parotid saliva and incubated, PBS-inoculated parotid saliva were run on every gel. Destained gels were imaged with a ChemiDoc MP system (Biorad, Watford, UK) and analysed in ImageLab 5.2.1 (Bio-Rad Laboratories, Hercules, CA, USA). Total lane density of sample lanes and PBS-inoculated lanes relative to the unincubated parotid saliva lanes were measured. Bacterial pellets were retained for analysis confirming the protein changes observed in saliva (Supplementary Figure 2).

1H-NMR spectroscopy

Centrifuged samples were prepared and analysed using internal standard as described (Gardner et al. 2018). All reagents and consumables were purchased from sigma. NMR buffer was prepared with 0.5 mM trimethylsilyl-[2,2,3,3,-2H4]-propionate (TSP) standard, 0.2 M Na2HPO4 and 44 mM NaH2PO4 in 50% deuterium oxide (D2O) by volume. Sample (440 µl), centrifuged as for the protein analysis, was mixed with NMR buffer (110 µl) in 5 mm external diameter NMR tubes to give a final concentration of 0.1 mM TSP and 10% by volume D2O. Using a 600 MHz spectrometer (Bruker, Karlsruhe, Germany), operating at a proton frequency of 600.2 MHz, spectra were acquired at 25 °C using a Carr-Purcell-Meiboom-Gill (CPMG) spin-echo pulse sequence with presaturation to supress macromolecule resonances from the spectra. The total echo time was 64 ms with relaxation delay of 4 s and acquisition time of 2.32 s. Following four dummy scans, 128 transients were collected with 64,000 data points and spectral width of 20 ppm (~5 to 15 ppm). Spectra were automatically phased and baseline corrected with further manual adjustment where required. The control samples described for the protein analysis were also analysed by 1H-NMR spectroscopy. An additional non-incubated sample of parotid saliva with 4% pooled WMS added was prepared to control for baseline metabolite content of the inoculum.

Spectra were analysed by targeted manual quantification of known metabolites. Peak assignments were made using HMDB (hmdb.ca), Chenomx 8.3 (Edmonton, Canada) and literature values. Spectra were integrated into 0.01 ppm buckets from δ 0.7 to 8.5 ppm, excluding δ 4.5 to 5.5 ppm buckets, using MestreC (Santiago de Compostela, Spain), normalised to the standard peak, centred and Pareto scaled and then analysed by principal component analysis and k-means cluster analysis in Knime v.3.4.2 (Konstanz, Germany).

Investigation of oral microbial metabolism in the presence of exogenous nutrients and associations with host taste sensitivity in-vivo

Sample collection and study design

Food-grade sucrose solutions (Sigma) were prepared at 0.25 M in Buxton (Nestle, York, UK) mineral water. Experiments were conducted between 2:00 and 3:00 p.m., at least one hour after the last exposure to exogenous substances. Volunteers were administered 10 ml of mineral water as a control and instructed to passively hold the liquid in the floor of the mouth for 30s. The water was expectorated, and WMS collected into pre-weighed universal tubes over two minutes. This process was repeated with 0.25 M sucrose.
Participants rated their maximum perceived intensity of the sucrose sweetness on generalised labelled visual analogue scales (gIVAS, Supplementary Figure 3). Participants were first familiarised with the use of the scale via verbal and written instructions. Fifty-two participants were screened for taste sensitivity. Inclusion/exclusion criteria were as described for biofilm donors and included no reported deficiency in salivary flow or taste function. Salivary samples from sensitive and relatively insensitive sucrose perceivers, defined as rating sweetness as greater or less than one standard-deviation from the mean, were selected for further analysis, (n = 9 per group). Conformity with STROBE guidelines (ISPM, Bern, CH) for case-control studies was ensured.

**Salivary analyses**

Salivary flow rate was calculated in g/min by dividing the mass of saliva collected by the collection time. Samples were analysed by 1H-NMR spectroscopy as described and targeted metabolite concentrations were quantified. Biofilm metabolite output was calculated in (µmol/min) by multiplying metabolite concentration by flow rate. Differences in metabolite output relative to control following sucrose exposure were compared between the sensitive and relatively insensitive perceivers. Relative flow rate changes and relevant metabolite ratios were also determined and compared.

**Statistical analyses**

Data were primarily analysed in GraphPad prism 8 (La Jolla, CA, USA) and Knime v.3.4.2 (Konstanz, Germany). Following inspection for normality (Shapiro-Wilk test and Q-Q plots), data were analysed by appropriate statistical tests including ANOVA, single-sample t-test and two-tailed paired t-tests. CFU densities were logarithmically transformed prior to analysis. Adequate statistical power was confirmed post-hoc for the differences observed in the in-vivo study.

**Results**

**Oral bacteria consume salivary proteins**

Parotid saliva was minimally affected by incubation alone, displaying minor changes in statherin and low MW proteins. Inoculated samples universally showed considerable protein loss, with amylase typically the only residual protein. Lane density of samples were significantly reduced relative to controls for all inoculums. Tongue biofilm samples had significantly reduced protein relative to WMS inoculated samples. Log_{10} CFU of inoculated samples differed significantly between WMS and posterior tongue biofilms. A moderate correlation (R^2 = 0.62) was found between final CFU and protein consumption of the samples. These results are summarised in Figure 1.

**Oral bacteria generate metabolites from parotid saliva**

Metabolic content of parotid saliva inoculated with oral bacteria was considerably different to PBS inoculated parotid saliva. Typical spectra pre- and post-incubation are shown in Supplementary Figure 4. Changes in the metabolite concentrations are shown in Table 1. The majority of host derived metabolites present in parotid saliva at baseline were partly or wholly consumed by oral bacteria. Inoculated samples displayed considerable concentrations of SCFAs, amino acids and phenolic compounds. These tended to be most concentrated in the tongue biofilm inoculated samples. Spectral profiles of control samples were not significantly different to the baseline unincubated parotid saliva. The only measured metabolic difference between control and baseline parotid saliva was that phenylalanine was not detected in baseline parotid saliva. Multivariate
analysis found that there was a degree of separation between the metabolic composition of tongue biofilm and WMS inoculated samples, (Figure 2). The consumption of proteins correlated with the generation of several metabolites, notably acetate, butyrate, propionate and phenylacetate (Supplementary Figure 5). A comparison of inter-individual variability in metabolite profiles of inoculated samples and participant WMS found no differences in variation between the samples (Supplementary Figure 6). Importantly, endogenous salivary metabolites were also consumed from the baseline parotid saliva. Endogenous glucose was fully consumed in all cases. Citrate and urea were significantly consumed by all inoculums, however pyruvate and lactate were significantly consumed by tongue biofilm but were not significantly consumed by WMS bacteria.

Table 1: Summary of the concentrations of metabolites consumed and generated following 24 h anaerobic incubation of parotid saliva inoculated with oral bacteria relative to inoculation with sterile PBS. Significant results (p < 0.05) are presented in bold. NA = statistical test could not be conducted due to total consumption of metabolite from all samples yielding a S.D. of zero; n.s. = not significant. Sample means were compared to PBS metabolite concentrations by a one-sample t-test (n = 6).

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Post-incubation metabolite conc. (mM) for PBS inoculated PS (n=1)</th>
<th>Mean (S.D.) post-incubation metabolite conc. (mM) for PS inoculated with:</th>
<th>P-value between bacterial and PBS inoculum (one-sample t-test).</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tongue biofilm (ant.) (n=6)</td>
<td>Tongue biofilm (post.) (n=6)</td>
<td>WMS (n=6)</td>
</tr>
<tr>
<td><strong>Metabolites consumed</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urea</td>
<td>0.07</td>
<td>0.00 (0.00)</td>
<td>0.00 (0.00)</td>
</tr>
<tr>
<td>Lactate</td>
<td>0.18</td>
<td>0.03 (0.02)</td>
<td>0.02 (0.01)</td>
</tr>
<tr>
<td>Citrate</td>
<td>0.08</td>
<td>0.01 (0.01)</td>
<td>0.00 (0.00)</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.05</td>
<td>0.02 (0.01)</td>
<td>0.01 (0.01)</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.12</td>
<td>0.00 (0.00)</td>
<td>0.00 (0.00)</td>
</tr>
<tr>
<td><strong>Metabolites generated</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formate</td>
<td>0.004</td>
<td>0.77 (0.18)</td>
<td>0.92 (0.17)</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.02</td>
<td>0.08 (0.04)</td>
<td>0.11 (0.09)</td>
</tr>
<tr>
<td>Phenol</td>
<td>0.00</td>
<td>0.16 (0.05)</td>
<td>0.13 (0.04)</td>
</tr>
<tr>
<td>Proline</td>
<td>0.00</td>
<td>0.37 (0.11)</td>
<td>0.45 (0.17)</td>
</tr>
<tr>
<td>Valine</td>
<td>0.00</td>
<td>0.19 (0.10)</td>
<td>0.20 (0.12)</td>
</tr>
<tr>
<td>Phenylacetate</td>
<td>0.00</td>
<td>0.09 (0.05)</td>
<td>0.05 (0.04)</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.02</td>
<td>0.50 (0.21)</td>
<td>0.58 (0.32)</td>
</tr>
<tr>
<td>5-aminopentanoate</td>
<td>0.00</td>
<td>1.06 (0.41)</td>
<td>1.04 (0.43)</td>
</tr>
<tr>
<td>3-phenylpropionate</td>
<td>0.00</td>
<td>0.13 (0.11)</td>
<td>0.10 (0.10)</td>
</tr>
<tr>
<td>Putrescine</td>
<td>0.00</td>
<td>0.32 (0.09)</td>
<td>0.32 (0.06)</td>
</tr>
<tr>
<td>Succinate</td>
<td>0.04</td>
<td>0.05 (0.05)</td>
<td>0.09 (0.07)</td>
</tr>
<tr>
<td>Acetate</td>
<td>0.01</td>
<td>4.70 (1.02)</td>
<td>4.19 (1.02)</td>
</tr>
<tr>
<td>Butyrate</td>
<td>0.00</td>
<td>0.62 (0.25)</td>
<td>0.47 (0.25)</td>
</tr>
<tr>
<td>Propionate</td>
<td>0.00</td>
<td>2.33 (0.83)</td>
<td>1.97 (0.79)</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.02</td>
<td>0.10 (0.06)</td>
<td>0.12 (0.09)</td>
</tr>
</tbody>
</table>
Host sensitivity to sucrose is associated with different intra-oral bacterial sucrose catabolism in-vivo

Exposure of oral bacteria to sucrose causes significant changes in the salivary concentration and biofilm outputs of multiple metabolites, (Table 2). Data subdivided by sucrose sensitivity is presented in Supplementary Table 1. When comparing sensitive and relatively insensitive perceivers of sucrose, no significant difference in relative salivary flow rate was detected. Significant differences in lactate:pyruvate ratios and citrate:pyruvate ratios between sensitive and relatively insensitive perceivers were detected. Relatively insensitive perceivers had a significantly higher lactate:pyruvate ratio compared to sensitive perceivers (26.88 ± 5.63 vs. 16.04 ± 2.35, respectively), and a significantly lower citrate:pyruvate ratio (-0.32 ± 0.17 vs. 0.04 ± 0.04, respectively). Data are presented in Figure 3.

Table 2: A summary of the salivary concentrations and output changes of salivary metabolites post-sucrose exposure, relative to water control. * - glucose is measured as the sum of α- and β-glucose quantified in the sample. Data were analysed by paired t-test (n = 18). Significant p-values (p < 0.05) are included in bold.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Salivary metabolite concentration (mM):</th>
<th></th>
<th>Salivary metabolite output (µmol/ml):</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Post-control (water)</td>
<td>Post-sucrose (0.25 M)</td>
<td>p-value (paired t-test)</td>
<td>Post-control (water)</td>
</tr>
<tr>
<td>Formate</td>
<td>Mean ± S.D.</td>
<td>Mean ± S.D.</td>
<td>Mean ± S.D.</td>
<td>Mean ± S.D.</td>
</tr>
<tr>
<td>Sucrose</td>
<td>0.068 ± 0.118</td>
<td>0.036 ± 0.013</td>
<td>18.39 ± 9.90</td>
<td>0.023 ± 0.012</td>
</tr>
<tr>
<td>Citrate</td>
<td>0.046 ± 0.029</td>
<td>0.042 ± 0.028</td>
<td>0.021 ± 0.006</td>
<td>0.033 ± 0.015</td>
</tr>
<tr>
<td>Succinate</td>
<td>0.18 ± 0.13</td>
<td>0.72 ± 0.42</td>
<td>1.84x10^5</td>
<td>0.35 ± 0.25</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.18 ± 0.07</td>
<td>0.40 ± 0.19</td>
<td>4.31x10^5</td>
<td>0.33 ± 0.15</td>
</tr>
<tr>
<td>Acetate</td>
<td>4.94 ± 2.34</td>
<td>4.16 ± 1.79</td>
<td>0.023</td>
<td>9.53 ± 5.09</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.13 ± 0.06</td>
<td>0.15 ± 0.07</td>
<td>0.021</td>
<td>0.25 ± 0.13</td>
</tr>
<tr>
<td>Acetoin</td>
<td>0.06 ± 0.03</td>
<td>0.11 ± 0.08</td>
<td>0.004</td>
<td>0.12 ± 0.06</td>
</tr>
<tr>
<td>Lactate</td>
<td>0.53 ± 0.53</td>
<td>4.42 ± 2.84</td>
<td>1.31x10^5</td>
<td>0.92 ± 0.81</td>
</tr>
<tr>
<td>Propionate</td>
<td>0.98 ± 0.64</td>
<td>1.11 ± 0.62</td>
<td>0.019</td>
<td>1.85 ± 1.20</td>
</tr>
<tr>
<td>Butyrate</td>
<td>0.31 ± 0.13</td>
<td>0.27 ± 0.12</td>
<td>0.005</td>
<td>0.60 ± 0.30</td>
</tr>
<tr>
<td>Glucose*</td>
<td>0.15 ± 0.01</td>
<td>3.91 ± 2.06</td>
<td>0.006</td>
<td>0.30 ± 0.03</td>
</tr>
</tbody>
</table>

Discussion

Despite being recognised as an important nutrient source for oral bacteria (Ruhl 2012; Takahashi 2015), microbial metabolism of saliva has been sparsely studied. Carbohydrate moieties of salivary glycoprotein (MUC5B) have been identified as a target for microbial catabolism, however the present results demonstrate that salivary proteins are readily catabolised by oral bacteria in a non-specific fashion. Amylase was typically the only protein partly remaining at the experimental endpoint. Many of the metabolites generated from the microbial breakdown of salivary protein are present in WMS, including SCFAs, glycine and phenylalanine. A depiction of relevant metabolic pathways is shown in Supplementary Figure 7. A number of additional metabolites not typically seen in healthy WMS were generated in this in-vitro model. These included amino-acid degradation by-products such as putrescine and 5-aminopentanoate and phenolic compounds such as 3-phenylpropionate and phenylacetate. Phenylacetate has been implicated as a biomarker in periodontal disease (Liebsch et al. 2019). This indicates the importance of ecological niche to the pathogenicity of oral bacteria. While all participants harboured oral bacteria capable of proteolysis
and generation of phenylacetate *in-vitro*, these metabolites were not detected in their baseline WMS samples, indicating an environment-dependant shift towards proteolysis.

As predicted by Neyraud & Morzel (2019), the tongue biofilm generated an abundance of metabolites, some of which have the potential to manipulate taste and oral perception. These include SCFAs, which have previously been inversely associated with oral sensitivity to oleic acid (Mounayar et al. 2014). A number of other amino acids with the potential to alter taste perception were also observed. The concentrations of glycine, valine, leucine, phenylalanine and proline produced following 24h in-vitro incubation, while higher than those generally found in saliva, were still below the respective detection thresholds. Nevertheless, local concentrations of such metabolites within tongue biofilm *in-vivo* might theoretically reach higher concentrations (Feron 2019). Interestingly, there were differences between the metabolic patterns of WMS and tongue biofilm inoculums. These differences may be attributable to different bacterial loads (WMS inoculums yielded significantly lower Log₁₀CFU/ml than posterior tongue biofilms). This might explain quantitative differences, however, qualitative differences in spectral profile of WMS and tongue biofilm inoculated samples were detected by PCA. Therefore, microbial compositional differences between the inoculum sources, as well as the planktonic nature of WMS and biofilm structure of tongue samples may be more important in shaping the net metabolic activity of oral bacterial niches. Differences in endogenous metabolite consumption between WMS and tongue biofilm inoculums were also found, in particular relating to lactate and pyruvate consumption. Alongside the *in-vivo* findings, this highlights the complexity of host-microbiome interactions in the oral cavity. For example, salivary lactate concentrations are in constant balance between delivery rate from host-glandular fluid, microbial consumption under fasted conditions and microbial generation upon exposure to exogenous nutrient sources. There are several limitations of this in-vitro study. Firstly, the experimental design represents a static nutrient pool, whereas even during sleep when salivation is minimal a degree of flux would occur in the oral cavity. Secondly, measurement of microbial diversity would ideally complement the metabolomic data. Few studies have done so to date, and this approach represents a useful future direction (Zaura et al. 2017).

With respect to *in-vivo* intra-oral catabolism of sucrose, this work unveiled some interesting findings. A similar metabolomic approach to saccharide metabolism in plaque has been reported (Takahashi et al. 2010), however the different analytical techniques allow for different molecules to be analysed. In the present work, salivary concentrations and outputs of molecules not always conventionally associated with glycolysis were observed, including alanine and acetoin. These differences likely serve to underline the central role of pyruvate in the oral metabolome. Pyruvate can be converted into both alanine and acetoin (March et al. 2002; Owen et al. 2002), as well entering the citric acid cycle or being converted to lactate. These latter metabolic events appeared to be associated with host sensitivity to sucrose. Lactate:pyruvate ratio in plasma is used as a medical parameter indicative of adverse metabolic events when raised. Lactate:pyruvate ratio has previously been analysed in parotid saliva following ingestion of sugars (Kelsay et al. 1972), although the aim was to investigate how it correlated with plasma lactate:pyruvate ratio. We found a significantly higher lactate:pyruvate ratio in relatively insensitive sucrose perceivers compared to sensitive perceivers. Conversely, citrate:pyruvate ratios showed the opposite relationship. These metabolic differences might be explained by differences in oral microflora. Streptococci such as *S. mutans*, which are efficient oral lactate producers, feature altered or absent citric acid cycles with a limited role in energy production (Ajdić et al. 2002). Therefore, sensitive sucrose perceivers could have a less lactogenic oral microbiome. Whether sensitivity to sucrose is associated with intake is controversial, involving genetic and environmental factors (Eny et al. 2010; Keskitalo et al. 2007). While some studies report no association (Cicerale et al. 2012), certain patterns of sugar
consumption in the form of soft drinks have been demonstrated to reduce sucrose sensitivity (Sartor et al. 2011). Thus, as speculated (Alcock et al. 2014), taste sensitivity may be associated with the oral microbiome leading to enhanced consumption of refined sugars which could ultimately lead to negative oral and systemic health consequences. Future work into the nature of intra-oral metabolite mediated host-microbiome interactions could potentially be adapted into functional measures of caries risk assessment. Such knowledge may also help clinicians appreciate the complex biological factors in explaining health behaviours as the dental profession collectively moves away from “patient-blaming” models of disease aetiology.

**Author contributions**

All authors contributed to the conception and experimental design of the work. AG collected all data. All authors contributed to data analysis. AG drafted the manuscript and all authors revised and approved the final version of the manuscript.

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**References**


Figure 1: Summary of bacterial protein consumption from parotid saliva. a. shows an example Coomassie stained polyacrylamide gel. Lane i. = unincubated parotid saliva, ii. = PBS inoculated, incubated parotid saliva, iii. = WMS inoculated parotid saliva, iv. = anterior tongue biofilm inoculated parotid saliva, v. = posterior tongue biofilm inoculated parotid saliva. Samples from two representative donors are shown on this gel. gIPRP = glycated proline-rich protein, PRP = proline-rich proteins. b. Protein consumption from the inoculated samples, measured relative to the unincubated parotid saliva (dotted line). AT = anterior tongue, PT = posterior tongue. c. Final log_{10} CFU/ml from the inoculated samples. Data in b. and c. are mean ± SEM, analysed by Tukey’s multiple comparison test following ANOVA (n = 6 samples per group). d. Correlation between protein consumption and final log_{10} CFU/ml from all inoculated samples, n = 18, measured by Pearson’s correlation.
Figure 2: PCA plot with k-means cluster analysis of metabolite profiles of inoculated parotid saliva. Statistical clusters are indicated by colour, and inoculum is indicated by size. There appears to be a degree of separation between WMS and tongue biofilm inoculums, with only 1 posterior tongue and 2 anterior tongue samples being clustered with WMS inoculums. There is no evidence of a distinction between posterior and anterior tongue biofilm inoculums.
Figure 3: Summary of data comparing microbial sucrose catabolism between sensitive and relatively insensitive sucrose perceivers. 

- **a.** Shows the sensitive and relatively insensitive perceiver groupings based on their gIVAS intensity ratings of 0.25 M sucrose.
- **b.** Shows the post-sucrose flow rate relative to control for both perceiver groups, which did not differ.
- **c.** and **d.** Depict the difference between relatively insensitive and sensitive sucrose perceivers for citrate:pyruvate and lactate:pyruvate ratios, respectively. Bar graphs display mean ± SEM, p-values are for two-tailed t-test. Based on the present sample (n = 9) a β value of 0.81 was calculated, at α = 0.05. n.s. = not significant.