Salivary biochemistry of the healthy oral ecosystem
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CHAPTER 1

Introduction
The oral ecosystem in health and disease: homeostasis and dysbiosis

A healthy mouth is necessary for a number of important functions such as chewing, speech, taste and social interactions [1]. The human oral cavity displays a unique set of environmental conditions and is home to a complex community of microorganisms [1-5]. This resident microflora is constantly modulated by intrinsic, host-related factors (e.g. saliva, host immune cells) and extrinsic factors (e.g. food particles, xenobiotics, oral hygiene measures). The oral ecosystem is more stable compared to those from other ecological niches in or on the human body [6, 7]. Its microbial composition is better maintained across time under normal conditions and is more resilient to challenges (such as treatment with antibiotics) compared to the gut microbiome [6]. The oral microbiota shows less deviation and recovers faster than the gut, skin, or vaginal microflora [6, 7].

In spite of the aforementioned relative stability of the oral ecosystem, dysbiosis may occur and exert potentially severe negative effects on oral health. Dental caries and periodontitis – the most frequent oral diseases – do not fit the model of single microbe pathogenesis, but may rather be viewed as resulting from severe dysbiosis occurring within the mouth [4].

Dental caries is a multifactorial disease involving excessive demineralization and subsequent localized destruction of tooth hard tissue [8]. Cycles of demineralization and remineralization continuously occur under normal conditions, but certain factors may permanently upset this equilibrium and tilt the process towards an irreversible degradation of tooth structure (Fig. 1).
Figure 1. Illustration of ecological pressures altering the composition of oral microflora leading to oral disease (in this case, dental caries) (from Marsh, 2006 [9]).

Foremost among cariogenic factors is a diet rich in easily fermentable carbohydrates (e.g., sucrose). It favors the selection of acidogenic and aciduric bacteria and therefore shifts the oral microbiome towards a cariogenic type [10]. The bacterial biofilm attached to the surface of the tooth is directly responsible for cariogenesis. When provided with fermentable sugars, it produces and releases acids, causing a localized drop in pH and accelerating demineralization. Poor oral hygiene is also a major risk factor [11]. Dental caries is among the most prevalent chronic diseases, and therefore has a significant detrimental effect on well-being and imposes a considerable economic burden [12, 13].

Periodontitis is a bacterial biofilm-induced chronic inflammatory disease [14]. It can eventually destroy the connective tissue and alveolar bone surrounding and supporting the teeth (i.e. the periodontium) and ranks as the number one cause of tooth loss worldwide [15]. The tissue damage is caused not only by the action of a dysbiotic bacterial biofilm, but also by the host immune reaction to the microbial challenge, which may in fact be the primary effector [15]. An increase in the production of inflammatory cytokines disrupts bone homeostasis and is a major contributor to the loss of periodontium [14, 15]. Periodontitis is often preceded by gingivitis, a reversible inflammation of the gingiva caused by the accumulation of supragingival plaque [16].
Chapter 1

It can be ascertained that a healthy mouth depends on oral host-microbiome homeostasis. Homeostasis is defined by an intricate network of relationships and interdependencies. The next chapters will examine some of the main components of the system (i.e. salivary proteins and peptides, the oral microbiome, and the oral metabolome), determine their boundaries, and describe the interactions between them when at health.

**Saliva and oral health**

Saliva is a mucouserous fluid secreted by three pairs of major salivary glands (parotid, submandibular and mandibular), as well as by minor glands on the lower lip, tongue, palate and cheeks [17-19]. Saliva production begins within specialized acinar cells contained inside the glands, after which salivary secretions are transported and further modified along the salivary ducts [18]. Whole saliva is a complex mixture composed of secretions from the salivary glands, gingival crevicular fluid, food debris, and components derived from oral microflora as well as from exfoliating oral mucosal cells and host immune cells [18, 20].

Saliva is essential for oral health. This can be inferred by observing the consequences of insufficient salivary production. Salivary secretion impairment may be caused by Sjögren’s syndrome (an autoimmune condition), radiation therapy, but it most commonly appears as a side-effect of xerostomic medications [21]. Regardless of etiology, the effect is highly damaging: oral soreness and discomfort, loss of taste, difficulty in swallowing, increased caries, candidiasis [21].

One of the primary roles of saliva is to minimize and repair acid-induced demineralization of the tooth enamel [18]. The hydroxyapatite component of dental enamel begins to demineralize when the pH of saliva drops below 5.0 – 5.5. Saliva minimizes the effect of acid challenges through four different buffer systems: bicarbonate/carbonate, phosphate, urea, and via the side chains of the salivary proteins [1, 18]. However, the main buffering mechanism in saliva is the bicarbonate/carbonate system [22]. Salivary urea is metabolized by oral bacteria with production of ammonia and subsequent pH increase [23, 24]. Saliva is supersaturated in calcium and phosphate ions which remineralize the enamel when the salivary pH rises back to normal values and are crucial for the long-term preservation of the tooth integrity [25].

Saliva bathes and lubricates oral surfaces, providing both protection against mechanical wear and a superficial layer shielding against acids, toxins and bacterial proteases [26, 27]. The main component enabling this function is mucin MUC5B, a very large (>1 MDa), oligomeric and highly glycosylated protein, with covalently attached sugar moieties comprising 80% of its molecular mass [1, 22]. It is a major component of whole unstimulated saliva, making up 20-30% of the total protein content [22]. The functionality of MUC5B is directly linked to its structure. MUC5B’s large size and filament-like form, together with a large hydrophilic carbohydrate component, are responsible for the
characteristic viscoelastic, coating, and lubricant properties of saliva [1]. The other salivary mucin, MUC7, is smaller (around 200 kDa), monomeric, but also rich in carbohydrate side chains. MUC7 contributes to the aggregation and clearance of bacteria, as it is able to bind to a variety of oral bacteria [1, 22].

Saliva actively aids digestion. Amylase is one of the most abundant proteins in saliva, and its primary function is to initiate the digestion of starch [1, 28]. However, amylase is also a component of the dental pellicle and mediates bacterial adhesion to the tooth surface [28, 29].

Saliva contains a number of antimicrobial proteins and enzymes [1]. Secretory-Immunoglobulin A is the predominant immunoglobulin in saliva, where it binds and aggregates bacteria, viruses, and toxins [18, 30]. Lactoferrin is a multifunctional protein: it sequesters iron – a limiting factor for bacterial growth – thus providing an important bacteriostatic effect, but also releases bactericidal peptides when subjected to proteolysis, and may have other iron-independent antimicrobial functions [1, 31]. Lysozyme is an enzyme that hydrolyzes peptidoglycan, thus cleaving Gram-positive bacterial cell walls leading to cell lysis and death [1, 18, 32]. It is also thought to have non-enzymatic killing effects on Gram-negative bacteria [33]. The enzyme chitinase has a similar effect on yeast cell walls [34]. Cystatins, though not antimicrobial, may have a protective effect by inhibiting the damaging action of some bacterial proteases [35]. Many of the salivary antimicrobial proteins and enzymes act through several different mechanisms and have not only additive, but also synergistic effects, forming a complex defensive functional network [1].

Salivary albumin is not secreted by the glands, but originates from plasma via gingival crevicular fluid and mucosal leaking. It is therefore a useful marker for oral lesions and bleeding.

**Saliva as a research medium: opportunities and caveats**

As a research medium, saliva presents a number of advantages. Salivary collection is quick, simple, non-invasive, and poses no risk of adverse effects for the donor [36]. Indeed, several studies have focused on saliva in order to develop biomarkers and diagnostic tests for diseases ranging from periodontal disease and Sjögren’s syndrome to oral and breast cancers [36, 37]. Saliva is a prime candidate as a medium for the study of oral health, due to its capacity to supply quick and accessible biological information derived from multiple levels of the oral ecosystem.

However, using saliva as a research and diagnostic material involves some particular challenges. Salivary secretion is a process influenced by both the sympathetic and the parasympathetic branches of the nervous system [18, 19]. Salivary flow rate and composition can be drastically altered by stimuli such as mechanical movement (chewing,
speak) or gustatory triggers [19]. Moreover, other factors such as age, various
medications, length of time elapsed since the last stimulation, and phase of the circadian
rhythm can also have a significant effect [38-41]. It is therefore very important to carefully
control all these factors at the time of saliva sampling in order to obtain meaningful and
reproducible data.

Another caveat lies within the sequence of procedures used to process and store
saliva prior to analysis [20, 42, 43]. The varying turbidity of sampled saliva caused by the
presence of cellular debris and potential food residues means that centrifugation is
generally used to clarify saliva and remove these unwanted components as well as other
insoluble aggregates. The exact conditions of clarification may influence salivary
composition [20, 42].

Finally, saliva is prone to rapid changes and degradation if improperly processed
and stored. The presence of numerous proteases in saliva makes it inherently unstable at
room temperature. It is therefore crucial that saliva samples are collected and processed on
ice and subsequently frozen at -80°C as quickly as possible [20, 42].

**Oral bacteria and oral health**

Although the oral cavity is home to bacteria, archaea, fungi, protozoa, and viruses, most
research has generally focused on the bacteria, with far less information available on other
microorganisms [2, 44]. In this thesis, ‘microbiome’ and ‘microflora’ are subsequently used
to refer strictly to bacteria.

There are currently around 700 species/phylotypes of oral bacteria catalogued in
the Human Oral Microbiome Database (www.homd.org) [45]. Depending on the
methodology, the actual total number of species is estimated to be several thousand [46].
Although there is substantial variation between individuals with regard to the composition
of the oral microbiome, studies have found that some taxa form an oral ‘core microbiome’
[3, 7, 47]. These predominant taxa include Firmicutes (e.g. genus *Streptococcus, Veillonella*),
Proteobacteria (e.g. *Neisseria, Haemophilus*), Actinobacteria (*Corynebacterium, Actinomyces*), Bacteroidetes (*Prevotella, Porphyromonas*), and Fusobacteria (*Fusobacterium*) [3].

Commensal oral microflora play an important role in maintaining oral health [2,
48]. First of all, they inhibit colonization by pathogens, utilizing several mechanisms for
colonization resistance [2]. They also convert dietary nitrate to nitrite, which is
subsequently converted to nitric oxide, an anti-hypertensive molecule important for
vascular health [49].

The composition and behavior of oral bacteria is shaped by the conditions inside
the oral cavity: constant presence of liquid water (from saliva), extreme short-term
temperature fluctuations, and wide variations in the availability of carbon and nitrogen
substrates [50]. An important characteristic of oral bacteria is their tendency to form biofilms (e.g. dental plaque) [9, 51]. Oral biofilms are species-diverse and structurally and functionally organized [9]. The biofilms form in a sequence of stages: pellicle formation (from salivary components), reversible adhesion of initial colonizers (*Streptococcus gordonii*, *Streptococcus mitis*, *Streptococcus oralis*, *Streptococcus sanguinis*), followed by stronger, adhesion/receptor-mediated bacterial attachment [9, 50]. Subsequently, secondary colonizers also attach and exopolysaccharides are synthesized, strengthening the biofilm structure. There is extensive metabolic interaction, cell-signaling and cross-talk between the different bacterial species within a biofilm [52]. Fragments of the biofilm may detach from the surface and get swept up by saliva [9].

Oral bacteria use saliva as a primary nutrient substrate [53]. The major salivary glycoproteins (e.g. mucus) are an important source of carbon. Although the sugar groups on these proteins are highly diverse and thus require an extensive enzymatic apparatus in order to be fragmented, oral bacteria collaborate and work sequentially in order to catabolize them [53, 54]. The overall composition of available substrates and the functional profile of the bacterial communities metabolizing these substrates determine a distinctive signature of small molecules in saliva – the salivary metabolome.

The oral cavity contains several ecological sub-niches (e.g. teeth, tongue, cheeks, hard and soft palates), either on the surfaces of the teeth or on mucosa [3]. Each sub-niche may offer different conditions (pH, oxygen and nutrient availability, redox potential) and select for a specific type of bacterial community [3, 51]. In turn, the metabolic activity of the resident microbiota can modify conditions at the respective site [55]. Saccharolytic bacteria in supragingival sites produce acids and lower local pH. Non-mutans streptococci and *Actinomyces* dominate in healthy supragingival plaque. Some conditions (e.g. high frequency of carbohydrate exposure) can select for more acid-tolerant bacteria and more efficient acid producers such as *Streptococcus mutans*, lactobacilli or *Bifidobacterium*, disturbing enamel mineralization homeostasis and leading to irreversible demineralization and dental caries [9, 51]. On the other hand, asaccharolytic bacteria in subgingival sites secrete nitrogenous catabolites derived from gingival crevicular fluid, thereby raising local pH [55]. *Fusobacterium* and *Prevotella* in subgingival plaque increase pH and stimulate the flow of gingival crevicular fluid [55]. This favors acid-intolerant, proteolytic species associated with periodontitis [55].

The oral microbiome is characterized by high taxonomic diversity, complex networks of inter-bacterial and bacterial-host interactions, and functional complementarity and redundancy. These traits may partly explain the remarkable stability and resilience of the oral ecosystem [44]. However, the mechanisms associated with homeostasis and therefore with oral health have yet to be fully understood [44].
Objectives

This thesis aimed to describe the boundaries and interrelationship between the salivary proteins, the salivary microbiome, and the salivary metabolome of a healthy oral ecosystem, and to examine the changes occurring within these levels of the ecosystem when a challenge is applied (i.e. experimental gingivitis). The overall purpose was to increase our understanding of the oral ecosystem and to gain insights into the processes involved in maintaining oral health.
Thesis outline

In Chapter 2 the variation and relationships between a set of salivary proteins and enzymes with known relevance for oral health were analyzed using targeted antibody- and enzyme activity-based assays. Subgroups in the sample population of young healthy adults and sex-related differences in salivary biochemistry were also examined.

In Chapter 3 the variation in salivary peptide profiles in healthy adults was characterized using MALDI-TOF mass spectrometry. Subgroups of individuals were defined based on these profiles and the functional differences between them were assessed. The subset of peptides responsible for discriminating the subgroups was identified and the possible mechanisms were discussed.

In Chapter 4 an ecosystemic network of the healthy mouth was built by integrating and interrelating salivary functional biochemistry with salivary microbiome and salivary metabolome data. Metabolome data were acquired using a multi-platform mass spectrometry-based approach, while microbiome data were obtained by 16S rRNA gene amplicon sequencing.

In Chapter 5 the changes occurring in the salivary functional biochemistry and the salivary metabolome during a challenge intervention in healthy adults were examined. The challenge was a 2-week induction of experimental gingivitis. The effect of erythritol was also assessed in a randomized trial setting.

In Chapter 6 the changes occurring in the salivary peptide profiles during a 2-week induction of experimental gingivitis in healthy adults were examined. The effect of erythritol was also assessed in a randomized trial setting.

In Chapter 7 a summary of the findings was compiled together with a general discussion and an outlook for future studies.

The studies encompassed in this thesis were performed as part of the ‘Novel strategies to promote oral health’ project within the framework of TI Food and Nutrition. Data were acquired from two separate clinical studies: a cross-sectional observational study aimed at estimating the boundaries of a healthy oral ecosystem (Chapters 2, 3, and 4) and a challenge intervention, randomized clinical trial exploring the dynamic interactions in the oral ecosystem during the induction of mild gingival inflammation (Chapters 5 and 6).
Chapter 1

References


Chapter 1


