CHAPTER

7

Summarizing discussion
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General discussion

The oral cavity is the site of complex interactions between the oral microbiota, salivary components and the host immune system [1-3]. Co-evolution of the oral bacteria and the human host has created an ecosystem that exhibits significant temporal stability and resilience to challenges [4-6]. However, factors such as diet, smoking and an insufficient level of oral hygiene can shift the ecosystem towards dysbiosis [7]. Dysbiosis can manifest through either compositional or functional changes in the oral microflora. This can lead to dental decay or induce a maladaptive inflammatory response which destroys the periodontium and eventually may cause tooth loss (i.e. periodontitis) [7-10]. Maintaining oral homeostasis is therefore crucial for the preservation of oral health [11]. However, the mechanisms acting to maintain homeostasis are not well understood [11].

The aim of this thesis was to describe the molecular and bacterial composition of the oral ecosystem in a healthy state, to examine the network of interrelations between salivary proteins and peptides, oral bacteria and the oral metabolome, and to assess the changes occurring in the ecosystem when it is faced with a specific challenge (i.e. induction of experimental gingivitis).

The objective of Chapters 2, 3 and 4 was to obtain insights into the natural variation in saliva in a systemically and orally healthy population. These chapters contain data acquired from samples collected during a cross-sectional, observational clinical study - Dutch Trial Register (NTR3649) - conducted at the Academic Center for Dentistry Amsterdam (ACTA) in 2013. The study protocol is described in detail in Chapter 2.

In Chapter 2 the analysis of a set of salivary proteins and enzymes relevant for oral health was described. The study population were systemically healthy young adults (N = 268) screened for suitability according to the criteria as proposed for the Dutch Periodontal Screening Index (DPSI). Volunteers were included if they had a DPSI < 3-. Exclusion criteria were: presence of overt dental caries, inter-proximal restorations between the first and second, or second and third upper molars, apparent oral lesions, a habit of smoking, infections, recent use of antibiotics, and use of anti-inflammatory drugs. Concentrations of mucins MUC5B and MUC7, secretory-IgA, lactoferrin, cystatin S, and albumin were determined using ELISAs. Lysozyme, chitinase, amylase, and proteases were measured with enzymatic activity assays. Salivary pH, buffered pH, flow rate and total protein content were also measured. The subset of health-related salivary functional proteins and enzymes analyzed is not exhaustive. Other salivary components which play important roles in oral homeostasis like agglutinin, statherin and lactoperoxidase were not determined. A selection of analytical targets had to be made, taking into account limited sample volumes and available resources.
First, the network of correlations between the analyzed proteins and enzymes was examined. While most proteins correlated negatively with salivary flow rate and positively with each other and with total protein content, there were some intriguing, flow rate-independent, negative partial correlations between specific components, such as between lysozyme and chitinase. While lysozyme possesses bactericidal activity, chitinase lyses fungal cell walls [12, 13]. This network of positive and negative correlations between functional salivary proteins may suggest that feedback and/or balancing mechanisms are present in the regulation of salivary composition.

Using Spectral Clustering, we found two subgroups within the study population. The smaller subgroup (N = 22) was differentiated from the rest of the study population by lower salivary pH and buffered pH, and higher MUC7 levels and lysozyme activity. In addition, a number of significant differences were observed between males and females. Salivary pH, buffering capacity, protein content, MUC5B, secretory-IgA, and chitinase activity were all lower in females compared to males, while MUC7 and lysozyme activity were higher in females (P < 0.05, FDR set to 5%). We speculated that these differences could be due to physiological factors, primarily the influence of sex hormones on gene expression in salivary gland tissues, as well as sexual dimorphism in the size of the glands between males and females [14-16]. These sex-related physiological differences in saliva may be a factor contributing to the higher caries prevalence observed in women compared to men [17, 18].

The data from the salivary functional protein biochemistry analyses were also used in Chapter 4 to construct the salivary ecosystemic network.

In Chapter 3 we analyzed the salivary peptide profiles of the 268 healthy volunteers using MALDI-TOF mass spectrometry (MS). Salivary peptides were purified and concentrated using Zip-Tips and spectra were acquired for m/z values between 2 – 15 kDa.

Spectral Clustering identified four subgroups within the study population. An Unsupervised Feature Selection algorithm revealed that these clusters were differentiated by the variation in nine peptide peaks. Five of the nine peaks (two different ionization forms and three different fragments) were linked to the same molecular entity, an acidic proline-rich protein named P-C peptide. P-C peptide is formed from precursors (PRP1, PRP2, Pif-f and Db) which show different functionality compared to the free P-C peptide [19]. The precursors possess a domain which facilitates attachment to the tooth surface. They play a role in mediating enamel hydroxyapatite crystal growth, as well as in the formation of the dental pellicle and subsequent bacterial adherence [20-22]. On the other hand, P-C peptide (which lacks the aforementioned binding domain) is thought to have a protective function against dietary tannins [23]. The relative amount of precursors and P-C peptide, and the P-C peptide fragmentation profile are determined by proteolytic events taking place both pre-secretion (inside the salivary glands and ducts, under the action of host proteases), as well as post-secretion (in the mouth, due to microbial proteases). Thus,
the pattern of these peptides may provide information on the physiological state of the salivary glands as well as on the functional composition of the oral microflora. This hypothesis is supported by the observation that the four clusters of volunteers discriminated by their salivary peptide profiles differ significantly with regard to two antimicrobial enzymes - lysozyme and chitinase ($P < 0.001$). These findings suggest that MALDI-TOF MS salivary peptide profiles may relate information on the underlying state of the oral ecosystem.

In Chapter 4 the ecosystemic network of saliva of healthy volunteers was examined. Microbial DNA was extracted from saliva and processed for amplicon sequencing of the V4 hypervariable region of the 16S rRNA gene on an Illumina MiSeq platform. Salivary metabolome data were obtained from three separate platforms (two LC/MS-MS and one GC/MS) [24]. The salivary microbiome data were integrated with the salivary metabolome data and the salivary functional protein data (previously described in Chapter 2). The heterogeneity of the microbiome, of the metabolome, and the mutual interrelations between these datasets and the host-related biological and environmental parameters were assessed.

The salivary microbiome appeared heterogeneous, forming five sample clusters. These microbiome clusters related to characteristic biochemical properties of saliva. The presence of ‘oral ecotypes’ and the links observed between the microbiome clusters and salivary functional proteins suggest a role of host factors in shaping the microbial community. One of the factors that related to the oral microflora composition was salivary pH, a known driver of microbial community activity and ecological shifts [25, 26]. Other interesting associations were found between salivary lysozyme activity and microbial composition. Lysozyme is an antimicrobial protein capable of lysing the bacterial cell wall peptidoglycan [27]. It was therefore surprising to find a positive correlation between salivary lysozyme activity and streptococci, which are Gram-positive. However, lysozyme is thought to also possess additional, Gram-independent mechanisms of action, such as aggregation of bacteria, which are subsequently cleared by swallowing [28]. A previous study showed that lysozyme adsorbs to oral bacteria in a strain-specific manner [29]. The lowest adsorption of lysozyme was observed for Streptococcus mitis strains [29]. Interestingly, the highly abundant OTU with the strongest positive relation with lysozyme activity in saliva was classified as Streptococcus dentisani/infantis/mitis/oralis or mitis group streptococcus (OTU4). These mitis group streptococci are known as primary colonizers of oral surfaces and are associated with oral health [30]. Higher abundance of mitis group streptococci was not only associated with higher lysozyme activity, but also with lower salivary pH. Since the optimum activity of lysozyme depends on pH and ionic strength [31], lysozyme activity, measured ex vivo at pH 7, might indicate a compensatory over-expression of lysozyme at a physiological salivary pH below the functional optimum of the enzyme.
The large inter-individual heterogeneity seen in the salivary microbiome did not translate to the salivary metabolome profiles, which were much more similar. This may imply that metabolism in overnight fasted saliva of different individuals carrying different microbiota is driven by similar processes, in line with the previously described functional redundancy of human microbial communities [32]. However, about one fourth of the individuals showed different (e.g. more proteolytic activity-driven) and more diverse metabolome profiles. These profiles related with several biochemical parameters of saliva, such as higher pH and higher albumin concentration, and with microbial communities dominated by anaerobes and Gram-negative taxa associated with mature oral microbial communities and gingival inflammation [33]. The positive relation between salivary albumin and pro-inflammatory metabolites in these saliva samples may suggest the presence of inflammation [34].

A clear dichotomy was observed when relating the microbial taxa with the metabolites. Either strong positive (such as for OTU66, *Megasphaera micronuciformis*), or strong negative correlations (such as for OTU4, *Streptococcus mitis* group) with the majority of the metabolites were observed. This suggests that during overnight fasting, when saliva is the main substrate for oral bacteria [35], there are metabolite ‘producers’ such as megasphaeraceae and metabolite ‘consumers’ such as streptococci. The complex microbial interactions required to break down salivary glycoproteins were confirmed by visualization of the complex networks of megasphaeraceae co-occurring with other taxa compared to the isolated position of streptococci.

Both the microbiome and the metabolome of saliva were gender-associated. The higher abundance of streptococci in female saliva could be a result of an ecological pressure due to physiologically lower pH [36]. On the other hand, the higher abundance of streptococci could directly contribute to a lower salivary pH, since streptococci are the main acid producers in a healthy oral cavity [37].

Brought together, these findings indicate that a healthy oral ecosystem is based on concerted interactions of its heterogeneous and multifactorial components. Data described in Chapters 2, 3, and 4 were obtained from a cross-sectional, observational study. As such, mechanistic explanations for observed correlations and trends may be suggested but causal links cannot be clearly drawn solely on observational data. Deciphering the mechanisms which enable the stability of this ecosystem will only be possible through longitudinal studies.

**Chapters 5 and 6** contain data acquired from samples collected during a challenge intervention, randomized study - Dutch Trial Register (NL51111.029.14) – conducted at the Academic Centre for Dentistry Amsterdam (ACTA) in 2015. The study population were systemically healthy adults (N = 61). The exclusion criteria were: a dental pocket probing depth of ≥ 5 mm with bleeding on probing and attachment loss ≥ 2 mm (DPSI 3+/4) [38], >
40% bleeding on probing, overt dental caries, smokers, prescribed medication, and evidence of systemic disease.

The challenge intervention was based on a full-mouth modified experimental gingivitis protocol [39]. The study design was composed of a two-week baseline period, followed by a two-week gingivitis induction period in which all participants were asked to refrain from any form of oral hygiene, and finally a one week resolution phase. The aim of the study was to observe the changes occurring in the oral ecosystem as a result of plaque accumulation and subsequent induction of inflammation. The rationale was to gain insights into the early stages of a shift of the oral ecosystem away from homeostasis by measuring the changes occurring in the salivary microbiome, metabolome, and functional biochemistry.

The treatment group (N = 20) was asked to take erythritol every day during the entire duration of the study. The control group (N = 41) did not take erythritol. Unstimulated saliva samples were collected from all participants at seven time-points during the study. The study protocol is described in detail in Chapter 5.

In Chapter 5 the changes occurring in the salivary functional biochemistry and the salivary metabolome during a 2 week-long experimental gingivitis induction were analyzed. The effect of erythritol, a polyol proposed to have a beneficial effect for oral health, was also examined in a randomized trial setting. Salivary functional proteins were measured using ELISAs and enzyme activity assays as previously described in Chapter 2 [40]. Salivary metabolome data were acquired on four different LC-MS/MS platforms, each optimized for a subset of metabolites with different physicochemical characteristics. Univariate mixed-design ANOVA models were constructed for each variable analyzed, with the challenge intervention (experimental gingivitis) as a within-subject factor and erythritol as a between-subject factor. FDR was used to adjust for multiple comparisons (FDR set to 5%).

Bacterial amino acid catabolites (cadaverine, N-acetylcadaverine, and α-hydroxyisovalerate) were found to increase, pointing towards an intensification of plaque metabolism. End-products of alkali-producing bacterial pathways (both γ-aminobutyrate and N-α-acetyltornithine) also increased, suggesting that some bacteria within the oral biofilm employed these pathways in order to adapt to a presumably lower pH in the maturing dental plaque. Increased bacterial amino acid catabolites and increased products of bacterial alkali-producing pathway suggest an up-regulation in oral plaque metabolism as the biofilm matured.

Mevalonate increased by 50%. The mevalonate pathway is known to be involved in anti-inflammatory and immunomodulatory effects [41, 42]. Significant changes were also found in a set of salivary metabolite ratios involving 13 host cell membrane lipids (eight sphingolipids and five glycerophospholipids) potentially involved in cell signaling, host responses to bacteria, and defense against free radicals [43]. The increase in
mevalonate – occurring within the first 2 days of experimental gingivitis induction – together with the changes in lipid ratios may be indicative of an early anti-inflammatory reaction by the host in response to changes in the quantity and functional profile of oral plaque.

No significant effects of the experimental gingivitis induction were found on any of the functional salivary proteins and enzymes analyzed. Similarly, no significant effects of erythritol on the salivary metabolome or on the functional salivary proteins were found. 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 - 3 years - was much longer [44, 45]. It is possible that any effect that erythritol may have on the oral ecosystem is dependent on longer exposure than used in the present study. Moreover, the present study was designed to observe changes during the induction of gingivitis, not to monitor caries progression.

In Chapter 6 the salivary peptide profiles of healthy volunteers were examined during a 2-week induction of experimental gingivitis. MALDI-TOF spectra were acquired as previously described in Chapter 3 [46]. The effect of erythritol was also assessed in a randomized trial setting. Mixed-design ANOVA models were constructed to analyze changes in each peptide peak.

No significant changes were found in any of the 80 peaks analyzed, with no significant effects of the experimental gingivitis challenge or of the erythritol treatment.

**Future research**

The studies described in the present thesis provide an extensive - yet by no means complete - description of the oral ecosystem and the network of interactions between its different components (Chapters 2, 3 and 4), as well as a glimpse into the dynamics of this system when subjected to a challenge (Chapters 5 and 6). It is clear, however, that our understanding of the intricate complexity underlying the oral ecosystem remains limited. An obvious next step in the continuation of the analyses presented here would be the integration of the data from the experimental gingivitis induction study (i.e. linking salivary microbiome data with metabolome and functional biochemistry data). This could provide more insights into the mechanisms involved in oral plaque maturation and adaptation and into the corresponding changes in plaque-host interactions. It is becoming more and more apparent that examining the composition of the oral microbiota is not sufficient, and that the key lies in examining microbiota functionality. Indeed, results presented in this thesis (Chapter 4) suggest that oral microbiomes with large differences in composition may in fact have very similar functional profiles. It is this functionality that needs to be thoroughly understood in order to be able to effectively manipulate the system into a desired direction.
or to determine the optimal way to prevent an unwanted shift. This may be achieved through the integration in future longitudinal and prospective cohort studies, combining metabolomic and metatranscriptomic analyses with clinical measurements and targeted functional biochemistry assays. In such studies, particular focus should be given to microbial pathways dealing with carbohydrate metabolism, protein and peptide degradation, and alkali production, as they appear to be of primary importance in determining oral ecosystem homeostasis [47]. The ultimate goal is to reach a level of understanding that can be successfully applied in the clinic: one can imagine personalized therapies for periodontitis patients or caries-prone individuals, designed to restore the specific dysfunction diagnosed in their oral ecosystem. The analytical technology needed is already available, yet rigorous study design and improved data processing methods and statistical approaches are needed to fully benefit from the wealth of data that can be produced.
References


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