Growth and treatment of oral biofilms

Fernandez y Mostajo, M.

Publication date
2017
Document Version
Final published version
License
Other

Oral microbiology - Periodontology

Amsterdam
20 April 2017

Growth and treatment of oral biofilms

Mercedes Fernandez y Mostajo

Growth and treatment of oral biofilms

Mercedes Fernandez y Mostajo

Oral microbiology - Periodontology

Growth and treatment of oral biofilms

Mercedes Fernandez y Mostajo

Paranymphs:

Joyce van de Horst

Wendy de Wit

The thesis was financed with a commission from ACTA Dental Research BV.

ACTA Research BV received financial support from NGen Oral Pharma N.V. (Curacao) for the role of the Departments of Periodontology and Preventive Dentistry of ACTA in this project. NGen Oral Pharma N.V. provided study products. The author reports that this company had no influence on the design, content, results and publication of any chapter included in this thesis.

Publication and ceremony of this thesis has been made possible due to the contribution of

Research Institute

Title: Growth and treatment of oral biofilms

Cover design and layout: Pilar Montesinos (pilar.mro@gmail.com) based on www.webdesignhot.com template.

Printed by: GVO drukkers & vormgevers B.V.

ISBN: 978-94-6332-164-8

Growth and treatment of oral biofilms

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor

aan de Universiteit van Amsterdam

op gezag van de Rector Magnificus prof. dr. ir. K.I.J. Maex

ten overstaan van een door het College voor Promoties ingestelde commissie,

in het openbaar te verdedigen in de Agnietenkapel

op donderdag 20 april 2017, te 14:00 uur

door

Mercedes Fernandez y Mostajo

geboren te La Paz, Bolivia

Promotiecommisie:

Promotores:

Prof. dr. W. Crielaard	Universiteit van Amsterdam
Prof. dr. G. A. van der Weijden	Universiteit van Amsterdam

Copromotores:

Prof. dr. E. Zaura	Vrije Universiteit Amsterdam
Prof. dr. W. Beertsen	Universiteit van Amsterdam

Overige leden:

Prof. dr. B. G. Loos	Universiteit van Amsterdam
Prof. dr. D. Wismeijer	Vrije Universiteit Amsterdam
Prof. dr. C. van Loveren	Universiteit van Amsterdam
Dr. S.A.J. Zaat	Universiteit van Amsterdam
Prof. dr. G. N.Belibasakis	Karolinska Institutet, Stockholm Sweden

Faculteit: Tandheelkunde

Table of Contents

Chapter 1

General introduction

GENERAL INTRODUCTION

✓ Polymicrobial oral biofilms

The human microbiome is the collection of micro-organisms that live in and on the body, and contains representatives from all domains of life: Archaea, Bacteria and Eukaryota. One may not realize that our body contains approximately as many human cells as bacterial cells (1:1 ratio) (Sender et al., 2016). Bacteria of the human microbiome are diverse and many species have beneficial functions, e.g. protect the symbiotic balance between the host and oral bacteria from exogenous organisms. Although there is an anatomical connection for bacteria from one site to another within the body (mouth, gut, skin) the microbiome of these environments is different. This is a clear example of how a specific niche with specific physico-chemical properties and biological characteristics will lead to a different microbial composition.

In this thesis we focused on bacteria of the oral cavity, which form a community of organisms in conjunction with nonliving components (teeth, implants, prosthetic devices), interacting as a system. The mouth is a nutrient-rich, warm and humid environment in which polymicrobial biofilms (consortia) are formed in an organized manner. Biofilms form on all surfaces, i.e. both on teeth and oral implants (Marsh et al., 2011). The availability of nutrients and the niche in this ecosystem (teeth, sulcular crevice, periodontal pocket, tongue, and other mucosal surfaces) will influence and determine the bacterial composition (Marsh, 2004). It has been demonstrated that the composition of bacterial communities varies among individual sites and different micro-niches, e.g. lingual and vestibular sides of a tooth (Simon-Soro et al., 2013).

✓ Decades of limited insight due to incomplete microbial detection techniques

For decades researchers have been limited in studying this ecosystem due to the microbial detection techniques available at the time. Traditional techniques for microbial identification, so called 'targeted' techniques, have the "bias" of detecting what was known based on culturing. Cultivable species provided at first the basis for the selection of the probe panel needed for molecular techniques, restricting to a pre-selection of target organisms, e.g. checkerboard DNA-DNA hybridization, PCR, quantitative PCR. The emergence of 'open-ended' techniques offered to overcome this bias. These new techniques, called Next Generation Sequencing (NGS), like 454 pyrosequencing, have recently contributed, and greatly enhanced our understanding of oral polymicrobial communities. These techniques are in continuous development and improve along with progression in bioinformatics software (Zaura, 2012). New technologies have provided novel insights into how dental plaque functions as a biofilm.

✓ Living in harmony with the host - symbiosis

Symbiosis is a close and often long-term interaction between two or more different biological species (Lamont and Hajishengallis, 2014). Throughout life the oral microbiome lives in close

relationship with the host and it is exposed to environmental changes that have an impact on its composition i.e. due to aging or changes in diet. The ability of the (oral) ecosystem to adapt to changes is known as oral allostasis (Zaura and ten Cate, 2015). Moreover, the oral microbiome is continuously exposed to host and environmental factors, such as saliva, oxygen, diet and oral hygiene measures. The healthy oral microbiome has the ability to deal with these perturbations and recover; this is known as resilience (Rosier et al., 2014). In chapter 2 an overview of the healthy oral microbiome will be provided, describing how NGS approaches have changed the concept of the diversity and composition of the human oral microbiome.

✓ The broken balance - dysbiosis

The healthy oral ecosystem (host-microbe homeostasis) is able to recover from daily challenges (resilience), such as diet. Currently it is recognized that several host – and microbial factors contribute to this homeostasis (Meyle and Chapple, 2015). Poor control of the supragingival biofilms (dental plaque) for prolonged periods of time results in inflammation of the soft tissues (Lang et al., 2009). In the dento-gingival area this results in gingivitis (Fig. 1.1). In the implanto-mucosal area, this inflammation is termed mucositis (Renvert and Polyzois, 2015). These diseases can remain for a prolonged period of time.

The progression of the periodontal diseases is highly depending on susceptibility of the host and risk factors and a multitude of immune-inflammatory interactions. In some individuals gingivitis does not progress to periodontitis. From the ecological perspective this is called “incipient dysbiosis”. On the other hand, in highly susceptible patients only mild biofilm accumulation appears to exceed the threshold necessary to trigger a destructive host response and subsequent tissue damage.

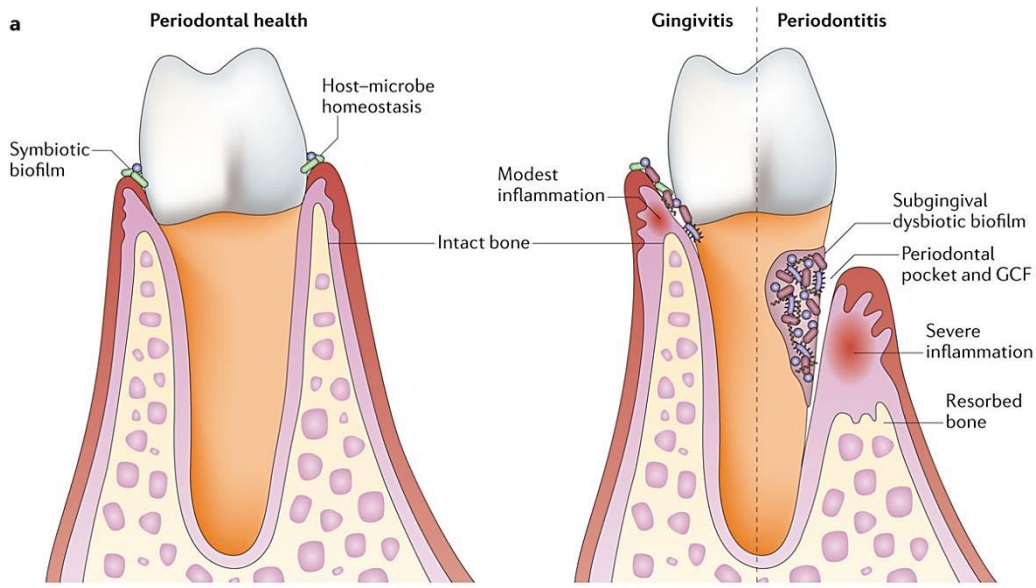


Figure 1.1. Polymicrobial synergy and dysbiosis in periodontitis.

Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology From: Periodontitis: from microbial immune subversion to systemic inflammation; Author: George Hajishengallis. 15, 30–44 (Hajishengallis, 2015), copyright 2015

✓ Dysbiotic periodontal microbiome

Experimental research has demonstrated that - clinically - gingivitis manifests itself after days or weeks of biofilm accumulation (Theilade, 1996, Loe et al., 1965), while changes in the underlying periodontal tissues (e.g. bone level) may occur in months or years (depending on the susceptibility of the host) and result in the development of periodontal pockets (Lang et al., 2015, Lang et al., 2009). While clinically the periodontal pocket in combination with clinical attachment loss represents the severity of periodontal tissue breakdown, from the ecological perspective it is a protective niche for microbial growth.

The availability of nutrients, redox potential and oxygen levels depend on several factors (among which pocket depth and local immune response) and will affect the microbial composition (Zhuang et al., 2014). Although periodontitis is initiated and sustained by dysbiosis in the subgingival microbiome, the progressive nature of the disease is determined by the susceptibility of the host (Meyle and Chapple, 2015).

Besides expanding our knowledge on the diversity of complex ecosystems, NGS techniques have also changed our concepts of the pathogenesis of periodontal diseases, in which bacteria play an important role. Culture-dependent and targeted methods for the characterization of the periodontal microbiota have focused on 'periodontal pathogens': a triad of oral anaerobic bacteria that comprises *P. gingivalis*, *Treponema denticola* and *Tannerella* forsythia which have traditionally been considered as "putative periodontal pathogens", based on their virulence properties and strong association with diseased sites (Socransky et al., 1998). It is of note that, with open-ended techniques, the same species appear to be associated with periodontal disease (Griffen et al., 2012), a more holistic concept of bacterial communities in contrast to the single-pathogen concept has provided a more ecological view with respect to the etiology of oral diseases.

✓ Dysbiotic peri-implant microbiome

Osseointegrated dental implants are currently a well-established treatment option in dentistry. As soon as the (healing) abutment on an implant is exposed to the environment, a so-called 'pristine pocket' is formed and immediately colonized by microorganisms attaching to the implant abutment surface, resulting in a polymicrobial biofilm. A climax community is reached within 2 weeks (Quirynen et al., 2006).

Polymicrobial biofilm accumulation on implants is considered a risk factor for peri-implant mucositis (Renvert and Polyzois, 2015). Although peri-implant diseases are microbial induced diseases, the pathogenesis of the disease is complex. Other factors have been shown to contribute to disease etiology such as the surface material of the dental implants, topography, surface roughness, surface tension, alloy composition and biocompatibility (Renvert and Quirynen, 2015). Even though these factors can be interrelated, each of them can independently influence not only bacterial colonization but also the reaction of the host cells. Other risk indicators, e.g. smoking, play an important role in the pathogenesis of peri-implant diseases (Renvert and Quirynen, 2015).

It is generally accepted that mucositis is the precursor of peri-implantitis (Jepsen et al., 2015) as gingivitis is for periodontitis. Therefore, the prevention and treatment of peri-implant mucositis is of great interest (Jepsen et al., 2015).

✓ Periodontal versus peri-implant microbiome

The inflammatory reactions surrounding dental implants are clinically visualized by a change in mucosal color and contour and bleeding on gentle probing (<0.25 N) based on which the diagnosis of peri-implant mucositis can be established. Many bacterial species of the identified bacteria, cultivable and non-cultivable, have the potential to trigger an inflammatory state in susceptible individuals. The inflammatory state can further progress and affect the bone. The more severe form of inflammation called 'peri-implantitis' is described as "inflammatory reactions associated with the loss of supporting bone around a functioning implant" (Zitzmann and Berglundh, 2008). A meta-analysis estimated a weighted mean prevalence of peri-implant

mucositis of 43% (CI: 32–54%) and for peri-implantitis of 22% (CI: 14–30%) (Derks and Tomasi, 2015). Since mucositis is the precursor to peri-implantitis, the prevention and treatment of peri-implant mucositis is of primary interest (Jepsen et al., 2015).

It has been shown that the subgingival microbiomes of periodontal and peri-implant niches are compositionally different and that the microbial diversity in periodontitis is higher than in peri-implantitis (Dabdoub et al., 2013, Kumar et al., 2012). Although periodontitis and peri-implantitis are considered different diseases (Robitaille et al., 2016), the current treatment strategies for peri-implantitis are based on the knowledge related to the treatment of periodontal diseases. Hence, there is an urge to increase our knowledge of the etiology and treatment of peri-implant diseases.

Given the fact that periodontitis and peri-implantitis biofilms have different microbial composition, treatment approaches based on the outcomes of the treatment of the periodontal disease might be debatable for peri-implant disease.

✓ Restoring the balance

Thus, understanding the composition of the periodontal and peri-implant microbiota and their interaction with the host will give new insights into the role these microbial communities play in health and disease. Possibly this could lead to novel antimicrobial or therapeutic strategies aimed at correcting dysbiosis and restoring the healthy periodontal or peri-implant microbiome.

Regular mechanical removal of biofilms from all non-shedding oral surfaces is the primary prerequisite to prevent disease. Toothbrushing, the mechanical removal of dental plaque, is the most important tool available to control the outgrowth and maturation of oral biofilms. A recent systematic review (Slot et al., 2012) reported that a 42% plaque score reduction was achieved using a manual toothbrush. Rosema et al. (Rosema et al., 2014) evaluated the effectiveness of a single powered brushing episode, which resulted in an average weighted mean plaque score reduction of 46%. These results taken together and the fact that a powered toothbrush is not for all individuals affordable, there is an urge to improve our methods in controlling dental plaque (biofilms). To further improve the efficacy of plaque removal, interdental brushes could be advised (Van der Weijden and Slot, 2015). Chemical agents can be used as an adjunct to self-performed oral hygiene when proper toothbrushing cannot be achieved or because of inadequate skills or lack of compliance (Van der Weijden et al., 2015).

From an ecological perspective, an antimicrobial agent should not eradicate the commensal oral microbiota. Instead, it should maintain the microbiota of the mouth at the level and composition that is compatible with oral health, this way preserving the beneficial functions of resident microbes (Marsh,2012; ten Cate and Zaura, 2012). Currently, there is a wide range of over the counter mouthwash products available, containing various active ingredients with for each of these specific indications. Some of these mouthwashes have no scientific background for their claims.

✓ Studying antimicrobial strategies *in vitro*

The evaluation of novel active agents and formulations in human in properly designed randomized clinical trials (RCT's) is time-consuming and has many difficulties as it relies on the compliance of the volunteers. It is also impossible to test new compounds in humans from which no evidence of effectiveness or safety is available.

Consequently, *in vitro* biofilm models have been developed, which are easier to control when testing a specific variable. Various supragingival and subgingival plaque biofilm models have been described to study biofilm formation and antimicrobial approaches. *In vivo*, oral biofilms are of polymicrobial nature. Therefore in this thesis we focused on polybacterial biofilm models. There are supragingival and subgingival biofilm models that use a pre-selected consortium of cultivable bacteria (Shapiro et al., 2002). However *in vivo*, oral biofilms are far more complex. To overcome this problem, several workers in the field have used saliva as inoculum (Kistler et al., 2015). Recently it has been shown that saliva poorly represents the bacterial composition of biofilms grown on teeth. Therefore we have developed a biofilm model using dental plaque as inoculum.

✓ ACTA active attachment model (AAA-model)

For studying subgingival biofilms, various insert materials have been used, placing into periodontal pockets of periodontitis patients (Zijnge et al., 2010). This approach represents an invasive method in a periodontal pocket, which can be of a problem when recruiting volunteers and has ethical limitations. This AAA-model has been developed at ACTA (Fig. 1.2), primarily to study caries-related biofilms (Deng et al., 2009, Exterkate et al., 2010). It is a model which allows for complex polymicrobial biofilm formation depending on the active attachment of bacteria to a substratum. One advantage of this model is that various types of substrata can be used for the screening or comparison of many antimicrobial compounds at the same time.

Figure 1.2. Active attachment model

Outline of this thesis

The overall aim of this PhD research was to study the biodiversity of oral microbial communities both *in vivo* and *in vitro* with an underlying focus on antimicrobial treatment of periodontal diseases.

The following questions will be addressed:

1. To what extent have NGS approaches changed our concept of the diversity and composition of the human oral microbiome? (**chapter 2**)
2. Currently, there is a wide range of over the counter mouthwash products available, containing various active ingredients with for each of these specific indications. Can we use a microcosm biofilm model to assess the effect of "new" mouthwashes on bacterial composition? (**chapter 3**)
3. Does an oxygenating mouthwash (Ardox-X, commercially known as O7 active®) have the potential to affect the microbial composition of dental plaque in healthy volunteers? (**chapter 4a**)
4. In the dental office, the presence of dental plaque is commonly made visible to the patient using a disclosing solution (i.e. erythrosine or a two-tone plaque disclosing solution). The use of a QLF-camera has been recently introduced as a diagnostic method in the dental office (20). Can red autofluorescing plaque be used to assess dental plaque? (**chapter 4b**)
5. Given the high diversity of the periodontal microbiota, can we develop a reproducible subgingival microcosm biofilm model? (**chapter 5**)
6. Can we further develop a subgingival peri-implantitis-derived biofilm model?

   Will this model be appropriate for antimicrobial testing of new compounds? (**chapter 7**)
7. In European countries such as the Netherlands, dental care professionals use an available targeted method to determine the subgingival composition of periodontal pockets from periodontitis patients in order to guide their (antimicrobial) therapy. The question that is discussed is: Does the routine analysis of subgingival microbiota in periodontitis patients using traditional techniques (not open ended) contribute to patients benefit? (**chapter 6**)

Chapter 2

The oral microbiome

This chapter has been published as:

Zaura, E., Fernandez y Mostajo, M., Koopman, M. J. & Crielaard, W. (2014)

In: *The Human Microbiota and Microbiome*, (ed.) Marchesi J.R., pp. 20 - 31. Cardiff CAB International. ISBN-13: 978-1780640495

INTRODUCTION

The human oral microbiome is defined as all the microorganisms that are found on or in the human oral *cavity* and its contiguous extensions such as tonsils and pharynx (Dewhirst et al., 2010). The oral cavity includes several distinct microbial habitats, such as dental surfaces and gingival crevice or sulcus, tongue (its papillae and crypts), and different keratinized (gingival and hard palate) and non-keratinized (cheeks, lips, soft palate) mucosal surfaces. Each of these habitats contains specific ecological niches that select for distinct microbiota (Aas et al., 2005; Zaura et al., 2009).

The oral microbiome is the most extensively studied part of the human microbiome. In 1683, Antonie van Leeuwenhoek in his letter to the Royal Society described dental plaque as "an unbelievably great company of living animalcules" (van Leeuwenhoek 1683). Since the discovery of van Leeuwenhoek we have learned how to culture, isolate and characterize hundreds of oral microbial species. With the advent of culture-independent techniques based on molecular phylogeny of microbial DNA we have obtained information on (so far) unculturable oral microbiota. This chapter will give an overview on diversity and composition of the human oral microbiome in the healthy setting.

Diversity of human oral microbiome

Bacteria

One milliliter of human saliva from a healthy adult contains around 100 million bacterial cells. At normal salivary flow rate, which is 750 ml per day, about 8 x 10^{10} bacteria are shed from the oral surfaces every 24 hours (Curtis et al., 2011). Approximately 280 bacterial species from the oral cavity have been isolated in culture and formally named, while about 360 oral species or phylotypes have been identified only by cloning and sequencing of bacterial 16S rRNA gene (Dewhirst et al., 2010). These bacteria are classified based on the phylogenetic similarity with their culturable relatives. Their sequences are deposited in publicly available databases, *e.g.*, the Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu/) which consists of over 2.1 million aligned and annotated bacterial and archaeal 16S rRNA sequences (Release 10.28) (Cole et al., 2009) or the NCBI GenBank Nucleotide Collection (http://blast.ncbi.nlm.nih.gov/) containing 2019 bacterial and 106 archaeal full genomic sequences (25 January 2012). Next to these general databases several curated database projects specific for oral microbial ecosystem have been funded. The Human Oral Microbiome Database (HOMD, http://www.homd.org/) (Chen et al., 2010), developed at The Forsyth Institute, contains 640 microbial taxa (URL at http://www.homd.org/ accessed on 25 January 2012) that have been associated with the human oral cavity and is based on a curated 16S rRNA gene-based provisional naming scheme. This database interlinks phenotypic, phylogenetic, genomic, clinical and bibliographic information for each taxon in the database. A similar project by Ohio State University has resulted in the curated 16S rRNA gene database CORE (http://microbiome.osu.edu/) (Griffen et al., 2011) with 1043 16S rRNA gene sequences of the

so called “core human oral microbiome”, based on taxa from published surveys of oral bacterial communities conducted using 16S rRNA gene sequencing. These diversity numbers (640 and 1043 taxa in HOMD and CORE, respectively) are all but final. In each new publication involving cloning and sequencing of clinical samples, new bacterial phylotypes are discovered. However, due to the laborious and expensive methodology, traditional cloning and sequencing studies are limited to describing of the predominant taxa in the community.

The problem of under-sampling in traditional cloning and culturing studies can be overcome using a next generation sequencing (NGS) approach where relatively short 16S rRNA gene amplicons are sequenced at unprecedented depth and throughput (Chapter 11 of this book” *The Human Microbiota and Microbiome”*). The major shortcoming of the current NGS technology is the limited taxonomic resolution. Due to short read length most sequences can only be assigned to genus or a higher taxon. Instead of classification into species and strains, NGS data results into OTUs – operational taxonomic units that are defined as a cluster of sequences at a certain (*e.g*., 97%) similarity threshold.

Studies using NGS show that the diversity of the oral microbiome, as assessed by culture and cloning, is greatly underestimated (Keijser et al., 2008; Yang et al., 2012; Zaura et al., 2009). Individual oral cavities have been shown to harbor between 540 – 650 (Zaura et al., 2009) and 600 - 4200 (Yang et al., 2012) OTUs. The number of OTUs (taxa) observed in a community increases with sampling effort until all taxa are observed. By assessing the relationship between the number of OTUs observed and the sampling effort, information about the total diversity of the sampled community can be obtained. This way it was demonstrated that the oral microbiome is still highly undersampled (Keijser et al., 2008).

Furthermore, it was shown that of about 8000 OTUs that were found in pooled dental plaque from 98 individuals, the 1000 most abundant OTUs represented 95% of all sequences. In other words, the majority of the taxa that contribute to the immense diversity of oral microbiome are present at a very low abundance. Based on the rank abundance curve, the overall species richness, i.e., how many types there would be found until the accumulation curve reaches the plateau, can be calculated. This way it has been estimated that overall diversity of oral microbiome would plateau above 10,000 OTUs (Keijser et al., 2008).

The sequences from the oral microbiome CORE database (Griffen et a., 2011) belong to 14 phyla and 152 genera (Fig. 2.1). Over 57% (365 of the 636 species-level OTUs) of the oral microbiome from the CORE database belong to yet uncultivated phylotypes (Fig. 2.2). None of the members of five phyla or so called candidate divisions (TM7, SR1, *Chloroflexi*, *Nitrospira*, OP11) have been identified and isolated by culture. The largest candidate division in this group is TM7 with 20 species-level OTUs. The DNA of these ubiquitous microorganisms has been found in a variety of terrestrial, aquatic and clinical habitats. Lack of cultured isolates makes determination of the functions of these organisms a challenge. Single-cell genome amplification of the TM7 cells isolated from subgingival crevice by use of a microfluidic device allowed assembly of over 1000 genes providing the first insights into the physiology of the members of this phylum (Marcy et al., 2007). Of the major phyla, the *Firmicutes* has the highest prevalence in the oral cavity (Keijser et al., 2008; Zaura et al., 2009) and contains one

of the most studied groups of oral microbiota - oral streptococci. Nevertheless only about 80 of the nearly 200 species-level OTUs that are classified as *Firmicutes* have been isolated by culture (Griffen et al., 2011).

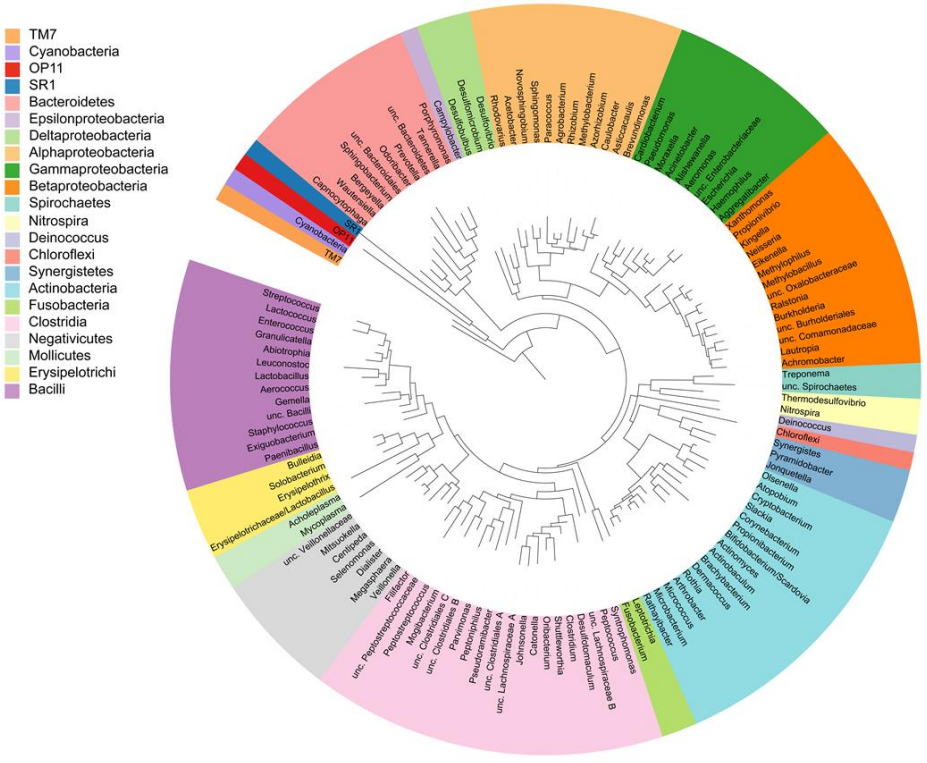


Figure 2.1. Circular phylogenetic tree at level of genus of taxa included in the CORE database of "core human oral microbiome" (Griffen et al., 2011). Genera are color-coded by phyla, except for the *Firmicutes* and *Proteobacteria*, which are shown at the level of class (Figure 1 from (Griffen et al., 2011).

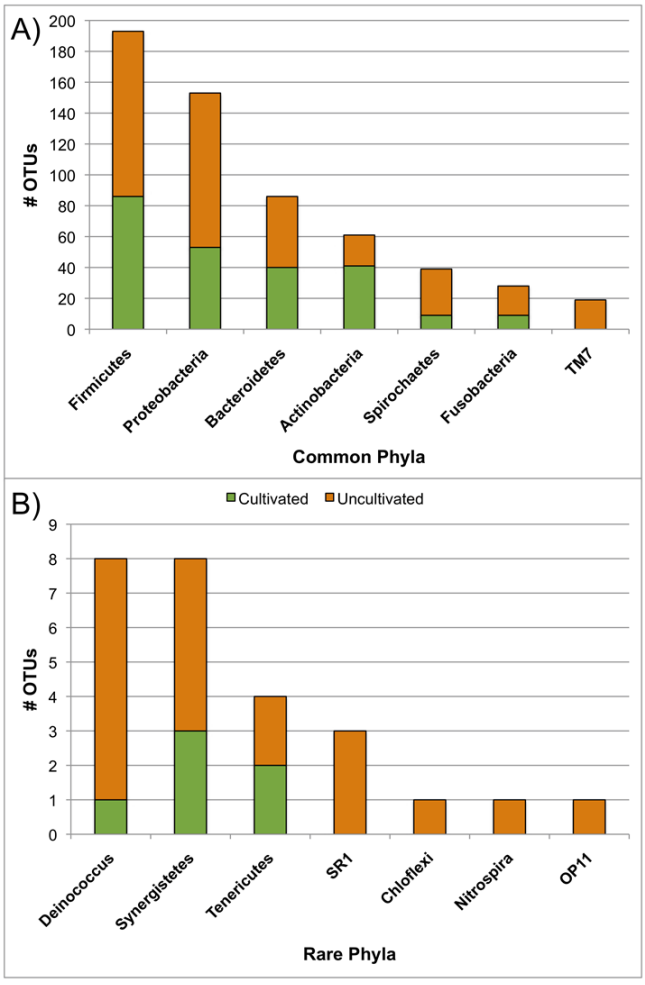


Figure 2.2. Numbers of species-level OTUs by phylum in the CORE database (Griffen et al., 2011). Number of OTUs assigned to each of the 14 phyla observed in the oral cavity and pharynx: A) Common phyla B) Rare phyla (<10 species-level OTUs). The fraction of OTUs for which a cultivated member has not been reported is indicated in orange, while cultivated members are presented in green (Figure 3 from Griffen et al., 2011).

Archaea

Archaea (originally named *Archaebacteria*) are prokaryotic microorganisms that, next to *Bacteria* and *Eucarya*, form the third elementary domain of life (Woese et al., 1990). Although *Archaea* were originally discovered in extreme environments and were thought to be the most primitive life form on Earth (hence the name *Archaea*), these microorganisms are ubiquitous and are found in various non-extreme environments, including humans (Dridi et al., 2011). Even though *Archaea* and *Bacteria* look similar under the light microscope, *Archaea* possess genes and metabolic pathways that are more closely related to those of eukaryotes. A distinct group within the *Archaea* is composed of organisms that produce methane from various substrates, such as H_2 and CO_2, acetate, and methylamines, and are therefore called methanogenic *Archaea* or methanogens. Representatives of this group, genus *Methanobrevibacter,* have been detected by molecular methods and were isolated from the oral cavity (Dridi et al., 2011; Matarazzo et al., 2011). Several studies have shown the association of *Archaea* with periodontal disease (Lepp et al., 2004; Vianna et al., 2008) or endodontic infections (Vianna et al., 2009; Vickerman et al., 2007). The metabolic capacity of methanogens to consume molecular hydrogen and the formation of methane may support the growth of fermenting bacteria, including opportunistic (*e.g*., periodontal) pathogens (Horz and Conrads, 2010). The role of *Archaea* as members of the commensal oral microbiome remains to be elucidated.

Fungi

Fungi are members of the *Eucarya* domain of life and are considered opportunistic oral pathogens responsible for several diseases, *e.g*., *Candida*-induced oropharyngeal thrush in newborns and mucositis in denture wearers and immuno-compromised patients. *Candida* species have been associated with caries (Raja et al., 2010; Signoretto et al., 2009), periodontal disease (Urzua et al., 2008) and endodontic infections (Siqueira and Rocas 2009). In a recent metagenomic study, pyrosequencing of the pan-fungal internal transcribed spacer (ITS) region showed that an oral cavity of a healthy adult individual contains 9 – 23 fungal species (Ghannoum et al., 2010). The diversity of fungi was unexpectedly high (74 culturable and 11 non-culturable genera, representing 101 species). Next to *Candida* species, which are relatively often found in the oral cavity, other genera such as *Cladosporium*, *Aureobasidium*, *Saccharomycetales*, *Aspergillus* and *Fusarium* were found in more than 30% of the samples. Clinical relevance of this high oral fungal diversity as disclosed by NGS technology remains to be explored.

Viruses

Viruses, especially from the herpesvirus family, are common in the oral cavity. Herpesviruses establish a lifelong persistent infection, and some herpesvirus species infect as many as 90% of the adult population. The great majority of systemically healthy adults continually shed herpesvirus DNA into saliva, reaching levels of DNA copies between 10^6 and 10^9 per ml saliva (Slots and Slots, 2011). Cytomegalovirus and Epstein-Barr virus (both belonging to the

herpesvirus family) have been associated with the symptomatic periapical pathology of endodontically infected teeth (Siqueira and Rocas 2009) and with aggressive periodontitis (Slots 2011). Other relatively less common viruses, such as hepatitis A, B, C and G are detectable in saliva during the acute or chronic phase of the disease (Slots and Slots 2011). Human papillomaviruses (HPV) have been found in the saliva of 25% of healthy individuals and in 26% of gingival biopsies from periodontitis lesions (Slots and Slots 2011).

So far the only oral virome metagenomics study (Willner et al., 2011) describes an entirely different profile from the results above that were obtained using targeted PCR probes. The majority of viral metagenomic sequences in the pool from oropharyngeal samples of 19 healthy adults were identified as bacteriophages, also called phages (viruses of bacteria). The Epstein-Barr virus was the single eukaryotic virus that was found. As commented by the authors, the low sensitivity for detecting larger, eukaryotic viruses could be due to the methodology that still requires improvement. Nevertheless, the estimated diversity of the pooled virome was 236 viral species. Interestingly, the phages that dominated the metagenome were *Escherichia coli* phages T3 (widely used for experimental purposes in laboratory strains), *Propionibacterium acnes* phage PA6 and streptococcal phages, including *Streptococcus mitis* phage SM1. The SM1 phage is a temperate phage - a virus that does not immediately lyze the host cell, but integrates its DNA with that of the host. SM1 contains the genes *pblA* and *pblB* that encode platelet-binding factors that have been shown to contribute to the virulence of *S. mitis* in the endocardium, but had not been previously detected in the oral cavity (Willner et al., 2011). The oral cavity is thus a rich reservoir of bacteriophages, potential agents in horizontal gene transfer and antibiotic resistance, of which the clinical role in maintaining health still needs to be disclosed.

Acquiring the oral microbiome

For an individual, birth is a borderline between sterile intrauterine life and extrauterine existence with a continuous exposure to microorganisms. Microbiota are acquired via other individuals, animals and the local environment (Kononen 2000). A recent metagenomic study demonstrated that the mode of delivery (vaginal or caesarean section) determines the initial oral and intestinal microbiome of newborns (Dominguez-Bello et al., 2010). Vaginally delivered infants had microbiomes that resembled the vaginal microbiota of their mother, while microbiomes of Caesarean-delivered infants resembled the skin microbiota of their mother. The microbial community is further shaped by *e.g.* diet, personal oral hygiene and exposure to antibiotics. In infants, only mucosal surfaces are available for bacterial attachment and colonization in the oral cavity. A pyrosequencing study on edentulous infants (4.6 months old) and their primary caregivers showed that the microbiomes of infants were dominated by streptococci, vellonellae and neisseriae (Cephas et al., 2011). Interestingly, this study found that the microbiomes of toothless infants were already highly diverse – 578 OTUs were found in saliva of infants, compared to 1012 OTUs in the saliva of their caregiver. Eruption of teeth has a major impact on the microbiome by increasing the number of ecological niches (*e.g.*,

gingival crevices, occlusal pits and fissures and interproximal dental surfaces) for microorganisms to thrive, further increasing the diversity of oral microbiome.

Health-associated oral microbiota

For decades oral microbiologists have focused their attention on pathogens and overlooked the healthy oral microbiome. The healthy oral microbiome research era was initiated by the landmark study from researchers at the Forsyth Institute where the oral microbiome was profiled in five healthy individuals (Aas et al., 2005). Since then oral microbiomes of a number of healthy individuals at various ages and samples from different intraoral locations have become available (Bik et al., 2010; Crielaard et al., 2011; Nasidze et al., 2009; Zaura et al., 2009). The US National Institutes of Health (NIH) has given a large financial boost to healthy human microbiome studies. In 2007 the NIH launched a five-year Human Microbiome Project (HMP, http://commonfund.nih.gov/hmp) with a total budget of $115 million. The HMP represents a broad multi-institutional project generating unprecedented amounts of sequences, annotations and metadata: 300 healthy individuals are being sampled longitudinally at multiple body sites, including the oral cavity where different niches are currently being characterized at taxonomic (16S rRNA gene-based) and functional (metagenome) level (Turnbaugh et al., 2007). Until the HMP data are released, small-scale crossectional studies indicate that at genus level several taxa are found across unrelated oral microbiomes (Fig. 2.3), of which the genera *Streptococcus, Prevotella, Haemophilus, Veillonella, Neisseria, Rothia* and *Actinomyces* dominate in most of these reports. The exception is a study on Batwa Pygmies (a hunter-gatherer group in Uganda) (Nasidze et al., 2011), where the most predominant genus was *Enterobacter* (20% of sequences were classified as this genus), including other members (*Klebsiella* – 2.4%, *Serratia* – 2%) of the *Enterobacteriaceae* family of the *Gammaproteobacteria* class. The taxa from the *Enterobacteriaceae* family are not considered commensal oral microbiota, and their high abundance suggests the effect of an unusual lifestyle of this group of people on their microbiomes.

Although the amount of data on the overall oral microbiota is vast, there is little evidence-based information on which of the oral microbiome members has a strong association with oral health. Below we will summarize the current knowledge on health-associated microbiota. So far this information is based on small-scale clinical studies without the use of a unifying and standardized definition of oral health. Since a wide range of both targeted and open-ended methodologies is used in the included studies, the results are not directly comparable and involve several technology-related biases, which will not be addressed here.

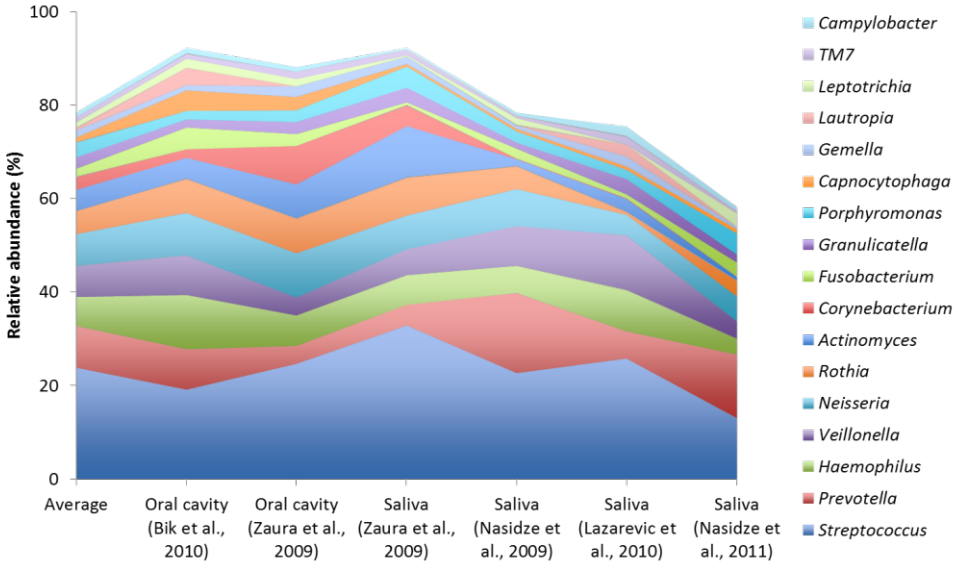


Figure 2.3. Relative abundance of predominant oral bacterial genera from five microbiome studies (Bik et al., 2010; Lazarevic et al., 2010; Nasidze et al., 2009; Nasidze et al., 2011; Zaura et al., 2009) contributing to 58 – 92% of all sequences. Two studies (Bik et al., 2010; Zaura et al., 2009) have sampled various intraoral habitats and are presented as "Oral cavity". Saliva data from the study by Zaura et al., (Zaura et al., 2009) is a subset of the total "Oral cavity" dataset. Study by Nasidze et al., (Nasidze et al., 2011) sampled saliva of Batwa Pygmies. The micrcobiomes of this hunter-gatherer group from Uganda were dominated by genus *Enterobacter* (20% of sequences), not included in this list of the predominant oral taxa. The legend indicates genera genera from top to bottom in the figure.

From the control groups used in the studies on dental caries and various stages of periodontal disease, numerous microorganisms are associated with the healthy control groups. Relatively few microbial taxa (listed in table 2.1) are associated with health in studies on caries and studies on periodontal disease. Interestingly, certain caries-associated bacteria appear to be health-associated regarding periodontal disease, *e.g.*, *Streptococcus mutans* (Iwano et al., 2010; Koll-Klais et al., 2005b; Kumar et al., 2005; Tanner et al. 1998), *Lactobacillus fermentum* (Koll-Klais et al., 2005a) and *Atopobium* species (Kumar et al., 2003). The opposite is true for certain periodontal pathogens, *e.g.*, *Campylobacter rectus* (Gross et al., 2010), *Selenomonas noxia* (Preza et al., 2008) and *Fusobacterium nucleatum* (Aas et al., 2008; Preza et al., 2008; Preza et al., 2009a), being more associated with health regarding dental caries. Most likely, the explanation for these "bad guys with a good conscience" is the antagonistic ecology behind the two diseases – high pH in the inflamed periodontal pockets or low pH at the sites with an active caries process. This way the microbiota may be harmful at one condition, but become beneficial at the other. For instance, in case of *S. mutans* and lactobacilli, these aciduric bacteria, if present in the periodontal pockets, will decrease the local pH and inhibit the periodontal pathogens that thrive at a pH above 7 (Marsh 1994).

Future research on the health-associated microbiome should involve standardized, hypothesis-driven clinical trials with a clear definition of oral health. High inter-individual variability among microbiomes of unrelated individuals (Lazarevic et al., 2010; Nasidze et al., 2009; Zaura et al., 2009), due to individual host and environmental factors, makes interpretation of cross-sectional microbiome studies difficult and renders the results to being mainly descriptive. Longitudinal studies are required to provide data for analysis of microbial shifts in the association with the health-disease equilibrium. Currently available small-scale descriptive studies should be used for power analyzes and sample size calculations. This would allow appropriate statistical analyses and would lead to evidence-based conclusions.

Table 2.1. Microbial taxa associated with oral health.

Phylum, Class	Final Taxon	References	
		Health versus caries	Health versus periodontal disease
Actinobacteria, Actinobacteria	*Actinomyces naeslundii*	Brailsford et al., 2001, 2005; Marchant et al., 2001; Becker et al., 2002	Tanner et al., 1998; Colombo et al., 2009
	Actinomyces odontolyticus	Marchant et al., 2001	Teles et al., 2007
	Corynebacterium matruchotii	Preza et al., 2008; Gross et al., 2010	Paster et al., 2001; Kumar et al., 2003
Bacteroidetes, Bacteroidia	*Bacteroides* sp. clone BU063	Aas et al., 2008	Leys et al., 2002; Kumar et al., 2003
	Porphyromonas catoniae	Crielaard et al., 2011	de Lillo et al., 2004
Bacteroidetes, Flavobacteria	Genus *Capnocytophaga*	Preza et al., 2009a	Kumar et al., 2005
	Capnocytophaga gingivalis	Gross et al., 2010	Kumar et al., 2005
	Capnocytophaga sputigena	Aas et al., 2008; Preza et al., 2009b	Colombo et al., 2009
Firmicutes, Bacilli	*Streptococcus mitis* group	Corby et al., 2005; Preza et al., 2008; Gross et al., 2010; Hart	Koll-Klais et al., 2005b
	Streptococcus oralis	Marchant et al., 2001	Tanner et al., 1998
	Streptococcus sanguinis	Caufield et al., 2000; Marchant et al., 2001; Becker et al., 2002;	Kumar et al., 2005; Colombo et al. 2009
Firmicutes, Costridia	*Selenomonas* sp. oral clone DS051	Preza et al., 2008	Kumar et al., 2005
Proteobacteria, β-*proteobacteria*	*Kingella oralis*	Aas et al. 2008; Preza et al., 2008	Colombo et al., 2009
Proteobacteria, ε-*proteobacteria*	*Campylobacter concisus*	Preza et al., 2009a	Macuch and Tanner 2000

Beyond the microbiome

For the analysis of microbial community ecology and the functioning of ecosystems, the determination of functional diversity is of great importance (Konopka 2009). Current oral microbiome studies focus on “who is there” (taxonomic diversity), while discovering “what are they doing” (functional diversity) will be the next logical step. Two pioneering oral metagenome studies (Belda-Ferre et al., 2012., Xie et al., 2010) demonstrate both, the opportunities and the challenges of this approach on such a complex microbial community as the oral microbiome. Xie et al., (2010) assessed a single plaque sample metagenomically and showed that only 51% of the assembled sequences could be assigned a functional role. Belda-Ferre et al., (2012) directly sequenced the metagenomic DNA from supragingival plaque of two caries-free, two caries-active and two individuals with caries experience in the past, as well as two carious lesions. Their findings indicate that functional profiles of the metagenome of the caries-free individuals are distinct from the profiles of the caries-prone individuals, with certain groups of genes (e.g., bacteriocins and stress response genes) being over-represented in the samples from healthy individuals. To conclude, we are at the doorstep of entering a new and exciting era in oral ecology studies where next generation sequencing on functional diversity will not only give us information on how the oral microbiome initiates infectious diseases, but also how it actively sustains oral health.

Chapter 3

Effect of mouthwashes on the composition and metabolic activity of oral biofilms grown *in vitro*

This chapter has been published as:

Fernandez y Mostajo, M., Exterkate, R. A.M. , Buijs, M. J., Crielaard, W. & Zaura, E. (2016) *Clin Oral Investig*. doi:10.1007/s00784-016-1876-2.

ABSTRACT

Objective

To determine the effect of an oxygenating mouthwash compared to two other established mouthwash products on bacterial composition and metabolic activity of oral biofilms *in vitro*.

Material and methods

Twelve healthy subjects participated as donors. Plaque-saliva mixture inoculated biofilms were grown and treated with 3 different chemotherapeutic mouthwashes [Amine fluoride/Stannous fluoride (MD), Oxygenating agent (AX), Chlorhexidine 0.12% (PA) and water (W)]. Effects of treatments were assessed on biofilm composition (16S rRNA gene amplicon sequencing), production of organic acids (formate, acetate, lactate, propionate, butyrate using capillary electrophoresis) and viability of the remaining biofilm (CFUs).

Results

Microbial profiles of biofilms clustered per inoculum donor and were dominated by the genera *Veillonella*, *Streptococcus* and *Prevotella*. Microbial diversity was only reduced after PA treatment. Significant changes in composition occurred after treatment with AX, resulting in lower proportions of *Veillonella* and higher proportions of non-mutans streptococci. Production of all organic acids after PA and lactate after MD was significantly lower as compared to W.

AX resulted in reduction of acetate, butyrate and propionate and increase in lactate production ($p<0.05$). Viable counts were significantly lower after PA and AX treatments compared to W, while no significant reduction was observed after MD.

Conclusions

All studied mouthwashes affected the *in vitro* biofilms differently. The effects of the AX treatment were the most prominent which resulted in changes of the bacterial composition and metabolism.

Clinical implications

Awareness by the dental team that mouthwashes can change the bacterial composition and metabolism is important when advising its use.

Key words: biofilm, microcosm, organic acids, mouthwashes, antimicrobials

INTRODUCTION

Dental plaque biofilms are communities of microbial cells in the oral cavity that are embedded in an extracellular polymeric matrix. Oral biofilms are among the most complex microbial communities in nature. Dental plaque affects all humans worldwide. Failure to deal with plaque in the early stage of formation may lead to the development of oral diseases of which the most prevalent are dental caries, gingivitis and periodontitis (Palmer R J, 2011). Since the mouth is the gateway to the human body, establishing and maintaining oral health is important.

Various strategies have been employed to reduce or eliminate dental biofilms. Mechanical disruption of dental plaque biofilms such as toothbrushing is worldwide the most common approach. The effectiveness of this is however limited (Chapple et al., 2015, Van der Weijden and Slot, 2015). Therefore also chemotherapeutic strategies to disrupt these biofilms have been considered, such as the use of mouthwashes. Over the past decades, the use of these has become customary, usually following mechanical plaque biofilm control (Van der Weijden et al., 2015).

Currently, there is a wide range of over the counter mouthwashes products available, containing various active ingredients with for each of these specific indications. It is the role of the dental teams to advice the use of mouthwashes when indicated, which is justified when its efficacy has been proven by studies based on clinical evidence.

A recent meta-review (Van der Weijden et al., 2015) has shown that, to date, the scientific body of evidence for the use of specific agents such as chlorhexidine is substantial. However, for most commercially available mouthwashes the scientific evidence it is considered to be moderate, weak or underreported. For instance, the underlying data retrieved in a systematic review for oxygenating agents (OAs) did not allow for a meta-analysis (Hossainian et al., 2011).

Serrano and colleagues (Serrano et al., 2014), have analyzed the scientific evidence for the efficacy of adjunctive anti-plaque chemical agents in managing gingivitis. These authors concluded that the use of specific agents showed statistically significant improvements in terms of gingival, bleeding and plaque indices. OAs were not included in their meta-analysis.

Nevertheless, most mouthwashes, among which also OAs, are available without a prescription and their use is common practice. Oxygenating agents, such as hydrogen peroxide (H_2O_2), buffered sodium peroxyborate, and peroxycarbonate, have been recommended for short-term use as disinfectants (Hossainian et al., 2011, Van der Weijden et al., 2015). Recently a mouthwash containing an oxygenating agent (peroxoborate) has been introduced in the market and the result of a pilot study has shown that this product has the potential for selective inhibition of oral bacteria. Twice-daily exposure for one week to this mouthwash resulted in a shift in the microbial composition towards a less diverse and less mature plaque (Fernandez y Mostajo et al., 2014).

Oral microbes contribute to the healthy homeostasis within the oral cavity (Marsh, 2012). Therefore, besides addressing a clinical efficacy of a mouthwash, it is important to investigate its effects on the oral microbiome (ten Cate and Zaura, 2012). For this purpose, *in vitro* biofilm models offer an approach to elucidate the microbiological and ecological effect of potential products at an early stage of product development (Eick et al., 2012, Exterkate et al., 2010, Filoche et al., 2008, Rudney et al., 2012, Shapiro et al., 2002, Zollinger et al., 2015).

The aim of this study was to assess and determine the effect of an oxygenating mouthwash compared to two other established and commercially available mouthwashes products and a negative water control on the bacterial composition and metabolic activity of oral biofilms *in vitro*.

The null hypothesis was that the treatment effect of an oxygenating and a fluoride-based mouthwash will not differ in terms of bacterial composition and metabolic activity than compared to a negative control (water) or a positive control (chlorhexidine).

MATERIALS AND METHODS

Selection of inoculum donors

Twelve individuals participated as donors of saliva and dental plaque after reading and signing an informed consent form. All donors were in good general health and had not been exposed to antibiotics and professional dental prophylaxis within the previous 3 months. Exclusion criteria were: visible caries or a Dutch periodontal screening index (DPSI) of smaller or equal to score 3 minus (which translates to periodontal pockets ≤ 5mm in absence of gingival recession)(Mantilla Gomez et al., 2001). Prior to sample collection subjects were asked not to perform any oral hygiene 24 h before the appointment and to refrain from any food or drink consumption for at least 2 h before sampling (Ntrouka et al., 2011).

Ethical approval of the protocol related to plaque-saliva collection and experimental research was provided by the Medical Ethical Committee from the Vrije Universiteit Medical Center Amsterdam (reference 2011/236).

Sampling

Sampling took place in the morning between 08:30 and 09:30. Supragingival dental plaque was collected with sterile plastic curettes along the gingival margin of all buccal and lingual surfaces in the second and fourth quadrants. The samples were placed in a previously weighed Eppendorf vial containing 100 µl of cysteine peptone water (CPW), vortexed for 30 s and briefly centrifuged. Then the vials were weighed again and the difference in weight was calculated. CPW was added normalizing to a proportion of 10 mg plaque: 1.3 ml CPW and stored on ice. Subsequently participants chewed on a piece of a parafilm for one minute. Approximately 1 ml of -stimulated saliva was collected in a sterile universal vial being kept on ice.

Biofilm model and production of the biofilms

An active attachment biofilm model was used as previously described (Exterkate et al., 2010). In brief, 24-well polystyrene culture plates (Greiner Bio- One, Alphen a/d. Rijn, the Netherlands) were used. The lid of the plate was replaced by a custom-made stainless-steel lid, onto which 24 nylon clamps were fixed allowing for various substrata to be inserted. The clamps were positioned in such a way that the inserted substrata would fit into the wells of the culture plate. Since the substrata were positioned vertically, bacterial attachment plays a key role in this model. By transferring the lid with the substrata to a new plate, the medium could be refreshed. In this study, standardized 12-mm hydroxyapatite (HA) disks (Himed, Old Bethpage, USA) were used as substratum. The assembled model was autoclaved at 121°C.

McBain medium (McBain et al., 2005) with 50 mmol/l PIPES at pH 7 (Exterkate et al., 2014) was used. After autoclaving, the medium was supplemented with filter-sterilized 0.2% sucrose and sterile heat-inactivated 5% fetal bovine serum (FBS, F4135, Sigma-Aldrich, USA). The suspension of dental plaque samples was dispersed using a sonicator (Vibra Cell; Sonics & Materials Inc, Newtown, CT) for 3 s with 1 s pulses at amplitude of 40 W and vortex-mixing for 30 s just before inoculation.

The inoculation medium was immediately prepared using a mixture of dental plaque 1:15 (1.25 ml of suspension in 20 ml of CPW) and saliva 1:50 (0.4ml of saliva in 20ml of CPW) as inoculum. Next 1.5 ml of the inoculation medium was added to each well of a standard polystyrene 24-well plate (multiwell plates; Greiner Bio One, Alphen aan den Rijn, The Netherlands).

For each donor an individual plate was used. The model was subsequently incubated anaerobically (10% CO_2, 10% H_2, and 80% N_2) at 37 °C. After an initial 24 h of incubation, the medium was refreshed with sterile medium twice a day based on a schedule of 8 and 16 h, up to 96 h.

To analyze the bacterial composition of the inocula, 100 µl of the plaque-saliva suspension was stored at -20ºC for DNA extraction and analysis.

Treatment of the biofilms

After 96 h, the biofilms were treated by transferring them to a new plate containing the treatment solution (1.8 ml/well) for 10 min at room temperature with one of the following compounds:

- Perioaid® (Dentaid Benelux, Houten, the Netherlands) containing 0.12% chlorhexidine (PA).
- Meridol® (GABA International, Basel, Switzerland), an amine fluoride/stannous fluoride (AmF/SnF_2)-containing mouthwash (MD).
- Ardox-X-technology®, commercially known as O7-active (NGen Oral Pharma N.V., Curacao) an oxygenating agent (AX).
- Sterile demineralized water as a negative control.

Triplicate biofilms were treated with the above-mentioned compounds and a negative water control resulting in four treatment groups for each donor.

Following treatment, biofilms were rinsed with CPW (1.9 ml/well) moving the lid 10 times up and down in the plate (Exterkate et al., 2014). This rinsing procedure was repeated 3 times with fresh CPW to remove excess treatment solution.

Biofilm incubation for acid formation

Subsequently, the lid was placed on a plate containing 1.5 ml buffered peptone water (BPW) with 0.2% sucrose per well and incubated anaerobically for 3 h at 37°C. After the incubation BPW was collected and stored at –80° C.

Harvesting of the biofilms

Each HA disc with biofilm was removed using sterile forceps and placed in a sterile vial containing 2 ml of phosphate-buffered saline (PBS). All samples were kept on ice, dispersed by sonication for 2 min at 1 s pulsations at the amplitude of 40 W (Vibra Cell; Sonics & Materials Inc, Newtown, CT) and vortexed for 30 s.

Determination of number of CFUs

Serial dilutions of the dispersed biofilms in PBS were made and plated on trypticase soy agar blood (TSAB) plates for total viable counts. The plates were incubated for 7 days at 37°C under anaerobic conditions (10% CO_2, 10% H_2 and 80% N_2) and the number of colony-forming unit (CFU) counts was determined.

Propidium monoazide (PMA) treatment

To assure that only DNA from the cells with intact membranes (undamaged cells) was processed for sequencing, the dispersed biofilms were treated with PMA as described previously (Exterkate et al., 2014). In brief, 2.5 µl PMA (Biotum Inc., Hayward, Calif., USA) was added to 500 µl suspended biofilm, incubated in the dark for 5 min and then exposed to intense light for 2 min using a 650 W halogen lamp placed 25 cm from the samples. The samples were kept on ice during this procedure.

Organic Acid Analysis

The concentration of organic acids in BPW following incubation with sucrose was determined by capillary electrophoresis (Waters Capillary Ion Analyzer Milford, Mass, USA) as described previously (Gerardu et al., 2003). As an internal standard, 0.12 mmol/l oxalate was included in all samples. Succinate, formate, acetate, lactate, propionate and butyrate were determined and expressed as mmol acid/disc. The total concentration of acid excreted by the biofilms to the environment (BPW) was calculated as the sum of all acids per biofilm.

DNA extraction, amplicon preparation and sequencing data analysis

DNA from the inocula and biofilms was extracted as previously described (Crielaard et al., 2011). Barcoded amplicon libraries of the small subunit ribosomal RNA gene hypervariable region V5–V7 were generated for each of the individual samples, pooled and sequenced using Genome Sequencer FLX Titanium system (Roche, Basel, Switzerland) as described previously (Koopman et al., 2015). The sequencing data were processed using Quantitative Insights Into Microbial Ecology (QIIME) (Caporaso et al., 2010) version 1.5.0 as described previously (Koopman et al., 2015).

To allow comparisons among different samples, the data set containing Operational Taxonomic Units (OTUs) was randomly subsampled at an equal depth. Hierarchical clustering based on the Bray Curtis similarities index (BC) was used to assess the similarities among the biofilms per group - a non-linear coefficient between every pair of OTUs. Shannon diversity index was calculated, which takes into account the abundance of each OTU, as well as the number of OTUs. To visualize microbial profile data, non-metric multidimensional plots (n-MDS) based on the BC were used.

In addition, taxonomy of the representative sequences of the most abundant OTUs was identified by microbial nucleotide BLAST search against a Human Oral Microbiome Database (HOMD) reference set (version 13.2). The possible alternatives for the species identification were then provided.

Statistical analysis

Statistical analyses were performed on median values of triplicate biofilm data. Non-metric multidimensional scaling (nMDS) plots were based on the Bray-Curtis coefficient to visualize similarity between samples. Stress <0.2 (Kruskal's stress formula 1) was used as an acceptable threshold (Clarke, 1993). Similarity percentage (SIMPER) was used to identify the OTUs with the highest contribution to dissimilarity between the treatments. The differences in composition between treatment groups were assessed using one-way permutational multivariate analysis of variance (PERMANOVA). The p-values were corrected for multiple comparisons using Bonferroni correction. P-values <0.05 were considered statistically significant. All calculations were performed using PAST version 3.0 (Hammer, 2001).

The software package IBM SPSS Statistics version 20.0 (2011, IBM Corp., Armonk, NY, USA) was used to perform all other statistical analyses. For comparisons among the treatment groups based on CFU counts, the median of triplicate biofilms was calculated, based on which the percentage of the remaining biofilm relative to the water-treatment was determined. Almost all groups were normally distributed (Shapiro Wilk test) except for the AX group. Pairwise comparisons were performed using Paired sample T-tests, and corrected for multiple comparisons using Bonferroni corrections.

Differences among the organic acid profiles per treatment group were analyzed regardless of the donor using one-way-ANOVA with Games-Howel post-hoc test; p-value corrected for multiple comparisons. Additionally, in order to determine relationships between the concentration of organic acids and the relative abundance of the most abundant genera (table 3.1), a Pearson correlation was calculated.

For the comparisons of mouthwashes in relation to the bacterial composition, only the most abundant genera (on average, contributing to 94% of reads in the water-treated biofilms) were selected. Based on their distribution (Shapiro Wilk test) the paired T-test or the Wilcoxon Rank test was used.

RESULTS

Of the twelve participants that donated dental plaque and saliva, 7 were female and 5 were male with an age range of 22-45 years. One participant was a smoker of <10 cigarettes/day (donor 4).

The relative abundance of bacterial genera in the inocula differed to a large extent by donor (Fig. 3.1). The mean Shannon diversity index of the inocula (3.05; SD 0.3) was significantly higher ($p<0.01$) than the mean diversity index of the 96 hours water-treated (W, negative control) biofilms (1.57; SD 0.5).

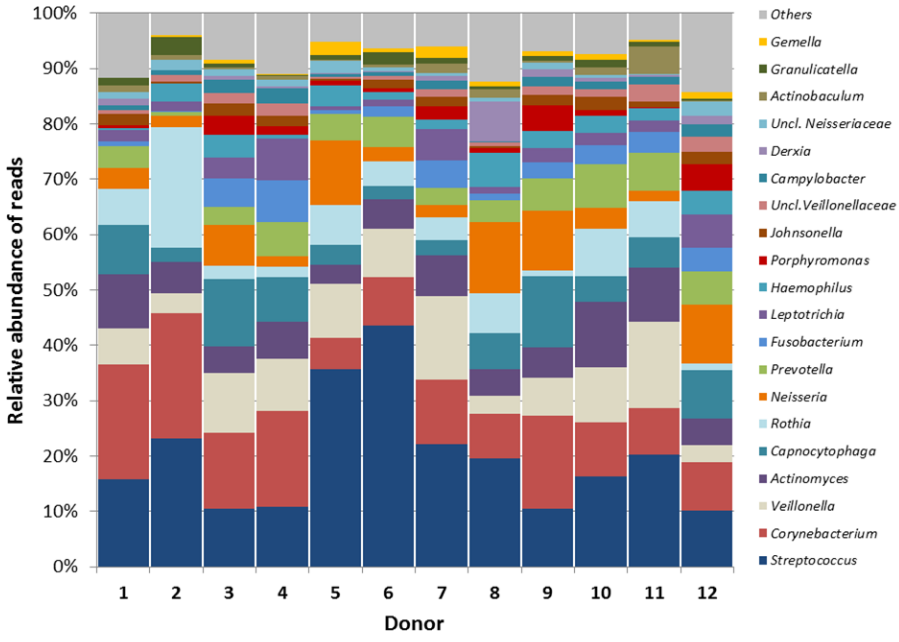


Figure. 3.1 Relative abundance of the major bacterial genera in the inoculum (plaque-saliva mixture) per donor. Others – the sum of the remaining taxa

All biofilms were dominated by genus *Veillonella* (OTU 190), *Streptococcus* (OTU 20) and *Prevotella* (OTU 296). These OTUs were blasted against a HOMD database for species-level taxonomical classification. *Veillonella* (OTU 190) was identified as *Veillonella dispar* (HOT 160) / *Veillonella rogosa* (HOT 158) at 99.7% blast ID and as *Veillonella atypica* (HOT 524) / *Veillonella parvula* (HOT 161) at 99.7% blast ID. *V. parvula* is phylogenetically similar to *V. dispar*, however *V. parvula* is catalase negative whereas *V. dispar* is catalase positive.

Streptococcus (OTU20) was identified as *Streptococcus oralis* (HOT 707) 100% blast ID *and as Streptococcus* species (HOT 071; HOT 070; HOT 064 and HOT 058) 100% blast ID. OTU 296 was identified as *Prevotella melaninogenica* (HOT 469) 100% blast ID.

The hierarchical classical cluster analysis based on the Bray Curtis similarity index among the biofilm triplicates showed that replicate biofilms clustered together (Data not shown).

The water-treated biofilms showed variability among donors in relative abundance of the genera *Veillonella*, *Streptococcus*, *Megasphaera*, *Peptostreptococcus* and *Prevotella* (Fig.3.2). *Veillonella* was the most abundant genus (40%-87% of reads), followed by *Streptococcus* (3%-27%) and *Prevotella* (3%-27%) with 4%-20% of the reads belonging to other genera.

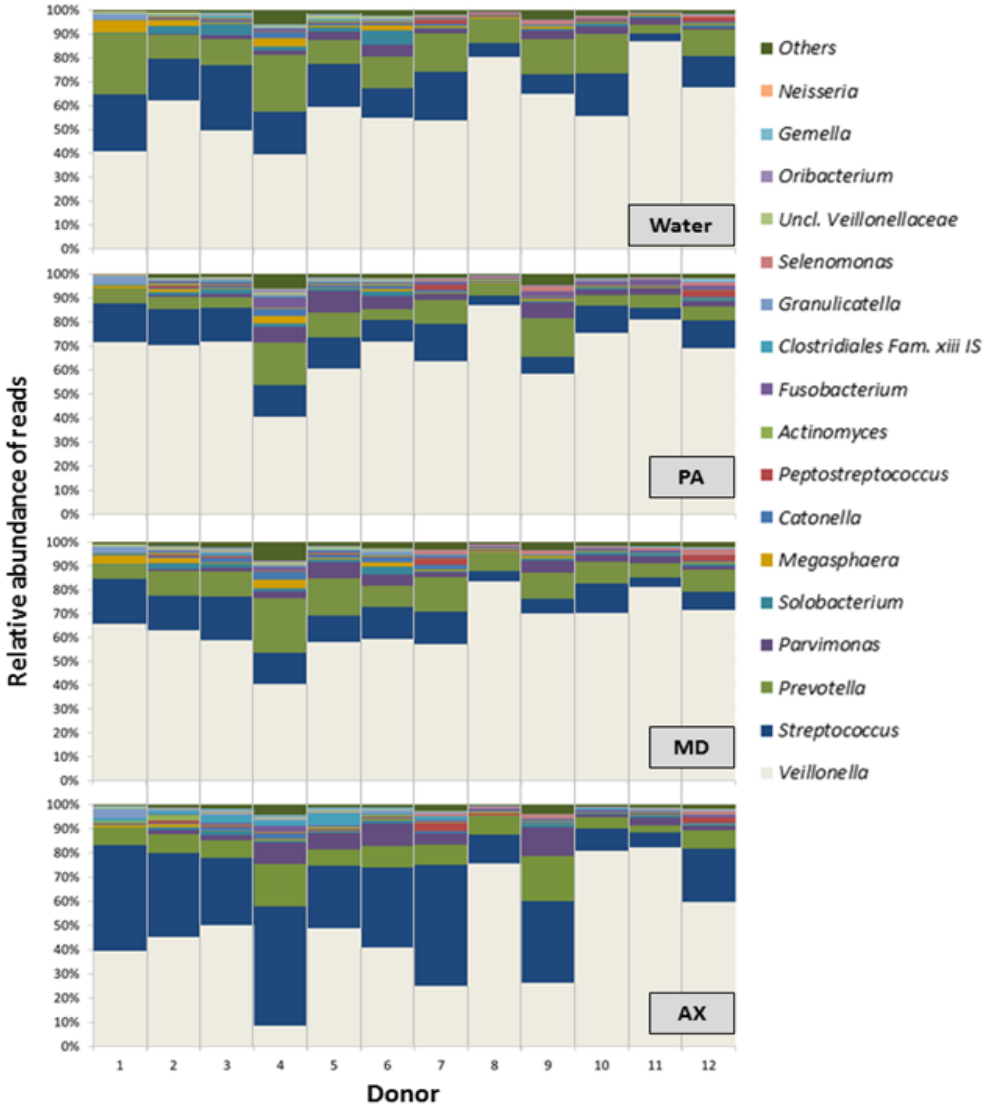


Figure. 3.2 Relative abundance of major bacterial genera per donor after 10 min treatment with water, PerioAid (PA), Meridol (MD) or Ardox-X (AX). The data shown are median values from triplicate biofilms

Ten minutes treatment with different compounds resulted in compositional changes in the remaining biofilm. As compared to the W-treated biofilms, the PA treatment resulted in a significantly lower diversity index (1.32, SD 0.4) compared to W-treated biofilms 1.57 (SD 0.5). The relative abundance of the genus *Streptococcus, Solobacterium* and *Megasphaera* was significantly lower in the PA-treated biofilms compared to the water control (Table 3.1, Fig. 3.2).

The diversity index for the MD treated biofilms (1.52, SD 0.4), was not statistically different from the water control. The relative abundance of genus *Streptococcus* was statistically lower in the MD treated biofilms than in the water control (Table. 3.1, Fig. 3.2).

Also AX treatment had no significant effect on diversity index (1.62, SD 0.4) in the biofilms. However, genus *Megasphaera* was significantly lower in the AX treated biofilms compared to the water control (Table 3.1, Fig. 3.2).

In order to assess the dissimilarities between the treatment groups we used the non-metric multidimensional scaling (n-MDS). This analysis showed that the dissimilarities among the treatment compounds were statistically significant only when AX-treated biofilms were compared to PA or MD treated biofilms ($p \leq 0.05$) (Fig. 3.3).

The effect of each of the tested compounds on the composition of the remaining biofilms had a clear pattern (Fig. 3. 2). Variability among the biofilms originating from different donors was visually observed on a 3-D plot. In particular, the respective samples from donors 8 and 11 clustered close together regardless the treatment group (Fig. 3.3). In contrast, biofilms from donors 2, 4, 6, 7 and 9 revealed a clear effect of the AX treatment (squares at the left side of the plot) except from donor 10 (square at the right side of the plot). The W-treated biofilms and AX-treated biofilms clustered close together in donors 1, 3 and 5.

Table 3.1 The relative proportion at genus level per mouthwash compared to the water treatment (n=12)

Genus (OTU ID)		Mean %	SD	*p-value*
	Water	62.2	(10)	
Veillonella[a]	MD	64.2	(10)	*0.081*
(OTU 190)	PA	68.0	(10)	*0.034*
	AX	48.2	(20)	*0.030*
	Water	15.6	(10)	
Streptococcus[a]	MD	11.4	(5)	***0.001***[c]
(OTU 20)	PA	11.1	(4)	***0.002***
	AX	28.7	(10)	*0.004*
	Water	14	(10)	
Prevotella[b]	MD	10.9	(4)	*0.084*
(OTU 296)	PA	7.8	(4)	*0.008*
	AX	8.7	(4)	*0.010*
	Water	2.1	(2)	
Parvimonas[b]	MD	2.6	(2)	*0.099*
(OTU 133)	PA	3.2	(3)	*0.075*
	AX	4.2	(4)	*0.015*
	Water	1.9	(2)	
Solobacterium[b]	MD	1.3	(1)	*0.050*
(OTU 6)	PA	0.9	(1)	***0.002***
	AX	0.8	(0)	*0.010*
	Water	1.5	(1)	
Megasphaera[b]	MD	1.3	(1)	*0.023*
(OTU 88)	PA	0.7	(1)	***0.002***
	AX	0.4	(0)	***0.002***

[a] Paired T-test

[b] Wilcoxon rank test (*p* values not corrected for multiple comparisons)

[c] *p* values (highlighted in bold) that remain significant after Bonferroni correction for multiple tests.

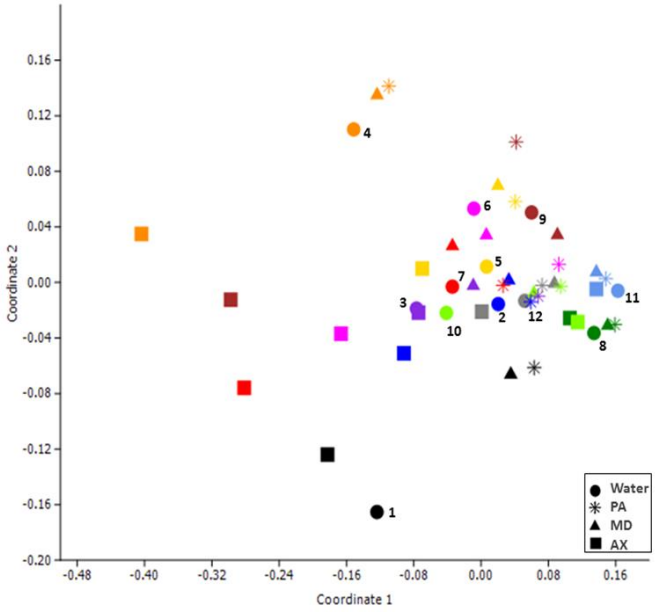


Figure 3.3 Non-metric multidimensional scaling (n-MDS) based on three-dimensional Bray-Curtis similarity (p=0.001; stress= 0.07; F=3.76), N=12. Colors indicate individual donors. The donors' number is shown next to the water treatment (dot). Shapes represent a treatment. Comparisons between groups were done using one-way PERMANOVA; W vs AX p=0.186; W vs PA p=0.394; W vs MD p=1; PA vs MD p=1; PA vs AX p=0.015; MD vs AX p=0.043

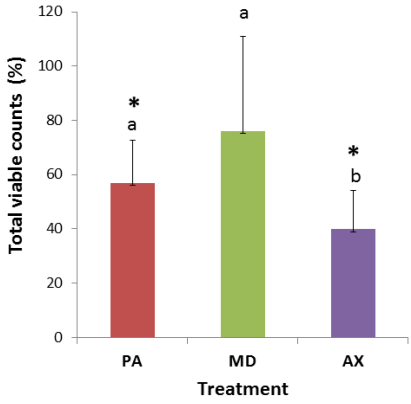


Figure 3.4 Effect of single treatment on viable counts expressed as a percentage of the water-treated control are described as the remaining biofilm. The data shown are the average and standard deviation (n=12). Different letters indicate statistically significant differences between the treatments PerioAid (PA), Meridol (MD) or Ardox-X (AX).* - statistically significant lower amount of remaining biofilms compared to the water treated control (p≤0.05), Paired sample T-test, corrected for multiple comparisons.

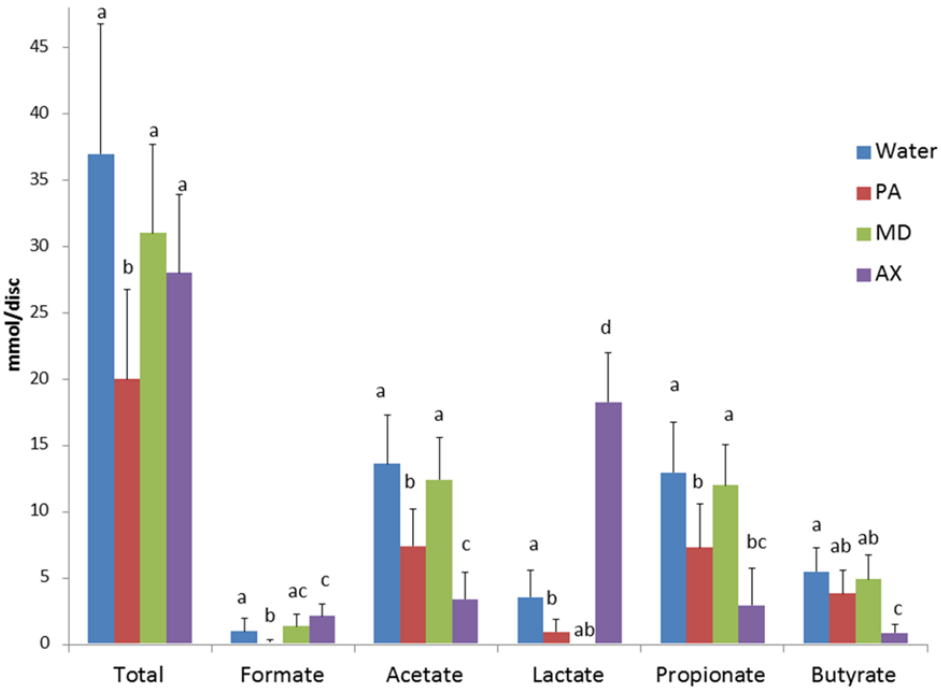


Figure 3. 5 Effect of single treatment on organic acids after a three-hour incubation with 0.2% sucrose following the respective 10-min treatment of plaque-saliva mixture-derived biofilms. The sum of all acids is represented as total. Different letters indicate statistically significant differences between the treatments ($p<0.05$, one-way-ANOVA, Games-Howel test; p-value corrected for multiple comparisons) per anion

Treatment effect on viable counts

W-treated biofilms yielded comparable average total viable counts ($5.9x10^9$ CFUs / biofilm, SD $9x10^9$) among all donors (p=0.176). Compared to the W-treated control, the percentage of remaining biofilm observed as viable counts after PA treatment was 57% (SD 16) and after AX treatment 40% (SD 14). This difference was statistically significant for both PA and AX treatment ($p<0.05$). After MD treatment viable counts were not significantly lowered : the percentage remaining viable cell counts was 76% (SD 35) relative to the W-treated control (Fig 3.4).

Organic acids

The concentration of succinate in biofilms was low and similar for all groups (data not shown). PA-treated biofilms had statistically significantly lower amounts of formate, lactate, propionate and acetate compared to water treated biofilms after a three-hour incubation with sucrose, while MD-treated biofilms had significantly lower lactate compared to W-treated biofilms (Fig. 3.5). In contrast, in AX-treated biofilms had a significantly higher amount of lactate and significantly lower amounts of acetate, propionate and butyrate ($p\leq0.001$)A positive correlation between between the relative abundance of genus *Veillonella* and the sum of acetate and propionate (Pearson r=0.7) excreted to the environment (BPW) was only observed for AX-treated biofilms. When all measured acids were added, PA had a significantly lower amount of total acid than the water control.

DISCUSSION

In this study an established microcosm biofilm model was used that allows studying the effects of mouthwashes from a microbiological and ecological perspective. We demonstrated that using this biofilm model, a single exposure to a mouthwash product resulted in different patterns in terms of bacterial composition and metabolic activity of the microcosm biofilm, depending on the chemotherapeutic agent used. Biofilms treated with AX caused major changes in bacterial composition, metabolism and killing effect. This study did however not evaluate the recovery of the biofilms after a single treatment exposure.

Since the compounds tested in the present study have different mechanisms, we did not attempt to determine the “best antimicrobial” but to characterize their effect with respect to bacterial composition of oral microcosms and their metabolic activity.

While current clinical studies have focused on the amount of plaque and the severity of gingival inflammation, this study highlights the importance of studying not only the “killing” capacity of mouthwashes or chemotherapeutic agents, but also the effect on bacterial composition and metabolic activity The most striking finding was the high amount of lactic acid produced after AX-treatment along with a reduction of the CFUs up to approximately 40% relative to the water control. This could be explained by the selective inhibition properties of this oxygenating compound (Fernandez y Mostajo et al., 2014). This is further supported by the observation that after AX treatment the relative abundance of genus *Veillonell*a was

significantly lower in the treated biofilm accompanied with the higher relative proportion of other genera mainly *Streptococcus*. Increase of streptococci on the other hand, is attributable to the proportionality of the data, because within 10 min no absolute increase that would imply bacterial growth is possible. Therefore the only explanation for this finding is that relative abundance of genus *Streptococcus* increases due to a decrease in other taxa such as *Veillonella*.

Veillonella which has an important role in biofilm development (Periasamy and Kolenbrander, 2010), is a gram-negative bacterium that lacks glucokinase and fructokinase. It is therefore unable to metabolize carbohydrates (Periasamy and Kolenbrander, 2010) and instead uses lactate as energy source. The fact that the *Veillonella* proportion was significantly reduced could explain the high lactate amount present in the AX-treated biofilms. These results are in line with the notion that the interaction of these genera can affect the metabolic activity reflected in lactate utilization by biofilms (Periasamy and Kolenbrander, 2010), which can potentially affect microbial community development (Keller and Surette, 2006).

Although both, PA- and AX-treatment resulted in a lower production of propionate, acetate and butyrate, this effect was greater for AX-treatment. Butyrate is mainly produced by proteolytic anaerobic bacteria (Pollanen et al., 1997, Tsuda et al., 2010) and has been associated with the inflammation found in periodontal disease (Qiqiang et al., 2012). AX has the potential to contribute to the treatment of periodontal diseases by further reducing the concentration of acetic, propionic and butyric acid and by increasing the lactic acid within the GCF. In this way, AX can contribute to the resilience of the subgingival microbiome towards disease. On the other hand, the possible consequences of the higher concentrations of lactate triggered by the AX-treatment need to be elucidated. When produced by probiotics, lactic acid has been described as a natural antimicrobial that is beneficial as an adjunctive to the treatment of periodontitis (Teughels et al., 2013). Although the results achieved here with AX are promising, *in vivo* studies are needed to assess this potentially beneficial effect on the non-surgical treatment, or in the maintenance of periodontal diseases.

MD-treatment proved to have the ability to inhibit the lactic acid formation by the biofilm without significantly reducing the total viable counts. Our findings that MD significantly lowered the relative abundance of genus *Streptococcus* and the lactate production are in agreement with previous *in vitro* studies (Exterkate et al., 2010).

In this study we supplemented the growth medium with heated inactivated serum. This was to simulate gingivitis, prevalent in large part of health adult population (Chapple et al., 2015). Serum is an important component of the gingival crevicular fluid, and the use of 5% serum in the medium may have influenced the growth of bacteria. Being aware that in the oral environment there is a continuous interaction between supragingival plaque and saliva, we have used this mixture as inoculum. Considering as well, that after a single episode of tooth brushing, on average plaque scores are reduced by only 42% the plaque that remains on the tooth surfaces (Chapple et al., 2015) will lead to a recolonization of the cleaned areas.

Using the same biofilm model as in the present study, a recent paper describes using saliva from a single donor as inoculum for the production of biofilms, with an initial incubation period of 8 h and a total incubation period of 48 h (Exterkate et al., 2014). It was observed that the composition of the water treated control biofilms (PMA treated) was dominated by genera *Streptococcus* (62%), *Veillonella* (35%) and *Prevotella* (2%). In the present study we attempted to increase the diversity of the biofilms by using as inoculum source a mixture of saliva and dental plaque. Furthermore we increased the incubation period to 24 h and the total incubation period to 96 h, providing time to the slow growing bacteria to establish within the biofilms. Figure 3.2 illustrates the relative abundance of the biofilms per donor being the water treated control biofilms dominated by genera *Veillonella*, *Streptococcus*, *Megasphaera*, *Peptostreptococcus*, *Prevotella* with 4%-20% of the reads belonging to other genera.

Rudney and collaborators (Rudney et al., 2012) have shown that repeated plaque or saliva inoculums taken on different weeks, from the same donor, clustered together, suggesting that the microbial composition of the biofilms was consistent within subjects over time. The authors have shown that microcosms were relatively stable within subjects over time, but there clearly were differences in species composition between subjects. Subsequently we decided to use multiple donors.

Regarding the variability among donors, it was not only biofilms from the same donor that clustered together, but also the bacterial profiles after the mouthwash treatment. In particular biofilms derived from donor 11 (Fig 3.2) seemed not to be affected by any mouthwash agent as compared to the water treatment. Also where donor 10 had minor changes after PA and MD treatment it seemed not to be affected by AX as compared to water treatment. Our results confirmed the variation among donors in *in vitro* biofilms and are in agreement with previous studies (Diaz et al., 2006, Filoche et al., 2008, Ledder et al., 2006, Rasiah et al., 2005). Our study supports the notion that pooling samples and averaging the data can mask the individual variability of results of the treatment and the conclusions that are based on them (Diaz et al., 2006, Ledder et al., 2006). The differences observed between the biofilms and their inoculum source are in agreement with previous *in vitro* studies (McBain et al., 2003, Rasiah et al., 2005).

A recent study has shown differences in bacterial composition at the site-level (Simon-Soro et al., 2013). Whether these differences found *in vivo* will lead to different microcosms derived from specific niches, and with-in or between donors still needs to be investigated. It is plausible that individual samples from certain sites in a given individual will lead to different results in bacterial composition.

A limitation of our study is that we assessed the effects of a single exposure to mouthwashes, whereas multiple exposures to a chemotherapeutic compound *in vitro* are known to lead to different results in viability (Ledder et al., 2014). This could explain in part, why in a previous clinical study we observed different changes in bacterial composition after twice-daily exposures for a week to AX (Fernandez y Mostajo et al., 2014). Oxygenating agents such as AX, are on the market while scientific evidence for their use is underreported (Van der Weijden et al., 2015). Therefore we underline the need of long-term randomized controlled trials testing

these compounds. Concerning “adjunctive” compounds for plaque and gingivitis control such as AX, the American Dental Association (ADA) requires an evaluation period of at least 4 weeks (ADA 1997, 2008) and for mouthwash products an evaluation period of 6 months.

All studied mouthwashes affected the *in vitro* biofilms differently. The effects of the AX treatment were the most prominent which resulted in changes of the bacterial composition, metabolism and in addition affected viability

Acknowledgements

We are very grateful, to Prof. Dr. Wouter Beertsen and Prof. Dr. Fridus van der Weijden for their valuable scientific contribution, to Ms. Wendy de Wit for her technical support and to those that donated the saliva and dental plaque.

Compliance with Ethical Standards:

Conflict of Interest: The authors declare that they have no conflict of interest pertaining to the data presented in this article.

Funding source: The study was financed with a commission from the Academic Centre for Dentistry Amsterdam (ACTA) Dental Research BV (grant number A10-0886). The Department of Preventive Dentistry of ACTA received financial support from NGen Oral Pharma N.V., Curacao.

This company had no influence on the design, content, results and publication of this study.

Ethical approval: Ethical approval of the protocol related to plaque-saliva collection and experimental research was confirmed by the Medical Ethical Committee from the Vrije Universiteit Medical Center Amsterdam (reference 2011/236).

Informed consent: All participants gave their informed consent prior to their inclusion in the study.

Chapter 4a

Effect of an oxygenating agent on oral bacteria *in vitro* and on dental plaque composition in healthy young adults.

This chapter has been published as:

Fernandez y Mostajo, M., van der Reijden, W. A., Buijs, M. J., Beertsen, W., van der Weijden, F., Crielaard, W. & Zaura, E. (2014)

Front Cell Infect Microbiol **4**, 95. doi:10.3389/fcimb.2014.00095

ABSTRACT

Introduction

Oral bacteria live in symbiosis with the host. Therefore, when mouthwashes are indicated, selective inhibition of taxa contributing to disease is preferred instead of broad-spectrum antimicrobials. The potential selectivity of an oxygenating mouthwash, Ardox-X® (AX), has not been assessed. The aim of this study was to determine the antimicrobial potential of AX and the effects of a twice-daily oral rinse on dental plaque composition.

Material and methods

In vitro, 16 oral bacterial strains were tested using agar diffusion susceptibility, minimum inhibitory and minimum bactericidal concentration tests. A pilot *clinical study* was performed with 25 healthy volunteers. Clinical assessments and microbiological sampling of supragingival plaque were performed at one month before the experiment (Pre-exp), at the start of the experiment (Baseline) and after the one-week experimental period (Post-exp). During the experiment individuals used AX mouthwash twice daily in absence of other oral hygiene measures. The microbiological composition of plaque was assessed by 16S rRNA gene amplicon sequencing.

Results

AX showed high inter-species variation in microbial growth inhibition. The tested *Prevotella* strains and *Fusobacterium nucleatum* showed the highest sensitivity, while streptococci and *Lactobacillus acidophilus* were most resistant to AX. Plaque scores at Pre-exp and Baseline visits did not differ significantly (p = 0.193), nor did the microbial composition of plaque. During a period of 7-days non-brushing but twice daily rinsing plaque scores increased from 2.21 (0.31) at Baseline to 2.43 (0.39) Post-exp. A significant microbial shift in composition was observed: genus *Streptococcus* and *Veillonella* increased while *Corynebacterium, Haemophilus, Leptotrichia, Cardiobacterium* and *Capnocytophaga* decreased ($p \leq 0.001$).

Conclusion

AX has the potential for selective inhibition of oral bacteria. The shift in oral microbiome after one week of rinsing deserves further research.

Key words: microbiome, selective inhibition, oxygenating agents, antimicrobials, Ardox-X®-technology

INTRODUCTION

Dental plaque biofilm is part of the oral microbiome that co-evolves in symbiosis with the human host (Marsh, 2012). Recently the importance and beneficial role of the oral microbiome in maintaining oral and general health has been brought forward (Hezel and Weitzberg, 2013, Marsh, 2012). On the other hand, undisturbed dental plaque accumulation is associated with an enhanced host inflammatory response and gingival inflammation (gingivitis) (Lee et al., 2012). Gingivitis is known to be associated with the onset of periodontitis (Schatzle et al., 2003), therefore the importance of maintaining gingival health is well understood.

Although regular mechanical plaque removal is recommended for prevention of periodontal diseases, the quality of self-performed mechanical plaque removal may not always be sufficient (Hioe and van der Weijden, 2005). When this fails or cannot be optimally maintained, for instance in physically or mentally disabled populations, a chemical approach, such as the use of an antimicrobial mouthwash, can be an alternative or an adjunct.

Anti-plaque agents should not eradicate the oral microbiota. Instead, they should maintain the microbiota of the mouth at the level and composition that is compatible with oral health, this way preserving the beneficial functions of resident microbes (Marsh, 2012, ten Cate and Zaura, 2012). This requirement is not met by so-called broad spectrum antimicrobial agents such as chlorhexidine (CHX). Interestingly, oxygenating mouthwashes containing peroxoborate are able to reduce the dental plaque amount and retard the colonization and growth of anaerobes (Binney et al., 1992, Moran et al., 1995, Wennstrom and Lindhe, 1979) and Gram-negative bacteria (Hernandez et al., 2013). Gram-negative anaerobes are generally associated with oral infections (*e.g*. periodontitis, peri-implantitis, endodontic infections).

Among oxygenating agents, boron-derived compounds such as sodium perborate (peroxoborate) generate active oxygen in aqueous solutions. This characteristic is the basis for their use as bleaching agents in detergents, cleaning products and cosmetic preparations, as well as a preservative in eye drops (Safety, 2010). In clinical dentistry, boron-derived compounds are used as a bleaching agent for teeth and as an adjunct to CHX to counteract extrinsic staining of the tongue and tooth surfaces (Feiz et al., 2014, Grundemann et al., 2000, Moran et al., 1995).

Ardox-X® technology (AX) was introduced to the market and promoted as a teeth whitening, anti-microbial, anti-fungal and anti-inflammatory compound (NGen Oral Pharma; www.ngenpharma.com) (Accessed 08 April, 2014). According to the manufacturer, the AX compound is formed by chemical complexation of peroxoborate with specific carriers such as glycerol and cellulose. This produces sodiumperborate-1,2-diol-glycerol/cellulose-ester adducts, i.e. single-reaction products containing all the atoms of all components. The manufacturer considers this to be a distinct molecular compound that provides controlled release of active oxygen without generating hydroxyl radicals. However, the scientific evidence for the antimicrobial efficacy of this compound is scarce. So far, only one *in vitro* study has

been published which showed that AX has an antimicrobial effect against polymicrobial biofilm (microcosm) grown on titanium surfaces (Ntrouka et al., 2011).

The aims of the current study were two-fold: first, to determine the antimicrobial effect of AX against oral bacteria *in vitro*; second, to evaluate *in vivo* the effect of AX containing mouthwash on the composition of undisturbed plaque accumulation in a one-week non-brushing model in healthy adults.

MATERIALS AND METHODS

In vitro study

Bacterial strains (Table 4a.1) were cultured on blood agar plates (Oxoid no 2, Oxoid, Basingstoke, UK) supplemented with 5% horse blood, 0.1% (w/v) haemin and 0.01% (w/v) menadione. For *Tannerella forsythia* Trypticase Soy Agar (TSA) (BBL, Beckton Dickson Microbiology Systems, Cockeyscille, MD) was used supplemented with 5% horse blood, 0.1% (w/v) N-acetyl muraminic acid (TSNAM plates), 0.05% (w/v) haemin and 0.01% (w/v) menadione (van der Reijden et al., 2006). All strains except *Staphylococcus aureus* HG386 were grown in anaerobic atmosphere containing 80% N_2, 10% CO_2 and 10% H_2. *S. aureus* was grown aerobically.

For this study, the manufacturer provided different concentrations of AX in standard equivalent units (SE) in a range 1 SE – 20 SE, where 1 SE contains 0.27% (w/v) of sodium perborate (SP), as well as the AX blank (AX without sodium perborate; (NGEN Oral Pharma N.V., Curacao; van den Bosch, US patent number 6.017.515) that was used as negative control. As positive controls, two different concentrations of over-the-counter chlorhexidine (CHX) products were used: Perio Aid (0.12% CHX) (Dentaid, Barcelona, Spain) and Corsodyl (0.2% CHX) (GlaxoSmithKline, Zeist, the Netherlands).

Agar diffusion tests were performed as described before (Wikler et al., 2009). For each strain, two blood agar plates were inoculated with 100 µl suspension of a single colony suspended in 5 ml phosphate buffered saline (PBS). The compounds were added in 5 mm holes punched in the agar. Five concentrations of AX (1.36%; 1.9%; 2.72%; 4.08%; 5.44%) together with a blank and two CHX concentrations were used. The blank and AX (2.72%) were included in each agar plate (twice per strain). Agar plates were incubated for 7 days at 37°C under anaerobic conditions (10% CO_2, 10% H_2, and 80% N_2). After incubation, plates were examined for growth inhibition. The inhibition zone around the holes was measured and expressed in mm from the edge to the nearest CFU.

Serial dilutions were used to determine the minimum inhibitory concentration (MIC) (Hecht, 2007) and the minimum bactericidal concentration (MBC) of AX. Two 24-wells plates were used per dilution set of 4 strains. The medium consisted of Brain Heart Infusion (BHI) broth (Oxoid, Basingstoke, UK) supplemented with 0.1% haemin and 0.01% menadione (h/m). For *Tannerella forsythia* filter-sterilized N-acetyl-muraminic acid (NAM) to a final concentration of 0.1% (w/v) was added. The compound was filter-sterilized (0.2 µm pore size; 7 bar max

Whatman, Germany). The initial dilution for the MIC was prepared using 0.5 ml AX at the highest concentration (54.4 g/l) in two-fold serial dilution series (range 40 - 40800 mg/l).

Inocula were prepared from a pure culture of each strain. A single colony was taken using a sterile cotton pick and suspended in 5 ml of PBS. Then 50 µl of suspension were dispensed into each labeled well. Plates were incubated for 7 days at 37°C under anaerobic conditions (10% CO_2, 10% H_2, and 80% N_2). After one week the MICs were determined by visual means. The procedure was performed in triplicate at different time points.

After MIC determination, 100-µl samples from the various dilutions were inoculated onto appropriate agar plates and incubated for 7 days at 37°C under anaerobic conditions (10% CO_2, 10% H_2, and 80% N_2). The concentration at which growth was visibly inhibited was defined as the MBC.

Table 4a.1. Bacterial strains and their abbreviations used in the text.

Strain	Abbreviation
Actinomyces naeslundii ATCC 12104	*An*
*Aggregatibacter actinomycetemcomitans HG 683**	*Aa*
*Campylobacter rectus HG 963**	*Cr*
Fusobacterium nucleatum ATCC 25586	*Fn*
Lactobacillus acidophilus ATCC 4356	*La*
*Parvimonas micra HG 1179**	*Pm*
*Porphyromonas gingivalis K- HG 91**	*Pg K-*
*Porphyromonas gingivalis K1 HG 66/W83**	*Pg K1*
*Porphyromonas gingivalis K6 HG 1691**	*Pg K6*
*Prevotella intermedia HG 110**	*Pi*
*Prevotella nigrescens HG 70**	*Pn*
Staphylococcus aureus ATCC 2592	*Sa*
*Streptococcus mutans HG 708**	*Sm*
*Streptococcus sanguinis HG 1471**	*Ss*
Tannerella forsythia ATCC 43037	*Tf*
*Veillonella parvula HG 318**	*Vp*

* Clinical isolates

Pilot clinical study

The study protocol was approved by the Medical Ethics Committee of the Academic Medical Center (AMC) of Amsterdam (NL37567.018.11) and registered at the Dutch trial register under the number NTR3145. The study followed the instructions based on the declaration of Helsinki. That statement acts as a starting point in subject recruitment.

Study population

Participation in this study was voluntary. Before enrollment all participants were given oral and written information about the products and the reason, aim, duration, demands of benefits and possible harm. After signing a declaration of informed consent, 26 systemically healthy participants meeting the inclusion criteria agreed to participate in the study.

All participants (non-dental students, ≥18 years) had to be dentate with at least 5 evaluable teeth per quadrant excluding prosthetic crowns. To include a population with high plaque scores at the start of the experimental period, participants were selected that had an overnight plaque score of 2 or higher as assessed according to Modified Quigley & Hein Plaque index (Paraskevas et al., 2007). Exclusion criteria were: oral mucosal lesions, orthodontic appliances, removable (partial) dentures, and overhanging margins of dental restorations (clinically assessed with a periodontal probe), the use of antibiotics during the last 6 months, Dutch Periodontal Screening Index (Mantilla Gomez et al., 2001) (DPSI) ≥3+ (periodontal pockets >5 mm with bleeding on probing and gingival recession), the use of medication possibly influencing normal gingival health, pregnancy and smoking.

Test compound

According to the manufacturer, the hydro-carbon-oxo-borate compound AX had the following ingredients: aqua, sodium lauryl sulfate, PEG-40 hydrogenated castor oil, sodium gluconate, cellulose gum, aroma, sodium citrate, magnesium sulfate, sodium perborate, sodium methylparaben, citric acid, sodium chloride, sodium fluoride, sodium saccharin (NGen Oral Pharma NV, www.ngenpharma.com; van den Bosch, US patent number 6.017.515).

Study design

The study started with a pre-experimental appointment (1 month prior to baseline) during which dental plaque was scored and sampled with the intention to assess the consistency of collected plaque scores and microbiological data relative to the baseline. At baseline, dental plaque was again scored and sampled. In addition, the level of bleeding on marginal probing was assessed as a descriptive of the oral health status of the included subjects. After the baseline measurements, a professional prophylaxis was performed by a dental hygienist as described in detail by Slot et al., (2010) in order to start the experiment with equally clean teeth. Following the prophylaxis, a one-week non-brushing experimental period of undisturbed plaque accumulation was started. With respect to oral hygiene the participants were only allowed rinsing with the distributed mouthwash (AX). Each subject received an

instruction form on how to use the intervention product and the first rinse was performed under supervision. Participants were instructed to rinse twice daily (morning and evening) for 1min and not to rinse, drink or eat for at least 30 min thereafter. No other form of oral hygiene during the subsequent week was allowed, including chewing gum (Keukenmeester et al., 2014) or any xylitol containing sweets or gum (Soderling, 2009).

Clinical assessments

In the study a partial-mouth model (Bentley and Disney, 1995) was used. Two contra-lateral randomly chosen quadrants (www.random.org) served for the collection of dental plaque biofilm that was not disturbed by scoring or the disclosing solution (one in the upper and one in the lower jaw; Heijnsbroek et al., 2006, van Leeuwen et al., 2015).

The two opposing contra-lateral quadrants were used for the clinical plaque assessments. All teeth in each of the two quadrants were examined except third molars. Scoring was performed by twoexperienced examiners each responsible for scoring one clinical parameter (plaque or bleeding) separately. For plaque scores teeth were disclosed with a 1% erythrosine solution. Plaque was assessed at six sites per tooth on a six-point scale using the Quigley & Hein (1962) plaque index as modified by Turesky et al. (1970) and further modified by Lobene et al., 1982, in which the absence or presence of plaque was recorded on a 0–5 scale (0 = no plaque, 5 = plaque covering more than two-thirds of the tooth surface) and described in detail by Paraskevas et al. (2007). At the baseline appointment the level of oral health was assessed in the two contra-lateral quadrants that had previously been sampled for supragingival plaque using the Bleeding on Marginal Probing (BOMP) score (Van der Weijden et al., 1994a, Van der Weijden et al., 1994b, Lie et al., 1998). Bleeding was elicited with a WHO-approved ball-ended probe (Ash Probe EN15, Dentsply International, York, PA, USA). The absence or presence of bleeding was scored within 30 s of probing on a scale of 0–2 (0 = non-bleeding, 1 = pinprick bleeding, 2 = excess bleeding).

Sampling procedure

Since it is imperative to characterize differences in microbial composition among specific oral locations, supragingival dental plaque was collected from the buccal sites of four pre-selected teeth being the same at all three assessments (first molar and canine, upper and lower jaw). Dental plaque was carefully collected by an experienced examiner with a sterile microbrush (Microbrush International, Grafton, USA) per tooth moving over the enamel surface from the mesial to distal curvature of the tooth crown along the gingival margin and tooth-surface border. The tip of each of the four microbrushes was clipped off and placed in a single vial containing RNAProtect Bacteria reagent (Qiagen, Hilden, Germany). Samples were coded, kept on ice until transfer within two hours to the laboratory.

DNA extraction, amplicon preparation and pyrosequencing

Of the 72 clinical samples belonging to 25 subjects, 6 samples were lost due to technical reasons. DNA was extracted with the AGOWA mag Mini DNA Isolation Kit (AGOWA, Berlin,

Germany) as described previously (Crielaard et al., 2011). Barcoded amplicon libraries of the small subunit ribosomal RNA gene hypervariable region V5–V7 were generated for each of the individual sample as described previously (Kraneveld et al., 2012), pooled and sequenced by means of the Genome Sequencer FLX Titanium system (Roche Molecular Diagnostics). The sequencing data was processed using Quantitative Insights Into Microbial Ecology (QIIME) (Caporaso et al., 2010) version 1.5.0. The reads were denoized using Denoiser version 1.3.0 (Reeder and Knight) and checked for chimeric sequences using UCHIME version 4.2.40 (Edgar et al., 2011). The results of the *de novo* and the reference-based approach were combined and reads marked as chimeric were removed. Sequences were clustered in operational taxonomic units (OTUs) at 97% similarity.

Statistical analyses

The statistical package SPSS software version 19.0 was used to perform statistical analyses. The effect of AX on bacterial strains in the agar diffusion assay was analyzed for each AX concentration relative to the effect of 0.2% CHX for each bacterial strain (Mann-Whitney test). Differences among strains per-compound were calculated using one-way ANOVA and Tukey B post hoc test.

For the clinical study, the mean plaque score and gingival bleeding score were calculated first per participant. Additionally the mean bleeding score at baseline for the sampled teeth was calculated. Plaque scores were tested for normality using the Shapiro-Wilk test. Non-parametric Wilcoxon Signed Rank test was performed to test for differences in plaque scores between the three visits: pre-experimental, baseline and post-experimental. To normalize the microbial data for comparisons among different samples and to avoid the effect of variable sample size on the diversity analyses, a randomly subsampled data set of 850 reads per sample was created. This resulted in exclusion of additional five samples with less than 850 reads/sample. PAST software (Hammer et al., 2001) was used to calculate Shannon diversity index, which takes into account the abundance of each OTU, as well as the number of OTUs. The normality of the diversity index data was assessed using Shapiro Wilk test. Paired samples T-test was used to compare the diversity indices between the three time points. OTU-significance paired samples T-test implemented in QIIME 1.5.0(Caporaso et al., 2010)was used to compare the abundances of OTUs at baseline and post-experimental samples. Only those OTUs that were present in at least 10 samples were included in the analyses, resulting in 75 comparisons. The p-values were corrected for these multiple comparisons using Bonferroni correction. P-values below 0.05 were considered statistically significant.

To visualize microbial profile data, principal component analysis (PCA) was used in PAST. The OTU abundances were log2 transformed to normalize the data distribution.

RESULTS

In vitro study

Based on the size of the inhibition zone (mm), the efficacy of CHX was greater than that of AX at all concentrations tested and for all bacterial strains ($p < 0.05$). AX showed high variation in inhibition (Fig. 4.1), which was statistically significant among various strains (Table 4a.2). *Streptococcus mutans, Lactobacillus acidophilus* and *Streptococcus sanguinis* were the least affected (inhibition zone 0 mm - 0.3 mm), while *Prevotella nigrescens* (9 mm) and *Prevotella intermedia* (9 mm) - the most $p < 0.05$ (Table 4a.2).

All tested strains were inhibited by CHX and the differences in inhibitory activity among strains were less pronounced than for AX (Table 4a.2). *Porphyromonas gingivalis K-* was the most affected (14 mm) and *Staphylococcus aureus* – the least (6 mm). None of the strains were inhibited by the AX blank (0 mm).

For most strains the MICs and MBCs for AX were ≤ 638 mg/l SP. Except for *L. acidophilus* - 2550 mg/l SP, *S. aureus* and *S. sanguinis* - 1275 mg/l SP (Table 4a.3). The MBCs and MICs for AX were nearly the same (Table 4a.3).

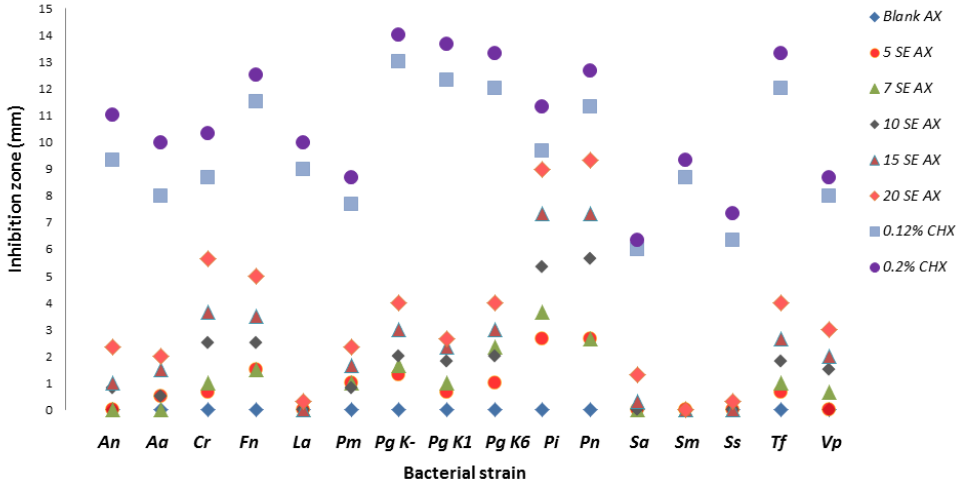


Figure 4a.1 Effects of Ardox-X®-technology (AX), Blank AX and Chlorhexidine (CHX) on inhibition of oral bacterial strains in agar diffusion assay. Inhibition zone size is expressed in mm (mean of triplicate experiment, except duplicate for *Aa* and *Fn*). The strains (Table 4a.1) used were: *Parvimonas micra (Pm); Porphyromonas gingivalis (Pg K1). (Pg K-). (Pg K6); Actinomyces naeslundii (An); Fusobacterium nucleatum (Fn); Campylobacter rectus (Cr); Staphylococcus aureus (Sa); Aggregatibacter actinomycetemcomitans (Aa); Lactobacillus acidophilus (La); Veillonella parvula (Vp); Streptococcus sanguinis (Ss); Streptococcus mutans (Sm); Prevotella intermedia (Pi); Prevotella nigrescens (Pn); Tannerella forsythia (Tf)*.

Table 4a.2 Results of the Agar Diffusion assay, performed in triplicate. Mean size of the inhibition zones (mm) and standard deviation.

Strain	Concentration of Ardox-X® technology																				
	0.2% CHX			0.12% CHX			5 SE			7 SE			10 SE*			15 SE			20 SE		
	Mean (SD)		Diff.	Mean (SD)		Diff.	Mean (SD)		Diff.	Mean (SD)		Diff.	Mean (SD)		Diff.	Mean (SD)		Diff.	Mean (SD)		Diff.
An	11	(0)	cd	9	(1)	cdef	0	NA	b	0	NA	d	1	(1)	cd	1	(1)	cde	2	(1)	def
Cr	10	(1)	cdef	9	(1)	def	1	(1)	ba	1	(1)	cd	2	(1)	b	4	(1)	b	6	(1)	bc
La	10	(2)	cdef	9	(0)	def	0	NA	b	0	NA	d	0	NA	d	0	NA	e	0	NA	fg
Pm	9	(1)	def	8	(1)	fgh	1	(2)	ba	1	(1)	bcd	1	(1)	cd	2	(1)	bcde	2	(1)	def
Pg K-	14	(1)	a	13	(1)	a	1	(1)	ba	2	(1)	bcd	2	(1)	bd	3	(1)	bcde	4	(1)	cd
Pg K1	14	(1)	a	12	(1)	ab	0.7	(0.6)	ba	1	NA	cd	2	(0)	bd	2	(1)	bcde	2.7	(0.6)	de
Pg K6	13	(1)	ab	12	(0)	abc	1	(1)	ba	2	(1)	abc	2	(1)	bd	3	(1)	bcde	4	(1)	cd
Pi	11	(1)	bcd	10	(2)	bcde	2.7	(0.6)	a	4	(1)	abc	5	(1)	a	7	(1)	a	9	(1)	a
Pn	13	(1)	bac	11	(1)	abcd	3	(1)	a	2.7	(0.6)	abc	6	(1)	a	7	(2)	a	9	(2)	a
Sa	6	(1)	f	6	(0)	h	0	NA	b	0	NA	d	0	NA	d	0.3	(0.6)	de	1	(1)	efg
Sm	9	(1)	cdef	9	(1)	efg	0	NA	b	0	NA	d	0	NA	d	0	NA	e	0	NA	g
Ss	7	(1)	ef	6	(1)	gh	0	NA	b	0	NA	d	0	NA	d	0	NA	e	0	NA	fg
Tf	13	(1)	ab	12	(0)	abc	1	(1)	ba	1	NA	cd	2	(1)	bc	3	(1)	bcde	4	(1)	cd
Vp	9	(1)	def	8	(2)	fgh	0	NA	b	1	(1)	d	2	(1)	bc	2	(0)	bcde	3	(0)	de
*Aa***	10	(1)		8	(1)		0	NA		0	NA		1	(1)		1	(1)		2	(1)	
*Fn***	12	(1)		11	(1)		1	(1)		1	(1)		2.5	(0.6)		3.5	(0.7)		5	(1)	

Mean size of the inhibition zones (mm) and standard deviation. Diff., Different letters indicate statistically significant difference among the different bacterial strains per-compound (within each column) ($p<0.05$; ANOVA, Tukey B test). * n=6; ** Excluded from analysis (n=2); NA - not applicable; SE - standard equivalent units.

Table 4a.3 MICs and MBCs of Ardox-X® technology for the 16 strains studied, expressed as sodium perborate (SP) concentration in the compound.

Bacteria strain	MIC		MBC	
	SP mg/l	range	SP mg/l	range
An	319	319 - 638	319	319 - 638
Aa	159	80 - 319	319	80 - 638
Cr	159	NA	638	319 - 638
Fn	159	159 - 319	159	159 - 319
La	2550	1275 - 2550	2550	1275 - 2550
Pm	638	638 - 1275	638	638 - 1275
Pg K-	319	NA	319	319 - 638
Pg K1	319	NA	638	NA
Pg K6	159	80 - 159	159	NA
Pi	80	40 - 80	80	80 - 159
Pn	80	80 - 159	80	80 - 159
Sa	1275	638 - 1275	1275	638 - 1275
Sm	638	638 - 1275	638	638 - 1275
Ss	1275	638 - 1275	1275	638 - 1275
Tf	319	159 - 319	319	159 - 319
Vp	159	NA	159	NA

Values are median (range) of experiment in triplicate. NA, not applicable due to equal values.

Table 4a.4 Subject demographics and their periodontal health

N	**25**
Female/Male	17/8
Age in years, mean (SD)	21.5 (1.9)
DPSI*	
1	1
2	11
3-	13
BOMP**, mean (SD)	1.15 (0.33)

***** Dutch periodontal screening index (Mantilla Gomez et al., 2001)

** Bleeding on marginal probing (BOMP) at baseline (Van der Weijden et al., 1994a, Van der Weijden et al., 1994b, Lie et al., 1998)

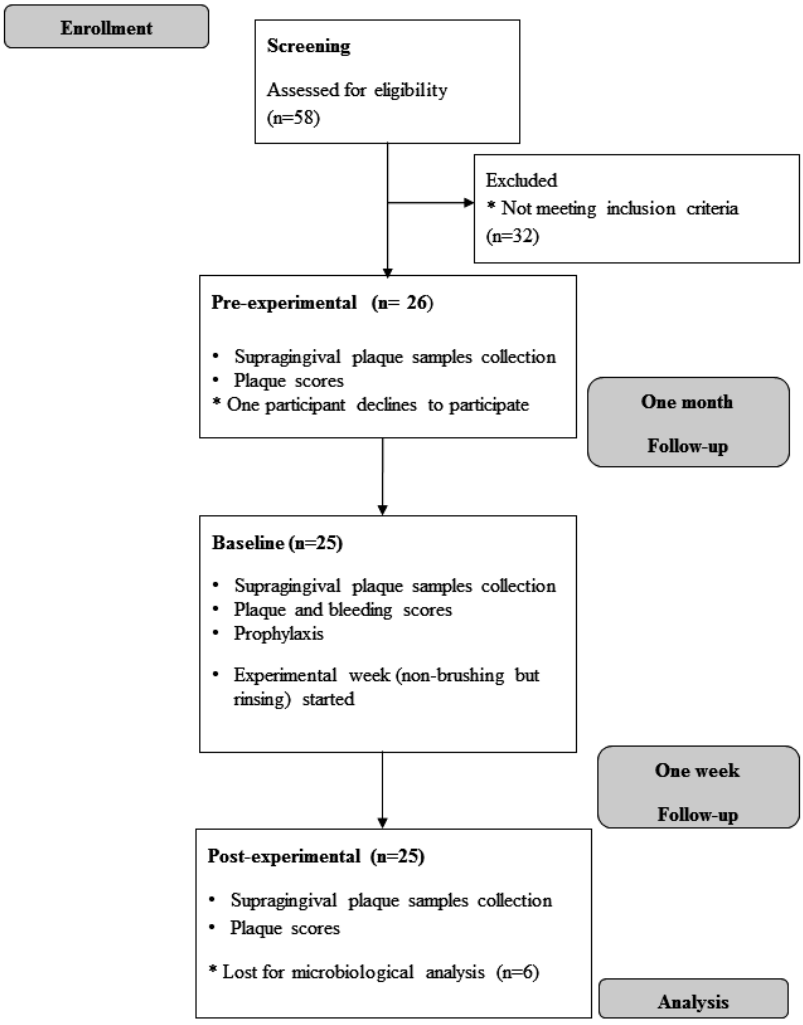


Figure 4a.2 Flow chart and timeline of the pilot clinical study

Pilot clinical study

Of 26 participants initially enrolled in the study, one participant dropped out for a reason unrelated to the study (Table 4a.4, Fig.4a.2). The duration of overnight plaque accumulation assessed at the pre-experimental and the baseline visits ranged from 10 h to 16 h with an average of 13 h (SD 2.9). At the baseline appointment, the mean level of gingival health of the participants, as assessed by Bleeding on Marginal Probing (BOMP) in two contra-lateral quadrants, was 1.15(SD 0.33) (Table 4a.4). The mean bleeding scores at the sampled teeth (total of 12 buccal sites from four pre-selected teeth) was 0.98 (SD 0.43), which corresponds to 51% bleeding (SD 23).

Plaque scores did not differ significantly between the pre-experimental visit and baseline (p=0.193), while plaque scores increased significantly (p= 0.014) during a week without any additional oral hygiene measures but with twice-daily use of AX mouthwash (Table 4a.5).

Compliance to the rinsing protocol was assessed by weighing the bottles at the baseline and 7 days later at the post-experimental visit. The difference was on average 140 g (± 14 g), which implied on average 14 servings of 10 ml complying with the individual instructions for use given.

In total 19 participants provided evaluable microbiological data. The data of three participants were excluded due to the technical reasons in sample processing, and another three – due to low reads (<850) per sample in one of the samples after the filtering steps of the sequencing data. The remaining 54 samples had on average 3135 reads/sample (SD 1047). The total of 169309 reads were classified into 15 phyla, with *Actinobacteria* (39% of the reads) and *Firmicutes* (31%) dominating the data, followed by *Proteobacteria* (19%), *Bacteroidetes* (7.5%), *Fusobacteria* (3.1%) and Candidate division TM7 (0.3%).

Table 4a.5 Mean (SD) and range of Plaque index scores

	Mean		**Min**	**Max**	***p-value****	
Pre-experimental	2.27	(0.34)	1.68	3.01	*0.193*	*Pre-experimental vs Baseline*
Baseline	2.21	(0.31)	1.60	3.12	*0.014*	*Baseline vs Post-experimental*
Post-experimental	2.43	(0.39)	1.63	3.26		

**Wilcoxon Signed Ranks test*

After subsampling at 850 reads/sample, the diversity and taxonomic comparisons among the three visits (pre-experimental, baseline and post-experimental) were performed. Shannon diversity index, taking into account the abundance of each OTU as well as the number of OTUs, significantly increased from pre-experimental to baseline visit from 2.67 (SD 0.29) to 2.79 (SD 0.29) ($p=0.02$) and significantly decreased at post-experimental visit to 2.09 (SD 0.39) ($p<0.001$).

Genera *Corynebacterium* (21% of reads) and *Streptococcus* (16-20%) dominated the pre-experimental and baseline samples. Both of these genera were significantly affected by the treatment period: *Corynebacterium* was reduced to 2% and *Streptococcus* – increased to 32% (Fig.4a.3). Additionally, genus *Veillonella* showed significant increase from 2-3% to 12% after the treatment, while genus *Derxia* showed significant decrease from 3% to 0.7%, respectively (Fig. 4a.3). Genus *Leptotrichia* was nearly absent after the experimental period, while it constituted approximately 2% of the reads at the pre-experimental and baseline visits. Genus *Prevotella* was present at a very low proportion – between 1 and 1.5% of the reads throughout the study and showed no significant effect of the treatment.

To identify the OTUs that contribute to the differences between the visits, an OTU-category significance test using paired samples T-test was performed, corrected for multiple comparisons (75) using Bonferroni correction. No OTUs differed significantly between the pre-experimental and baseline visits, while 10 OTUs differed between the baseline and post-experimental visit (Table. 4a.6). Two OTUs – OTU169, classified as *Veillonella*, and OTU113, classified as *Streptococcus* (blast: *Streptococcus sanguinis* SK1, 100% ID) increased. From the eight OTUs that showed a significant decrease, OTU197 was identified as *Streptococcus cristatus* (100% blast ID) and OTU183 – as *Leptotrichia hongkongensis* (100% blast ID), while the remaining six OTUs (Table 4a.6) could not be identified at species level.

Next, the microbiome profile data were ordinated by applying principal component analysis (PCA) (Fig. 4a.4). The first principal component (PC1) explained 27% of the overall variance among the samples and showed a clear separation of the pre-experimental (black dots, Fig. 4a.4) and baseline samples (blue dots, Fig. 4a.4) from the post-experimental samples (red dots in Fig. 4a.4). The second component explained 11% of the total variance and separated the samples belonging to subjects Nr 1 and Nr 31 from the rest (Fig. 4a.4).

Table 4a.6 Significantly differently abundant OTUs between baseline and post-experimental visit and their abundance in plaque samples.

	Number of reads (SD)					
OTU*	**Pre-experimental**		**Baseline**		**Post-experimental**	
113.Streptococcus	155	(52)	128	(53)	258	(93)
120.Corynebacterium	153	(93)	158	(84)	13	(15)
169.Veillonella	28	(43)	16	(14)	106	(61)
16.Corynebacterium	23	(21)	20	(15)	3	(6)
183.Leptotrichia	11	(8)	9	(7)	0.05	(0.23)
251.Capnocytophaga	7	(7)	9	(7)	2	(3)
197.Streptococcus	7	(8)	6	(4)	0.2	(0.5)
245.Cardiobacterium	6	(6)	8	(6)	2	(2)

Samples were randomly subsampled to 850 reads/sample. * - OTUs that remained significant after OTU-significance Paired samples T-test, Bonferroni correction, $p \leq 0.001$; N=19

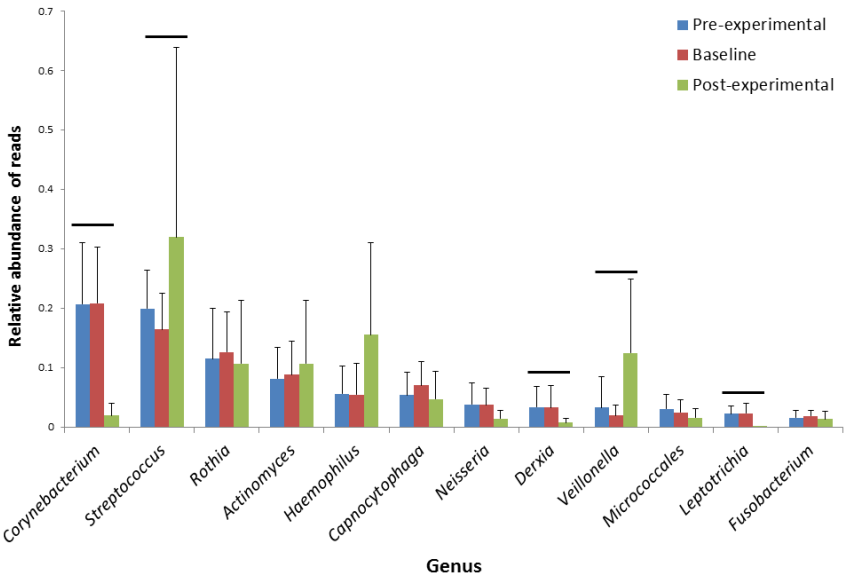


Figure 4a.3 Average proportions of major genera from dental plaque samples collected at pre-experimental, baseline and the post-experimental visit. Error bars – standard deviations. Horizontal lines indicate statistically significantly different proportions of reads of the respective genera between the post-experimental and the other two visits ($p<0.05$, Wilcoxon signed ranks test, after Bonferroni correction for multiple comparisons). N=19

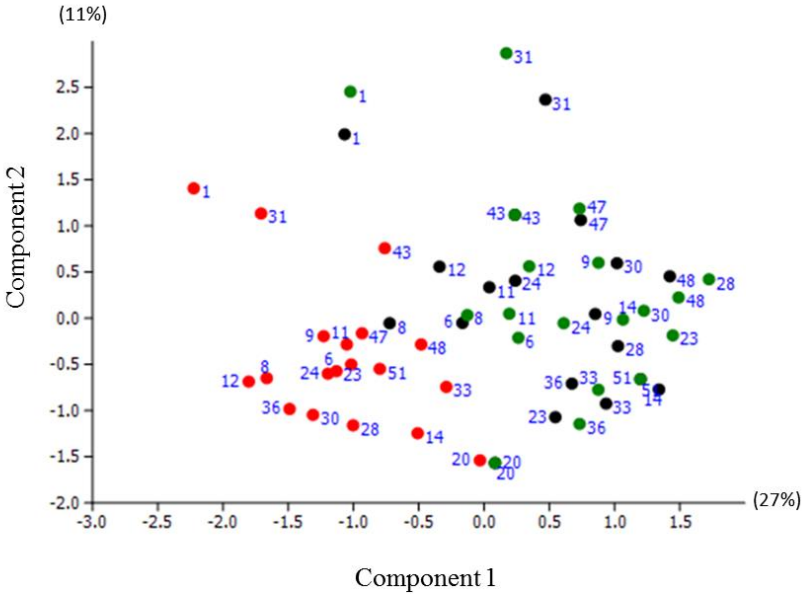


Figure 4a.4 Principal Component Analysis (PCA) plot of microbiome samples from pre-experimental visit (black dots); baseline of the experimental period (green dots) and post-experimental visit (red dots). The first component (PC1) explained 27% of the total variance, the PC2 - 11%. The same labels indicate samples that originated from the same individual.

DISCUSSION

The *in vitro* experiments of this study indicated that oxygen-releasing compound Ardox –X® technology (AX) selectively inhibits oral bacteria, with anaerobe Gram-negative species being the most sensitive. These promising findings were further tested *in vivo*, during a pilot clinical study with experimental period without any oral hygiene measures but twice-daily rinse with mouthwash containing AX. After a week of non-brushing, the plaque scores increased, while the microbial composition showed a shift towards compositionally less diverse plaque, dominated by primary colonizing genera *Streptococcus* and *Veillonella* compared to the *Corynebacterium* dominated plaque at the baseline.

It has been proposed that in order to study the effects of a mouthwash, a population with a high amount of plaque should be studied (Wennstrom, 1988). The study population therefore included individuals that proved to be good plaque formers at the screening visit. Moreover, it is known that the periodontal condition affects the rate of supragingival plaque forming (Hillam and Hull, 1977, Rowshani et al., 2004). Participants showed to have moderate gingivitis at baseline whereby 51% of the sampled sites were bleeding on marginal probing. The one-month interval between the pre-experiment assessment and baseline did not result in significant changes in plaque scores or in plaque composition of the study population. This is in line with previous studies, which have demonstrated the stability of the oral microbiome (Zhou et al., 2013). Microbial composition of the supragingival plaque in this gingivitis population one month before the experiment and at the baseline resembled mature plaque (Haffajee et al., 2009) and plaque associated with gingivitis (Huang et al., 2011). The major taxon in these samples was identified as *Corynebacterium*, a Gram-positive, facultatively anaerobe bacterium that resembles Gram-negatives with respect to the lipid layer in the cell membrane (John, 1984). *Corynebacterium* is associated with mature plaque and is found in dental calculus (Moorer et al., 1993).

The participants of the classical experimental gingivitis model (Loe et al., 1965, Theilade et al., 1966) received a prophylaxis and subsequently refrained from oral hygiene for 21 days inducing an acute stage of inflammation in otherwise healthy subjects. For the present study a seven-day model was chosen since the purpose was not to change the level of gingival health but to assess the effects on the microbial composition of undisturbed plaque. In the absence of oral hygiene, bacterial re-colonization increases after professional oral hygiene reaching or exceeding pre-prophylaxis levels at two days (Uzel et al., 2011) and *de novo* plaque formation reaches a stable microbial community between four to seven days (Uzel et al., 2011). Higher diversity of mature supragingival plaque compared to younger plaque has been found in a recent experimental gingivitis study (Kistler et al., 2013). In the present study after the baseline assessment, the participants received a thorough professional oral hygiene and were not allowed to brush their

teeth for one week. Instead, they were asked to perform a twice daily rinsing with AX-containing mouthwash. As expected, the plaque amount increased during the experimental period. The composition of the sampled plaque also changed impressively, whereby microbial diversity had decreased significantly, when compared to the pre-experimental and baseline visits. Genus *Corynebacterium* was considerably reduced , while streptococci, *Veillonella* and *Haemophilus* – all health-associated primary colonizers (Colombo et al., 2009, Simon-Soro, et al. 2013) - dominated the post-experimental plaque.

In vitro diffusion and susceptibility tests showed that AX is highly selective in inhibiting oral bacteria. The Gram-negative anaerobes such as prevotellas, but also *Veillonella*, *Tannarella*, *Campylobacter*, *Fusobacterium* and *Porphyromonas* were highly sensitive, while streptococci and lactobacilli, facultative anaerobe Gram-positive bacteria, were not inhibited even by the highest dose of AX tested in the diffusion test. In the clinical samples genus *Prevotella* were found at very low levels throughout the study and no effect of AX was discernible. However, other Gram-negative taxa such as genera *Derxia* and *Leptotrichia,* as well as OTUs classified as *Cardiobacterium* (OTU#245) and *Capnocytophaga* (OTU#251) were significantly reduced during the experimental period. Unfortunately, *in vitro* tests did not include any genus *Corynebacterium* species which allows only for speculation whether this Gram-positive bacterium with the characteristics of Gram-negatives (John, 1984) was also highly susceptible to exposure to AX. Alternatively its nearly complete elimination from the post-experimental plaque samples could have other reasons for instance its growth could have been inhibited due to ecological shifts in the entire community (Bradshaw et al., 1989). Another intriguing bacteria was *Veillonella* - anaerobe Gram-negative bacteria, associated with early supragingival plaque (Haffajee et al., 2009, Li et al., 2004). Although *Veillonella* was found to be susceptible to AX in *in vitro* testing, this genus showed a significant increase after the experimental period with twice-daily exposure to the AX mouthwash. This could be attributed to the 'pioneering' function of this bacteria; it is found in healthy individuals, in young supragingival plaque, in similar proportions with streptococci (Keijser et al., 2008; Haffajee et al., 2009). Veillonellae are secondary fermenters – they consume lactic acid produced during glucose fermentation by streptococci (Keller and Surette, 2006, Periasamy and Kolenbrander, 2010) and produce other, weaker acids such as acetic and propionic acid. By doing so, the environmental conditions are created that promote growth of both of these genera (Bradshaw et al., 1989). The most likely explanation of the increase of genus *Veillonella* during the experimental period, although sensitive to direct exposure to AX *in vitro*, could be related to this ecologically beneficial relationship with streptococci.

The selective inhibition of oral bacteria by AX is of interest with respect to gingival and periodontal diseases, since infections associated with Gram-negatives would

be selectively suppressed whereas the microorganisms regarded as more beneficial for periodontal health such as streptococci and lactobacilli would not. Several *in vitro* studies (Teughels et al., 2007, van Essche et al., 2012) and recent clinical studies (Iniesta et al., 2012, Teughels et al., 2013, Yanine et al., 2013) have suggested that these allegedly beneficial bacteria can cause antagonism towards Gram-negative bacteria.

In the agar diffusion assay, AX had significantly lower inhibitory effect than CHX. The activity of AX could have been limited to a short period right after its administration until the active component is broken down and oxygen is released. CHX is known to retain its activity for a longer time period after a single application *in vitro* (Carrilho et al., 2010). The only other study that has assessed the antimicrobial effect of AX, showed that the AX containing product had higher antimicrobial capacity than chlorhexidine towards monospecies bacterial biofilm and microcosm obtained from pooled saliva (Ntrouka et al., 2011).

So far, chlorhexidine (CHX) has proven to be the most effective antimicrobial agent in clinical dentistry and is considered as the "gold standard" disinfectant in dental research (Arweiler et al., 2001, Jones, 1997). Although it is widely used in periodontics and is among the most effective compounds preventing plaque formation (Addy, 1986), it has several side effects (Gurgan et al., 2006, Keijser et al., 2003) and therefore may result in poor rinsing compliance by patients (Cortellini et al., 2008, Addy and Moran, 1985, Van Strydonck et al., 2012). Optimizing anti-plaque agents, reducing their side effects while at the same time taking care that the oral microbiota are kept in balance with oral health has initiated interest in the development of other chemotherapeutical agents. Interestingly, AX showed selective inhibition of oral bacteria that may contribute to this demanding balance and deserves further investigation.

The lack of controls is a major limitation of this study that could have potential bias on the interpretation of this study outcomes. This pilot however indicates a potential rationale for more elaborate studies with a randomized clinical trial protocol that would include both a positive control such as CHX, and a negative control without any antimicrobial effects. The potential effect of AX on reductions in both the clinical manifestations of gingivitis and the inhibition of or reduction of plaque or plaque pathogenicity still needs to be demonstrated. For that purpose a 21 days experimental gingivitis model could be used or alternatively a 4-week trial among gingivitis subject as proposed by the American Dental Association in their Acceptance Guidelines of Chemotherapeutic Products for Control of Gingivitis (ADA, Acceptance Guidelines 2008).

In conclusion, a mouthwash containing the oxygenating agent Ardox-X® technology showed potential for selective inhibition of oral bacteria. Twice-daily exposure for one week to this mouthwash resulted in a shift in the microbial composition

towards a less diverse and less mature plaque. The clinical consequences of this shift in the oral microbiota need to be established.

Supplementary material

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fcimb.2014.00095/abstract

(1) seqs_rct_rep_set.fasta with the representative sequences of each OTU;

(2) otu_table_rct_not_subsampled.txt file with the OTU-table containing the list of OUTs and the irrespective taxonomical assignment and read abundances per each sample.

Funding source and Declaration of interests

The study was financed with a commission from ACTA Dental Research BV. ACTA Research BV received financial support from (NGen Oral Pharma N.V., Curacao) for the role of the Departments of Periodontology and Preventive Dentistry of ACTA in this project. NGen Oral Pharma N.V., Curacao, provided study products. The authors report that this company had no influence on the design, content, results and publication of this study.

Acknowledgements

We thank Nienke Hennequin-Hoenderdos and Dagmar Else Slot for coordinating the clinical study and Eveline van der Sluijs for her support during the process for the Medical Ethical Committee approval and the following persons for their contribution to the clinical study: Esther Martin and Guylaine van Anraat (Department of Periodontology Research group Prevention and Therapy of ACTA) and Wendy de Wit (Department of Preventive Dentistry at ACTA).

Chapter 4b

Comparison of red autofluorescing plaque and disclosed plaque

a cross-sectional study

This chapter has been published as:

Volgenant, C., Fernandez y Mostajo, M., Rosema, N. A. M.,

van der Weijden, F., ten Cate J. M. & van der Veen M. H. (2016)

Clin Oral Investig. doi:10.1007/s00784-016-1761-z

ABSTRACT

Objectives

The aim of this cross-sectional study was to assess the correlation between dental plaque scores determined by the measurement of red autofluorescence or by visualization with a two-tone solution. Clinical photographs were used for this study.

Materials and methods

Overnight plaque from the anterior teeth of 48 participants was assessed for red fluorescence on photographs (taken with a QLF-camera) using a modified Quigley&Hein (mQH)-index. A two-tone disclosing solution was applied. Total disclosed plaque was clinically assessed using the mQH index. In addition, total and blue disclosed plaque was scored on clinical photographs using the mQH-index.

Results

A strong correlation was observed between the total disclosed plaque scored on photographs and the clinical scores (r=0.70 at site level; r=0.88 at subject level). The correlation between red fluorescent plaque and total plaque, as assessed on the photographs, was moderate to strong and significant (r=0.50 at the site level; r=0.70 at the subject level), with the total plaque scores consistently higher than the red fluorescent plaque scores. The correlation between red fluorescent plaque and blue disclosed plaque was weak to moderate and significant (r=0.30 at the site level; r=0.50 at the subject level).

Conclusions

Plaque, as scored on white-light photographs, corresponds well with clinically assessed plaque. A weak to moderate correlation between red fluorescing plaque and total disclosed plaque or blue disclosed plaque was found.

Clinical relevance

What at present is considered to be matured dental plaque, which appears blue following the application of a two-tone disclosing solution, is not in agreement with red fluorescent dental plaque assessment.

Keywords: dental plaque, autofluorescence imaging, fluorescence, oral hygiene, dental plaque index; dental photography

INTRODUCTION

Caries and inflammation of the periodontal tissues are the most common oral diseases and are caused by the dental plaque present on the teeth (Marsh, 1994). Dental plaque becomes more pathogenic when present for a longer period on the tooth surface (matured plaque) (Kolenbrander et al., 2010). Therefore, prevention of oral diseases relies on frequent plaque removal (Axelsson and Lindhe, 1978).

Several plaque indices have been developed for research purposes to determine the area of the tooth that is covered with plaque. Clinically assessed scores as well as planimetric methods (Pretty et al., 2005) are frequently used after plaque has been disclosed. Often, a two-tone plaque-disclosing solution is used, which supposedly discloses 'young' plaque in a pinkish tone and 'old' or 'matured' plaque in a blueish tone (Gallagher and Cutress, 1977, Block et al., 1972). The pink dye adheres to all plaque, whereas the blue dye adheres and diffuses more easily into the denser / thicker plaque. Hence, the claim of the manufacturer is that young plaque stains pink and matured plaque stains blue-purple.

Dental plaque may also fluoresce red when excited with visible violet light (405 nm) (Heinrich-Weltzien et al., 2003, Thomas et al., 2008). This fluorescence is an intrinsic characteristic of plaque and is therefore called *auto*fluorescence. However, not all plaque fluoresces red. *In-vitro* studies on red autofluorescence from bacteria and biofilms assume that this fluorescence can be attributed to matured or cariogenic plaque (Kim et al., 2014, Thomas et al., 2008, van der Veen et al., 2006). While many bacteria that are associated with oral diseases are able to fluoresce red (Bjurshammar et al., 2012, Coulthwaite et al., 2006, Lennon et al., 2006a, Volgenant et al., 2013), modern dietary patterns may affect the autofluorescence of oral bacteria as well. Recent studies on plaque fluorescence indicate that either the volume or the age of the biofilm determines its red fluorescence or that fluorescence is activated by environmental triggers(Lee et al., 2013, Volgenant et al., 2013, Kim et al., 2014). In a clinical situation, red fluorescent plaque could thus be associated with a thick layer of plaque, maturation of the dental plaque, or the presence of inflamed gingival tissue. And more plaque formation occurs at sites in the oral cavity associated with periodontal inflammation as compared with that in healthy sites (Dahan et al., 2004, Rowshani et al., 2004). Plaque in some patients may fluoresce within 24 hours after professional tooth cleaning, while in others, no red fluorescing plaque is seen after 4 days without oral hygiene. This difference between patients makes it difficult to interpret red fluorescent dental plaque.

A clinical study about dental plaque fluorescence and its correlation with the plaque scores of the total disclosed plaque and the matured plaque portion (blue disclosed portion) could contribute to the understanding of the diagnostic value of red fluorescent plaque.

The aim of this cross-sectional study was to evaluate the correlation between clinically assessed combined (blue and pink) plaque scores and the combined plaque scores assessed on photographs. In addition on photographs, the correlation between scores of dental plaque as made visible by its red autofluorescence or by disclosing with a two-tone solution was assessed. Furthermore, the periodontal condition is known to be a defining factor in the rate

of plaque formation (Quirynen et al., 1991). Therefore, the level of gingival health was also assessed clinically, and the bleeding scores per site were compared with the obtained plaque scores to assess a possible relationship between the various methods of plaque assessments and gingival inflammation.

MATERIALS AND METHODS

Ethics approval and study participants

This cross-sectional study was performed as part of the baseline assessment of a clinical trial at the Periodontology department of the Academic Centre for Dentistry Amsterdam, which was approved by the Medical Ethics Committee of the Academic Medical Centre of Amsterdam (AMC) under registration number NL 37567.018.11. The trial was registered at the Dutch Trial Register under number NTR 3145. The study followed the instructions based on the Declaration of Helsinki (2008).

During the study-entry-assessment period, the participants received oral and written information about the study and could join the study after signing the informed consent. They were subsequently screened for inclusion and exclusion criteria approximately one month prior to the start of the study. The participants were considered eligible when they were 18 years of age and in good general health. They needed to have at least 6 evaluable anterior teeth and no prosthetics or crowns and bridgework in this region and should not have periodontitis as established by the Dutch Periodontal Screening Index (DPSI ≤ 3 minus) (Mantilla Gomez et al., 2001). This implies that participants did not have periodontal pockets deeper than 5mm and had no recessions. Prior to the screening visit, the participants abstained from any means of oral hygiene for at least 12 hours. Only participants with a modified Quigley & Hein plaque-index score ≥ 2 (clinically assessed) were included to pre-select participants who were able to form overnight dental plaque (‘heavy’ plaque formers). Throughout this study, the modified Quigley & Hein plaque-index was used(Quigley and Hein, 1962). This is a modification by Turesky et al. (Turesky et al., 1970) in the final description of Paraskevas et al. (2007) (mQH index). Participants wearing orthodontic appliances (except for lingual retention wires) or wearing removable (partial) dentures were excluded. Participants were also required to have no untreated cavities at the moment of inclusion, nor restorations with overhanging margins (when clinically assessed with a probe). Additionally, smokers and pregnant women were excluded from participation in this study.

Clinical procedures

The participants were instructed not to brush the night before the baseline study visit (to develop overnight plaque) and not to eat or drink (except water) 2 hours prior to the assessment. At the start of the study visit, the medical history was updated. Fluorescence photographs were taken of the vestibular aspect of the teeth in the upper and lower jaw (cuspid-to-cuspid) in an end-to-end position with a Canon 450-D SLR camera equipped with a Biluminator tube (QLF-D system, Inspektor Research Systems BV, Amsterdam, the

Netherlands) to assess the red fluorescence of plaque. Next, the plaque was disclosed using a cotton swab with a two-tone disclosing dye solution (Mira-2-Ton; Hager & Werken, Duisburg, Germany). The disclosing dye solution was applied on a fresh cotton swab till the swab was fully saturated. Subsequently the swab was gently applied on the tooth surfaces. Excess solution was washed away by allowing the participants to rinse with tap water once. An end-to-end photograph of the teeth after plaque disclosing was captured using an SLR camera with a ring flash (Canon Inc., Tokyo, Japan). Plaque was assessed clinically using the mQH index (Paraskevas et al., 2007). Inflammation of the gingiva was assessed using the bleeding on the marginal probing index (BOMP) as previously described (Van der Weijden et al., 1994). BOMP was determined on three upper front teeth and three contra-lateral lower front teeth.

Each clinical parameter was assessed by the same independent calibrated examiners; plaque assessment was performed by G.v.A. and bleeding assessment by E.J.C.M. Clinical parameters were scored from the vestibular aspect of the anterior teeth from the right cuspid to the left cuspid at the upper and lower jaw.

Photo assessment

The fluorescence photographs and the white-light clinical photographs were renamed with a unique identifier that blinded the examiner to the participant. The vestibular aspect of the anterior teeth (cuspid-to-cuspid, both upper and lower jaw) was assessed. Plaque was visually assessed on the clinical (white light) photographs using the mQH index. This index was applied combining both pink and blue disclosed plaque (*Combi-mQH*) as well as on the portion of blue disclosed plaque (*Blue-mQH*).

Red autofluorescence of dental plaque was visually assessed on the fluorescence photographs. To describe the extent of the red fluorescing plaque, the modified version of the mQH index was used (*QLF-mQH*, Figure 1).

All photographs were examined twice under similar circumstances by each of the following four independent and calibrated examiners: N.R., M.F., M.V. and C.V. Duplicate assessments of each examiner were performed at least one week apart. To analyse and compare plaque indices, consensus scores were derived for red fluorescing plaque, total disclosed plaque and blue disclosed plaque scores.

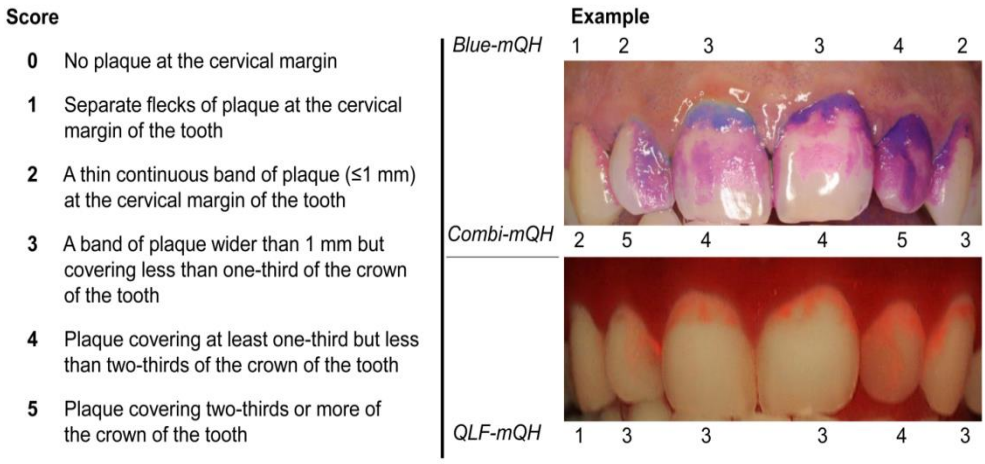


Figure 4b.1 An example of the plaque scoring system is shown for an upper jaw. The modified Quigley & Hein index (*mQH*) scores are described on the left. The buccal surfaces of the anterior teeth are divided into three surfaces (distal-buccal, mid-buccal and mesial buccal). The consensus scores for the mid-buccal surface of each tooth for blue disclosed plaque (*Blue-mQH*) are provided above the white light photograph. The consensus scores for the mid-buccal surface of each tooth for total disclosed plaque (*Combi-mQH*) are provided below the white light photograph. The consensus scores for the mid-buccal surface of each tooth for red fluorescing plaque (*QLF-mQH)* are provided below the fluorescence photograph.

Calibration

The four examiners were trained and calibrated using a set of 25 fluorescence photographs and 25 white-light clinical photographs, which were randomly selected from the participants' screening visit. A training session was organised for the assessment of disclosed plaque using the mQH criteria. Examiner N.R., experienced and calibrated for mQH assessments, trained the other three examiners. An identical procedure was performed for the assessment of red fluorescing plaque using the mQH criteria (Fig. 4b.1). Examiner M.V., experienced in QLF assessments, trained the other three examiners. During the training, each examiner scored the total disclosed plaque, blue disclosed plaque and red fluorescing plaque on 10 photographs. The scores from each examiner were compared and discussed to reach consensus scores. After training, the examiners independently scored the full training set in separate sessions at least one week apart.

Intra-and inter-examiner reliability

The intra-examiner reliability (Cronbach's alpha) was determined using the first and second assessments of the photographs included in the study. Inter-examiner agreement (Cronbach's alpha) was determined using the second assessments from each examiner. The intra-examiner consistencies were 0.92 - 0.99 for the scoring of red fluorescing plaque on the fluorescence photographs, 0.89 - 0.98 for the total disclosed plaque and 0.55 - 0.90 for the blue disclosed plaque. The inter-examiner consistencies were 0.45 - 0.73 for the red fluorescing plaque on the fluorescence photographs, 0.60 - 0.81 for total disclosed plaque and 0.45 - 0.70 for the blue disclosed plaque.

Data analysis

All statistical analyses were performed using SPSS (version 20, IBM Inc. USA). The BOMP scores were dichotomized as score 0 (no bleeding) and score 1 (combined score 1: pinprick bleeding and score 2: excessive bleeding).

The correlation coefficients at subject level between the different plaque scoring methods were calculated using the Pearson's *r*. For the site-level comparison, the partial correlation coefficient was calculated to correct for dependencies at subject level. A correlation of 0.1 to 0.3 was considered as a weak positive correlation; a correlation of 0.4 to 0.6 as a moderate positive correlation and a correlation of 0.7 to 0.9 as a strong positive correlation, according to criteria from Dancey & Reidy (Dancey, 2004). To test for differences in plaque scores between bleeding and non-bleeding sites, the Mann-Whitney U test was used. *P*-values < 0.05 were considered statistically significant.

RESULTS

Participants

Out of 83 volunteers, 32 did not meet the inclusion criteria, and consequently, 51 participants were enrolled in this study. Three participants dropped out after the screening due to other commitments that prevented them from attending the clinic at the baseline appointment. None of these commitments were related to the study. A total of 48 individuals (mean age 22.5; range 19 - 32) participated in the study of which 11 were men (mean age 22.1 years; range 20 – 26 years) and 37 women (mean age 22.6 years; range 19 – 32 years). The difference in age between men and women was not significant (t = - 0.48, p >.05; independent samples t-test). The mean clinical mQH index of the 12 anterior teeth of the participants was 2.0 (sd 1.0). The distribution of the DPSI among the participants is displayed in Table 4b.1. The mean level of gingival inflammation as assessed by BOMP in three upper front teeth and three contra-lateral lower front teeth was 0.55 (SD 0.50).

Table 4b.1 A summary of the characteristics of the participants and their level of gingival inflammation.

	DPSI* 0	**DPSI 1**	**DPSI 2**	**DPSI 3-**
Anterior teeth	0 (0%)	2 (4%)	44 (92%)	2 (4%)
Total mouth	0 (0%)	2 (4%)	21 (44%)	25 (52%)

*DPSI (Dutch Periodontal Screening Index) score 0 stands for a mouth with no pockets deeper than 3 mm, no bleeding on probing and no calculus and/or overhanging restorations present. DPSI score 1 has the same characteristics as score 0, but with bleeding on probing. DPSI score 2 has the same characteristics as score 1, but with calculus and/or overhanging restorations present. DPSI score 3- has pockets of a maximum of 5mm, bleeding on probing, supra- and subgingival calculus and/or overhanging restorations, but without recessions.

Table 4b.2 Cross-table visualizing the level of agreement between the clinically assessed total disclosed plaque (*Clinical*-mQH) and the total disclosed plaque assessed by photograph (*Combi-mQH)* at a site level. The grey boxes in the table show the value gradients.

		Clinical-mQH						***Total***
		0	1	2	3	4	5	
Combi-mQH	0	83	34	67	5	0	0	**189**
	1	65	75	100	8	0	0	**248**
	2	23	28	411	84	3	0	**549**
	3	1	1	138	244	10	0	**394**
	4	0	0	3	43	25	2	**73**
	5	0	0	1	8	13	3	**25**
Total		***172***	***138***	***720***	***392***	***51***	***5***	***1478***

Table4b.3 Correlation coefficients (at subject level Pearson's *r* and at site level the partial correlation coefficient) between the different plaque scoring methods

*: $p < 0.001$

	QLF-mQH		*Combi-mQH*		*Blue-mQH*	
	Subject level	Site level	Subject level	Site level	Subject level	Site level
Combi-mQH	0.70*	0.50*	-	-	-	-
Blue-mQH	0.50*	0.30*	0.66*	0.39*	-	-
Clinical mQH	0.74*	0.48*	0.88*	0.70*	0.56*	0.26*

Plaque scoring methods

Table 4b.2 illustrates the correlation of the *Clinical-mQH* with the *Combi-mQH*. As shown in Table 4b.3, the accompanying correlation at a site level was high ($r = 0.70$, $p<0.001$). A high correlation was also found between these two plaque scoring methods at the subject level ($r = 0.88$, $p<0.001$).

Table 4b.4a shows a moderate correlation at site level between *QLF-mQH* and *Clinical-mQH* ($r = 0.48$, $p<0.001$). At a subject level, the correlation between these two values was significant and strong ($r = 0.74$, $p< 0.001$, Table 4b.3). A similar result was found between *QLF-mQH* and *Combi-mQH* at a site level (illustrated in Table 4b.4b, $r = 0.50$, $p<0.001$) as well as at a subject level ($r = 0.70$, $p<0.001$). A weak correlation was found between *QLF-mQH* and *Blue-mQH* at a site level (Table 4b.4c, $r = 0.30$, $p<0.001$) with a moderate correlation at a subject level ($r = 0.50$, $p<0.001$).

The correlation between *Blue-mQH* and *Clinical-mQH* was weak at a site level ($r = 0.26$ $p<0.001$) and moderate at subject level ($r = 0.56$, $p<0.001$). Similar correlations were found between *Blue-mQH* and *Combi-mQH* (at site level $r = 0.39$, $p<0.001$; at subject level $r = 0.66$, $p<0.001$).

All correlation coefficients between the different plaque scoring methods (Table 4b.3) consistently showed numerically greater correlations at a subject level than at a site level.

Table 4b.4 (a, b, c) Cross-tables visualizing the level of agreement between red fluorescing plaque (*QLF-mQH*) and the other plaque scoring methods at a site level. The grey boxes in the tables show the value gradients.

a.

		Clinical-mQH						***Total***
		0	1	2	3	4	5	
QLF-mQH	0	163	123	398	125	12	0	**821**
	1	8	13	216	89	10	1	**337**
	2	1	2	92	120	14	0	**229**
	3	0	0	14	56	14	2	**86**
	4	0	0	0	2	1	1	**4**
	5	0	0	0	0	0	1	**1**
Total		***172***	***138***	***720***	***392***	***51***	***5***	***1478***

b.

		Combi-mQH						***Total***
		0	1	2	3	4	5	
QLF-mQH	0	165	200	320	116	19	1	**821**
	1	18	42	147	108	16	6	**337**
	2	4	5	80	117	17	6	**229**
	3	2	1	2	52	20	9	**86**
	4	0	0	0	1	1	2	**4**
	5	0	0	0	0	0	1	**1**
Total		***189***	***248***	***549***	***394***	***73***	***25***	***1478***

c.

		Blue-mQH						***Total***
		0	1	2	3	4	5	
QLF-mQH	0	556	182	83	0	0	0	**821**
	1	210	67	58	2	0	0	**337**
	2	99	43	80	7	0	0	**229**
	3	31	19	24	12	0	0	**86**
	4	2	1	0	0	1	0	**4**
	5	0	0	0	1	0	0	**1**
Total		***898***	***312***	***245***	***22***	***1***	***0***	***1478***

Bleeding scores

The Mann-Whitney U test showed that the plaque scores *Clinical-mQH, QLF-mQH* and *Combi-mQH* were significantly higher at bleeding sites than at non-bleeding sites (Table 4b.5). For the *Blue-mQH*, no differences were found in blue plaque at bleeding sites compared with blue plaque at non-bleeding sites.

Table 4b.5 Bleeding and non-bleeding sites in relation to plaque scores (Mann-Whitney U test), *: $p < 0.001$, ♦: $p=0.57$

		Average rank	*Z-score*
QLF-mQH	*no bleeding*	326	-5,54*
	bleeding	405	
Combi-mQH	*no bleeding*	328	-4.92*
	bleeding	403	
Blue-mQH	*no bleeding*	374	-0.57♦
	bleeding	366	
Clinical mQH	*no bleeding*	338	-4.09*
	bleeding	398	

DISCUSSION

In this study, the plaque scores on photographs showed a strong correlation with the clinical plaque scores of the matching surfaces at both the subject and site levels. This suggests that future clinical studies could use photographs to obtain an indication of the oral hygiene of the anterior teeth and to perform assessments by multiple examiners at a convenient moment, which could enhance the efficiency of a clinical study. The anterior teeth were studied for which it is relatively easy to obtain a good view at the buccal aspect from cuspid to cuspid in the upper and lower jaw with one single photograph. This selection of teeth can provide an indication of the oral hygiene at the full-mouth level.

Between red fluorescent dental plaque and the combined blue and pink disclosed plaque, moderate (site level) to strong (subject level) correlations were found upon clinical assessment and study of photographs. The plaque scores on the QLF-photographs were overall lower when compared with those of the combined blue and pink disclosed plaque, at subject and site levels. The mQH index used for retrieving these plaque scores was originally developed to describe the extent of surface coverage of plaque from the gingiva towards the incisal edges of the teeth and divides the vestibular and lingual surfaces into three areas. The mQH scoring system was applied in this study in a clinical situation. Therefore, a two-tone disclosing agent was used with a presumed discriminatory property to differentiate between 'new' and 'old' plaque according to the manufacturer. The exact mechanism of action of the dye is not known, but it is reportedly related to the pH and the thickness of the plaque biofilm (Gallagher and Cutress, 1977). The pink component of the dye adheres to all plaque that is present, whereas the blue component adheres and diffuses more easily into denser/thicker plaque, clinically resulting in two distinctive colours of the plaque. This is in contrast with the disclosing agent erythrosine, which stains all present plaque red (Paraskevas et al., 2005).

A weak (site level) to moderate (subject level) correlation was found between red fluorescent plaque and blue disclosed plaque. Both in the fluorescent plaque scores and in the blue disclosed plaque scores, the high plaque scores (scores 4 and 5) were underrepresented, which suggests that the scores are skewed towards 'old' plaque. Although blue stained plaque is assumed to be older (Block et al., 1972, Katayama et al., 1975), no clear conclusion can be drawn regarding the exact nature of 'blue' disclosed plaque. Red fluorescent plaque has been suggested to be matured plaque as well (Kim et al., 2014, Thomas et al., 2008, van der Veen et al., 2006), but its true nature could not be determined in this study design. However, the observed (weak to moderate) correlations between these two plaque scoring methods were not as expected.

At inflamed sites as indicated by bleeding on marginal probing higher red fluorescent plaque scores as well as total disclosed plaque scores (both clinical and on photographs) were found. Such an association was not observed between gingival inflammation and the blue disclosed 'older' plaque. This seems contradictory to what is generally accepted; dental plaque leads to gingival inflammation when it is present on the tooth surface for a longer period (Loe et al., 1965). One determining factor in the development of gingival inflammation is patient

susceptibility towards the presence of dental plaque (Fransson et al., 1999, van der Velden et al., 1985). Furthermore the assumption that only old plaque causes gingival bleeding does not take the microbiological characteristics of the plaque biofilm into account. Studies relating oral plaque microbiota to gingival inflammation do exist, but these evaluate total plaque or saliva rather than the old and young portions of plaque separately (Huang et al., 2014, Lie et al., 1995). A recent cross-sectional study in orally healthy participants has reported that the correlation between plaque and bleeding scores on average is low (Oliveira et al., 2015). These results could be explained by the assumption that bleeding on probing most likely represents the impact of the oral hygiene as performed over a longer period of time together with the immunological reaction on this hygiene level rather than being a reflection of the actual oral hygiene status. It can also be argued that blue disclosed plaque does not represent old plaque per se. This is supported by the absence of conclusive literature about the mechanism of action of two-tone dye (Block et al., 1972, Gallagher and Cutress, 1977, Katayama et al., 1975). Another influencing factor could be the relatively lower intra-examiner consistencies for scoring blue disclosed plaque compared, which indicates that it is more difficult to make an accurate assessment of the blue disclosed plaque.

Environmental factors, such as nutrition, are known to have an effect on autofluorescence of bacteria and of *in vitro* formed biofilms (Lee et al., 2013, Volgenant et al., 2013). When gingivitis develops during a period of non-brushing, a change in the oral environment occurs. The association, which was found between gingival inflammation and red fluorescent plaque, could be related to these environmental changes. New studies, preferably in a clinical setting, are needed to determine the relationship between red fluorescence and environmental factors. Although the partial evaluation of the BOMP of the anterior teeth provides an indication of the situation in the whole mouth, future studies should investigate whether similar associations are found when premolar and molar teeth are also included in the assessment of the inflammatory periodontal condition.

Overnight plaque development is representative for people who perform oral hygiene twice daily, resulting in 12 hours of plaque accumulation. The participants in the present study were preselected 'heavy' plaque formers (Simonsson et al., 1987) and were able to form a substantial amount of dental plaque overnight. These specific participants were selected because this study evaluated dental plaque indices for which participants with the full array of plaque scores (0-5) would contribute to a representative assessment of different plaque scoring methods. An adjustment in the exclusion criteria for the participants and a longer period of patient abstinence from oral hygiene habits could have influenced the study results. Similarly, the underrepresentation of sites with high plaque scores (*mQH scores* 4 and 5) could have influenced the study outcome.

The relationship between red fluorescent dental plaque and 'old' plaque on natural teeth has not been looked into before. Coulthwaite *et al*. (2009) performed a clinical study *on dentures* in which they also used a two tone plaque disclosing solution. They reported smaller red fluorescent plaque coverage compared with the clinical scores of the blue and pink disclosed plaque combined. Their study indicated that red fluorescent plaque is related to matured

plaque, although they did not correlate the blue disclosed plaque with the red fluorescent plaque. They observed no correlation between the presence of red fluorescent plaque and the amount of total disclosed plaque area on dentures. This could be the result of other characteristics and the composition of plaque in edentulous patients. They also used an earlier QLF-system (the QLF-CLIN system), which was not optimized for detecting red fluorescence. Due to the low contrast in red fluorescence on denture material, and the absence of red fluorescence from the denture material itself, the conclusions of this study are difficult to compare with studies on tooth enamel, such as the present study.

An *in situ* study on bovine enamel (Raggio et al., 2010) reported a correlation between red fluorescent plaque and blue disclosed plaque, although the blue disclosed plaque covered a larger area on the enamel than did the red fluorescent plaque. These results are however difficult to compare with those of the present study because the *in situ* administration of the plaque dyes differs (dipping instead of rinsing). Compared to their fluorescence camera (Vista Proof, Dürr Dental, Germany), the QLF-D camera used in the present study was optimized to better detect red fluorescence. Therefore, the detection level for red fluorescence is lower in the QLF-D system in comparison to the earlier QLF devices and the fluorescence camera use in the studies of Pretty et al. (2005) and Raggio et al. (2010), respectively. This may have been one of the reasons for differences between these studies.

Dental plaque forms gradually after cleaning of the tooth surfaces (Quirynen and van Steenberghe, 1989). Future research would therefore preferably comprise a longitudinal study to monitor the changes in the red fluorescence of plaque over time, which would then aid in a complete understanding of the correlation between the fluorescence and the matured plaque. The use of digitized photographs also allows the use of planimetric measurements of dental plaque as well as automatic plaque assessment. These assessments can be easily performed by lay people.

CONCLUSION

A strong correlation was found between the clinical plaque scores and the matching surfaces of the plaque scores on photographs. A moderate to strong correlation was found between the portion of red fluorescing plaque and total disclosed plaque, with total plaque scores that are consistently higher than red fluorescent plaque scores. Red fluorescent plaque and blue disclosed plaque showed similar scores, but the correlation between both was weak to moderate. Higher plaque scores were found at bleeding sites, except for blue disclosed plaque. Because no relationship was found between the blue disclosed ‘old’ plaque and BOMP, the interpretation of blue disclosed plaque as being old plaque can be called into question. Consequently, we can neither confirm nor reject the use of a fluorescence device to screen for matured dental plaque.

Conflicts of interest

Monique van der Veen is co-inventor on several patents relating to quantitative light-induced fluorescence (QLF). The authors declare that otherwise, there are no conflicts of interest pertaining to the data presented in this article.

Ethical standards

This study was approved by the Medical Ethics Committee of the Academic Medical Centre of Amsterdam (AMC) under registration number NL 37567.018.11. The trial was registered at the Dutch Trial Register under number NTR 3145. All procedures performed in this study were in accordance with the ethical standards of the institutional research committee and with the 1964 Declaration of Helsinki and its later amendments. All participants gave their informed consent prior to their inclusion in the study.

Acknowledgements

This research is supported by the Dutch Technology Foundation STW (project number 10948) and NGen Oral Pharma N.V., Willemstad, Curaçao. ACTA Dental Research B.V. receives financial support for their commitment to appoint this project to the Department of Periodontology Research group Prevention and Therapy of ACTA. Companies supporting this project had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

The authors thank N. Hennequin-Hoenderdos and D.E. Slot for coordinating the clinical study. For the clinical examinations, the authors thank G. van Anraat (clinical plaque assessment), E.J.C. Martin (assessment of the bleeding on marginal probing), E. van der Sluijs and S. Supranoto (professional prophylaxis).

Chapter 5

A reproducible microcosm biofilm model of subgingival microbial communities.

This chapter has been submitted to

Fernandez y Mostajo, M., Exterkate, R. A. M.,Buijs, M. J., Beertsen, W.,

van der Weijden, F.,Zaura E. & Crielaard W. (2016) *J Periodontal Research*.

ABSTRACT

Objective:

To develop a reproducible subgingival microcosm biofilm model.

Material and methods

Subgingival plaque samples were collected from four deep pockets (PPD≥6mm) in each of seven periodontitis patients and from shallow pockets (PPD≤3mm) in two periodontally healthy donors. An active attachment model and a peptone medium (Thompson et. al) supplemented with 30% serum was used. Biofilms were harvested at 2 and 4 weeks. DNA of dead cells was blocked for amplification by propidium monoazide (PMA) treatment. Composition was analyzed using 16S rRNA gene amplicon pyrosequencing. Similarities between the biofilm samples were assessed by non-metric multidimensional scaling (n-MDS) using Bray-Curtis (BC) similarity index and similarity percentage analysis (SIMPER). Data from duplicate experiments, different biofilm sources and different biofilm age were compared.

Results

The n-MDS revealed a strong clustering by the inoculum source, the donor and their periodontal status. Statistically significant differences were found between the sources of inoculum (p=0.0001) and biofilm age (p=0.0016). Furthermore, periodontitis biofilms (P) were distinct in composition from health-derived biofilms (H) by genera: *Porphyromonas* (P=19%; H=0%), *Filifactor* (P=10%; H=0%), *Anaeroglobus* (P=3%; H=0%), *Phocaeicola* (P=1.5%; H=0%), *Parvimonas* (P=19%; H=14%), *Fusobacterium* (P=2%; H=26%), *Peptostreptococcus* (P=20%; H=30%), *Veillonella* (P=7%; H=8%) and 57 other genera.

Similarity distances (BC) (mean 0.73, SD 0.15) and the Shannon diversity index (mean 2, SD 0.2) revealed no differences between duplicate experiments (p=0.121).

Conclusion

This model allows reproducible production of complex subgingival microbial communities.

Key words: biofilm, microcosm, subgingival, periodontitis

INTRODUCTION

Oral microbial communities differ in composition according to specific environments such as the surfaces of the teeth, mucosa, gingiva and the tongue. These various habitats have been shown to harbor a distinctive microbiota by both microbiologic culturing and molecular biology based analyses. (Aas et al., 2005, Simon-Soro et al., 2013). With respect to tooth surfaces, the gingival margin delineates two different habitats, the supragingival and subgingival environment. The ecological conditions of these habitats differ greatly, leading to different microbial communities (Marsh and Devine, 2011). Both are colonized by polymicrobial complex biofilms, which in dentistry are termed 'dental plaque'. Symbiotic microbiota covers the dental surfaces as a biofilm and thereby preventing the establishment of exogenous (and often pathogenic) species (Hajishengallis, 2015). On the other hand when dental plaque is not removed by means of proper daily oral hygiene in general dysbiosis will occur, resulting in an inflammatory reaction of the host, of which the first step is known as gingivitis. This inflammatory response to the accumulation of dental plaque has been observed in classical studies using a model in which periodontally healthy volunteers stop all oral hygiene measures for 2-3 weeks which results in a condition that is described as 'experimental gingivitis' (Kistler et al., 2013). In a susceptible individual, this established gingivitis may eventually progress to periodontitis, which is characterized by an inflammation that extends to the tooth supporting tissues and will result in breakdown of alveolar bone. The pathogenesis of this disease entity is multifactorial and complex (Knight et al., 2016).

Following the loss of attachment between the gingiva and the tooth a pathological periodontal pocket is formed which by definition starts by 4 mm probing depth but can extend up to the apex of the tooth. The environmental niche of the periodontal pocket is ideal for the colonization and growth of especially anaerobic species. Depending on the extent of attachment loss, it has been reported that the mean dentogingival surface area among individuals with periodontitis ranges from 8 cm^2 to 20 cm^2 (Hujoel et al., 2001) representing an important subgingival area which can be colonized by specific biofilms. The prevalence of bacterial species associated with periodontitis (e.g. *Porphyromonas gingivalis, Tannerella forsythia*) is highly dependent on the probing pocket depth, although they have also been found in shallow pockets (<4 mm) (Riep et al., 2009, Griffen et al., 2012, Li et al., 2014).

Over the last few years, advances in molecular biology based approaches for microbial identification e.g. high-throughput next generation sequencing technology, have modified and enhanced our understanding of the oral polymicrobial communities (Wade, 2011, Hajishengallis and Lamont, 2012). Next to the established periodontal pathogens, several non-cultivable and fastidious species are now recognized as being associated with the onset and development of periodontitis, thus enlarging the group of 'suspected periodontal pathogens' (Griffen et al., 2012, Hajishengallis and Lamont, 2012, Bizzarro et al., 2016). However, to date, the specific role that the majority of these novel genera and species play in the initiation and/or progression of chronic periodontitis remains unknown.

In the light of the increasing knowledge on the subgingival communities, researching such novel microorganisms originating from these specific niches in a microcosm and dynamic biofilm will further contribute to the development of our understanding of the human oral microbiome. Microcosms are artificial, simplified ecosystems that are used to simulate the behavior of natural ecosystems under controlled conditions. Microcosm studies can be useful to determine the ecological role of key species and to study the effects of disturbance of the biofilm (Exterkate et al., 2010). These microbiological models provide the opportunity to study the potential virulence of possible pathogens, the interactions with each other and to test the effect of potential antimicrobials (Exterkate et al., 2010, Koopman et al., 2015). Currently most of the subgingival biofilm models are limited to pre-selected laboratory species (10-35 species) (Guggenheim et al., 2009, Soares et al., 2015, Eick et al., 2012) lacking the hundreds of species recently identified under dysbiotic condition of this subgingival ecosystem (Abusleme et al., 2013, Camelo-Castillo et al., 2015b, Griffen et al., 2012, Bizzarro et al., 2016). Other studies have used subgingival dental plaque (Hope and Wilson, 2006, Shaddox et al., 2010, Thompson et al., 2015, Walker and Sedlacek, 2007) and biofilms were analyzed by traditional methods.

The aim of the present study was to develop a microcosm biofilm model of subgingival bacterial communities derived from periodontitis patients using next-generation sequencing techniques for compositional analysis.

MATERIALS AND METHODS:

Ethical approval of the protocol for subgingival plaque collection and experimental research was provided by the medical ethics committee of the VU University Medical Center Amsterdam (ref 2012/335).

Selection of donors

Patients referred to the Academic Centre for Dentistry Amsterdam (ACTA) for periodontal treatment were asked to participate as donors. The inclusion criteria were: patients diagnosed with periodontitis with at least 4 sites with probing pocket depth (PPD) ≥ 6 mm. Patients were excluded if they had used antibiotics or had received periodontal therapy within the previous 3 months, or if they were using antimicrobial mouthwash. All participants had to sign an informed consent prior to the study.

Subgingival plaque samples of patients diagnosed with periodontitis were collected from the four deepest sites (Timmerman et al., 2002, Mombelli et al., 1993).

Subgingival plaque samples from periodontally healthy donors (Dutch Periodontal Screening Index; DPSI ≤ 1); (Mantilla Gomez et al., 2001) were collected from the intracrevicular area from the right upper second molar to the right upper central incisor (first quadrant) and the left lower second molar to the left lower central incisor (third quadrant).

Sampling

Selected sites were isolated with cotton rolls and supragingival plaque was carefully removed with a universal curette (Hu-Friedy, Chicago, USA) and discarded. Subgingival plaque samples were collected using a new and sterile Gracey curette.

The procedure for harvesting the bacterial material for inocula from the deep periodontal pockets (≥ 6 mm) derived from patients having periodontitis was done prior to initial periodontal treatment, when local anesthetics were provided avoiding any discomfort to the patient.

Samples from the four different sites of the same donor were pooled and then transferred to a vial containing 500 µl reduced transport fluid (RTF), placed on ice and immediately transported to the laboratory. To protect the bacterial cells from cryodamage, 500 µl of sterile 60% glycerol was added (Exterkate et al., 2010). Then, samples were briefly sonicated with 3 pulsations (5 s) and vortexed for 10 s (Vibra Cell; Sonics & Materials Inc, Newtown, CT). Subsequently the samples were divided into 3 vials: two vials were used to duplicate the experiment (each of 450 µl) and one vial (100 µl) served to determine the composition of the inoculum. Samples were stored at –80 °C until use.

Biofilm model and production of biofilms

An active attachment biofilm model was used as previously described (Exterkate et al., 2010, Deng et al., 2009). In brief, 24-well polystyrene culture plates (Greiner Bio- One, Alphen a/d. Rijn, the Netherlands) were used. The lid of the plate was replaced by a custom-made stainless-steel lid, onto which 24 nylon clamps were fixed allowing for various substrata to be inserted. The clamps were positioned in such a way that the inserted substrata would fit into the wells of the culture plate. Since the substrata were positioned vertically, bacterial attachment plays a key role in this model. By transferring the lid with the substrata to a new plate, the medium could be refreshed. In this study, standardized glass disks (diameter 12 mm, Menzel, Braunschweig, Germany) were used as substratum. Each lid was assembled for triplicate biofilms of two donors for 2 and 4 week growth. The two middle columns of the 24 well plates were kept with no substratum and were not used in order to preserve sufficient distance between the biofilms. After assembly the models were autoclaved at 121°C. The duplicate experiment was operated at the same time but in a separate incubator.

The biofilms were grown using a basal liquid medium supplemented with Bacto proteose peptone (Thompson et al., 2015). This medium was chosen as the primary nitrogen source for the base medium because the wide range of peptide sizes makes it suitable for the culture of fastidious bacteria. The medium contained 0.25% pig gastric mucin (Sigma M-2378), 0.07% Bacto proteose peptone (Difco 0118-01-8), 0.1 mol/l potassium phosphate buffer (K_2HPO_4), 0.3% trypticase peptone (BBL 211921), 0.5% yeast extract (Bacto 212750), 0.25% KCl, 0.001% hemin (Sigma H-1652), 0.0001% menadione, 0.05% cysteine hydrochloride, 1 mmol/l lysine, 1 mmol/l glycine, 1 mmol/l urea, 5 mmol/l arginine. Serum was considered as an important component of the subgingival domain, because gingival crevicular fluid contains proteins

found in blood, e.g. albumin (Tew et al., 1985). The original peptone based (Thompson et al., 2015) contains 10% heat-inactivated fetal horse serum. On the other hand, the use of 50% of heat-inactivated human serum improves the stability of the biofilms resulting in significantly thicker biofilms (Ammann et al., 2012). Because the optimal serum concentration was unclear 'a priori', a pilot experiment was performed in order to test 10%, 30% and 50% of heat-inactivated fetal bovine serum (FBS). Results were analyzed with real time PCR (Bizzarro et al., 2013). *Porphyromonas gingivialis*, *Tannerella forsythia* and *Parvimonas micra* established in all three biofilms regardless of the serum concentration. *Aggregatibacter actinomycetemcomitans* grew with 30% serum, but not with 10% or 50% serum, whereas *Fusobacterium nucleatum* and *Prevotella intermedia* did not grow under the condition of 50% serum. On the other hand, *Treponema denticola* established only under a high serum concentration (50%). Apparently, the concentration of serum is critical for the growth of the various individual species. Therefore, based on the results of the pilot experiment, this medium (Thompson et al., 2015) was modified by adding 30% of FBS (F4135, Sigma-Alddrich Chemie, US) since this concentration promoted growth of most of the species tested. Moreover, it has been reported that *in vivo* the gingival crevicular fluid contains up to 35% of the albumin found in serum (Tew et al., 1985).

Immediately after the inocula were defrosted, the inoculation medium was prepared using subgingival dental plaque in a ratio of 1:27 (450 µl of suspension in 12 ml of medium). The models were subsequently incubated anaerobically (10% CO_2, 10% H_2 and 80% N_2) for 24 h at 37°C. After this initial inoculation period, the lids were transferred to new 24-well plates containing sterile fresh medium. Medium was refreshed every 3.5 days, with a total biofilm growth period of 4 weeks.

Harvesting

Biofilms were harvested at two weeks (2w) and four weeks (4w). The glass disks with biofilms were removed from the lid and transferred into 2 ml sterile phosphate-buffered saline (PBS) and kept on ice. All visible biofilms were dispersed by sonication for 2 min at 1 second pulsations at the amplitude of 40 W and vortexed for 30 seconds.

PMA treatment of dispersed biofilms

To assure that only DNA from the cells with intact membranes (undamaged cells) was processed for sequencing, the dispersed biofilms were treated with propidium monoazide (PMA) immediately after harvesting (Exterkate et al., 2014). A total of 1 mg PMA (Biotum Inc., Hayward, Calif., USA) was dissolved in 100 µl 20% dimethyl sulfoxide. Approximately 2.5 µl PMA was added to 500 µl of suspended biofilm, incubated in the dark for 5 min and then exposed to intense light for 2 min using a 650 W halogen lamp placed 25 cm from the samples. During this procedure the samples were kept on ice (Nocker et al., 2006).

The inocula from the donors were not treated with PMA, which implies that all 16S rDNA was sequenced including DNA from dead or damaged bacterial cells.

Assessment of microbial composition

DNA extraction, amplicon preparation and sequencing data analysis

DNA was extracted from the inocula and PMA treated biofilms (Crielaard et al., 2011). Barcoded amplicon libraries of the small subunit ribosomal RNA gene hypervariable region V5–V7 were generated for each of the individual samples, pooled and sequenced using Genome Sequencer FLX Titanium system (Roche, Basel, Switzerland) (Koopman et al., 2015). The sequencing data was processed using Quantitative Insights Into Microbial Ecology (QIIME) (Caporaso et al., 2010) version 1.5.0 as described previously (Koopman et al., 2015).

To allow comparisons among different samples with the inocula and between the groups the dataset containing Operational Taxonomic Units (OTUs) was randomly subsampled at 750 reads per sample based on the second lowest number of reads/sample and on rarefaction curves of the samples (data not shown).

The Shannon diversity index was calculated, which takes into account the abundance of each OTU, as well as the number of OTUs, using PAST software version 3.0 (Hammer Ø, 2001).

Reproducibility of all biofilms was analyzed using the hierarchical classical cluster analysis based on the Bray-Curtis similarity index. The Bray-Curtis coefficients were calculated between all pairs of samples to compare similarities within groups and to compare 2w and 4w biofilms from each donor. The Dissimilarity percentage (SIMPER) was used to identify the OTUs with the highest contribution to dissimilarity between the 2w- and 4w outcomes per donor.

Taxonomy of the representative sequences of the most abundant OTUs was identified by microbial nucleotide BLAST search against Human Oral Microbiome Database (HOMD) reference set (version 13.2). When the highest percentage of identification matched with more than one sequence, the possible alternatives for the OTUs identification were then provided. This was the case for OTU 89; *Streptococcus* sp (99.2% sequence identity) and for OTU 10; *Gemella* (99.5% sequence identity) (see table 5.1 at the supplementary data).

Nonmetric multidimensional plots (n-MDS) were used based on the Bray-Curtis coefficient to visualize similarity between samples. All calculations were performed using PAST software version 3.0 (Hammer Ø, 2001).

Statistical analysis

The non-metric multidimensional plots (n-MDS) (Clarke, 1993) based on the three-dimensional Bray-Curtis coefficient similarities (BC) were used to visualize similarities among donors and comparisons between 2w and 4w. Stress <0.2 (Kruskal's stress formula 1) was used as an acceptable threshold (Clarke and Ainsworth, 1993). One-way permutational multivariate analysis of variance (PERMANOVA) was used to test the differences in bacterial composition between biofilm age (2w and 4w), between inocula source and among donors. The p-values were corrected for multiple comparisons using Bonferroni correction. Calculations were performed using PAST software.

Triplicate biofilms led to 3 BC pair comparisons (BC-pairs) per group. To assess the differences between duplicate experiments per age group, the mean of all BC-pairs from 6 replicates (experiment 1 and 2) was calculated per donor and tested using Paired sample T-test (IBM SPSS version 20). The difference between the duplicate experiments was not statistically significant (p-value 0.509). Therefore, when the difference between biofilm age was assessed (2w and 4w), the mean BC-pairs from six replicates was used (regardless of the experiment, either 1 or 2) per donor and tested using Paired sample T-test.

RESULTS

Seven patients having periodontitis and two periodontally healthy individuals participated as donors of subgingival dental plaque (table 5.1). From 284 operational taxonomic units (OTUs) identified from the inocula of all donors, 126 OTUs established in the microcosm biofilms, ranging between 47 - 75 OTUs (avg 61, SD 8.2) per biofilm. The relative abundance of genera in the inocula showed a large variation among seven donors who were periodontitis patients as well as between two donors who were periodontally healthy (Fig.5.1).

In biofilms grown from these inocula, the non-metric multidimensional scaling plot (n-MDS) on composition data showed a strong clustering by donor visualized by different colours as shown in figure 5.2. Moreover, this analysis revealed that healthy donor-derived biofilms (triangles) and periodontitis-derived biofilms (dots) clustered evidently apart from each other revealing statistically significant differences among the periodontitis- and health-derived biofilms (PERMANOVA p=0.0001, F=25.7).

When a comparison among all donors was performed regardless of the inoculum source, the differences among donor sources were statistically significant at 2w (PERMANOVA p=0.0001, F=14) and 4w (PERMANOVA p=0.0001, F=18).

In addition, n-MDS was used to assess the similarities between 2w, 4w and among donors (Fig. 5.2). Pairwise comparisons between 2w (filled symbols) and 4w (empty symbols) demonstrated statistically significant differences in bacterial composition (PERMANOVA

p=0.001, F=4). The total average dissimilarity between 2w and 4w was 54% (SIMPER). The differences between 2w and 4w reflect the changes in composition between these two time points. BC similarities between 2w and 4w per donor showed some variability that ranged from 0.4 (SD 0.1) in donor 5 to 0.7 (SD 0.1) in donor 6 (table 5.2).

Reproducibility of all biofilms was analyzed using the hierarchical classical cluster analysis based on the Bray-Curtis similarity index (BC). This analysis showed high reproducibility of the 6 replicates which mostly clustered together according to the donor (supplementary data, Fig. 5.1) regardless of the experiment, being either 1 or 2. However, periodontitis derived biofilms clustered clearly together according to the donor whereas the health-derived biofilms (donor 8 and 9) were less reproducible. This could be attributed to the observation that during the first 2 weeks of growth, parts of the biofilm prematurely detached. This phenomenon was observed only for 2 biofilms out of 108, both belonging to health-derived biofilms (donor 9).

Bray-Curtis similarity index (BC) between the duplicate experiments (1 and 2) ranged from 0.5 (donor 9 at 2w) to 0.9 (donor 5 at 2w and donor 1 at 4w), average among donors of 0.73 (SD 0.1) (table 5.2). On figure 5.3, all replicates (n=6) for two donors (3 and 5) are presented, including those from experiment 1 (3 replicates) and experiment 2 (3 replicates). There were no significant differences (p = 0.121) in relative abundance of major genera among these duplicate experiments..

Differences among the replicates from experiment 1 and experiment 2 either at 2w or at 4w biofilms (table 5.3) were not statistically significant (p>0.05). Also the Shannon diversity index (mean 2, SD 0.2) revealed no differences between duplicate experiments (p= 0.121); see table 5.3.

Analysis of the bacterial composition of the biofilms was done at genus level - the OTUs were classified to genus or a higher taxon. Figure 5.3 shows the relative abundance of major genera of two donors (3 and 5). All replicates from 2 and 4 weeks biofilms are presented. Within donor 3, it can be observed that the differences between 2 weeks and 4 weeks are visually small, whereas this difference was more clear at donor 5. This observation was also confirmed quantitatively (table 5.2 and table 5.3).

Table 5.1. Descriptive clinical data from all donors

Donor	sex	age	DPSI*	nr of teeth	% BoP**	nr pockets > 5 mm
1	female	65	4	23	38	11
2	female	57	4	24	60	21
3	female	54	4	27	97	34
4	female	39	4	29	55	18
5	female	54	4	28	63	38
6	male	52	4	25	95	64
7	female	55	4	25	77	35
8	male	50	1	28	n. d.	0
9	female	35	1	27	n. d.	0

*DPSI (Dutch Periodontal Screening Index); ** BoP (Bleeding on probing); n. d. not determined

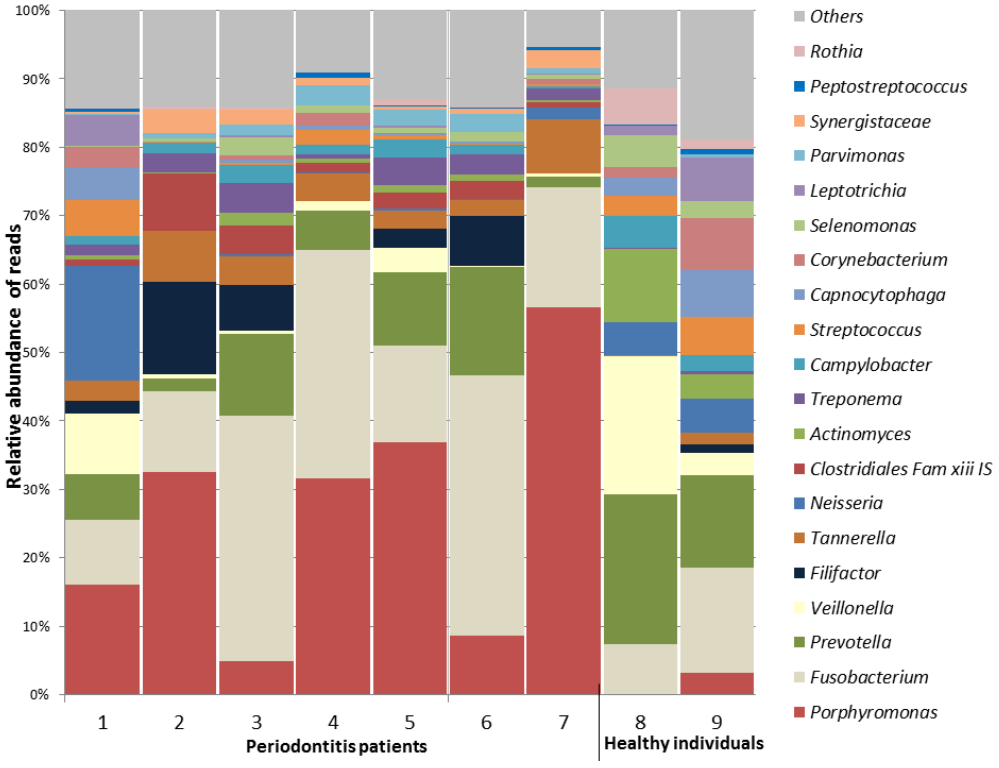


Figure 5.1 Relative abundance of major genera in the inocula (subgingival plaque) per donor.

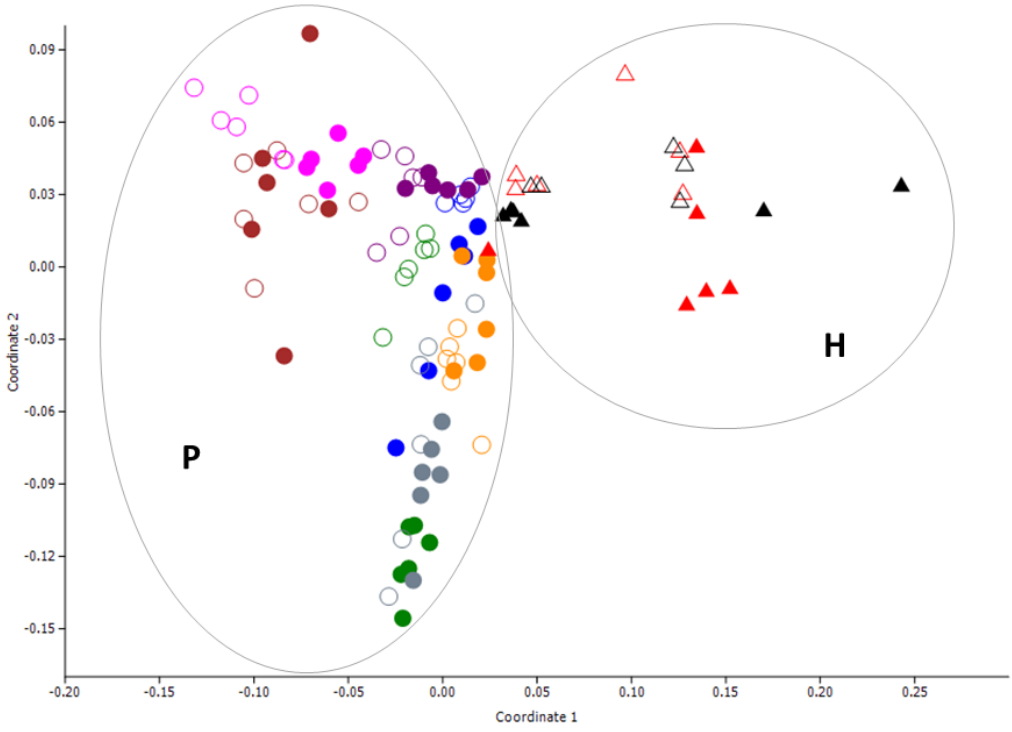


Figure 5.2. Non-metric multidimensional scaling (nmMDS) based on Bray-Curtis similarity index. Each color represents a different donor. Dots represent periodontitis-derived biofilms (P) and triangles healthy subject-derived biofilms (H). 2 week – old biofilms (2w) are filled shapes and 4 week – old biofilms (4w) empty shapes. The difference between 2w and 4w was statistically significant (PERMANOVA: p=0.001, stress=0.11, F=4). Pairwise comparisons between different inocula source (P and H) revealed statistically significant differences between biofilms (PERMANOVA: p=0.0001, stress=0.11, F=25.7) with a total average dissimilarly of 64% (SIMPER).

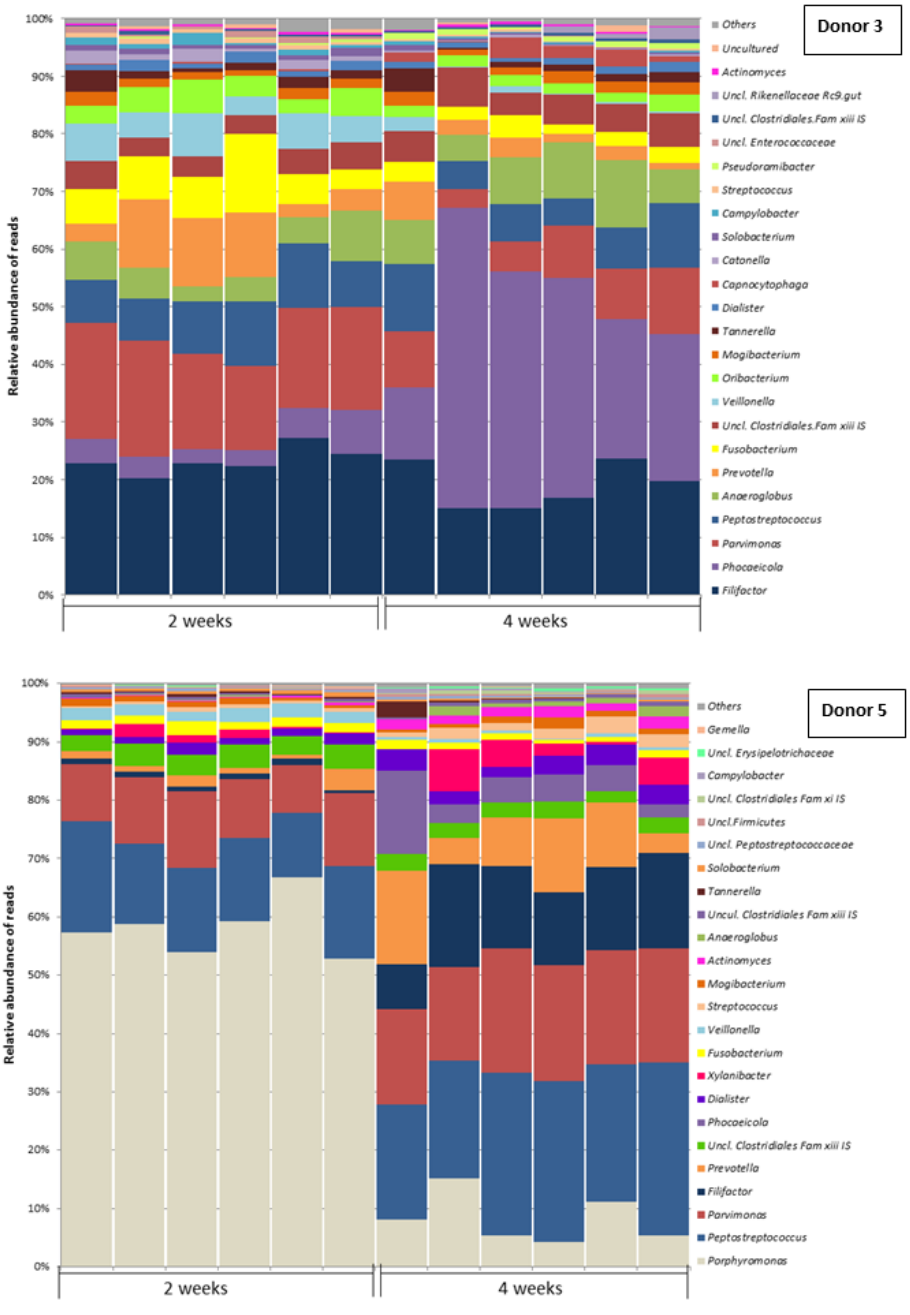


Figure 5.3 Relative abundance of major genera of all biofilms (6 replicates from duplicate experiments and two time points; 2 weeks and 4 weeks) from donor 3 and 5.

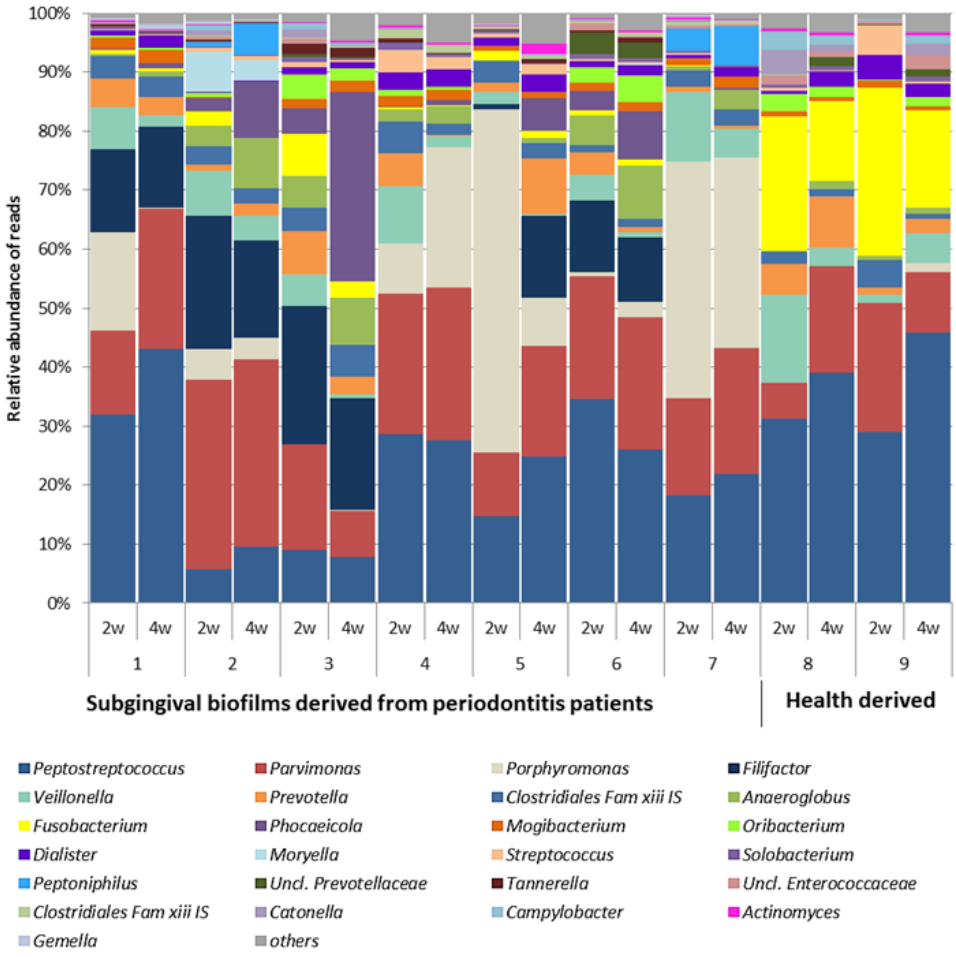
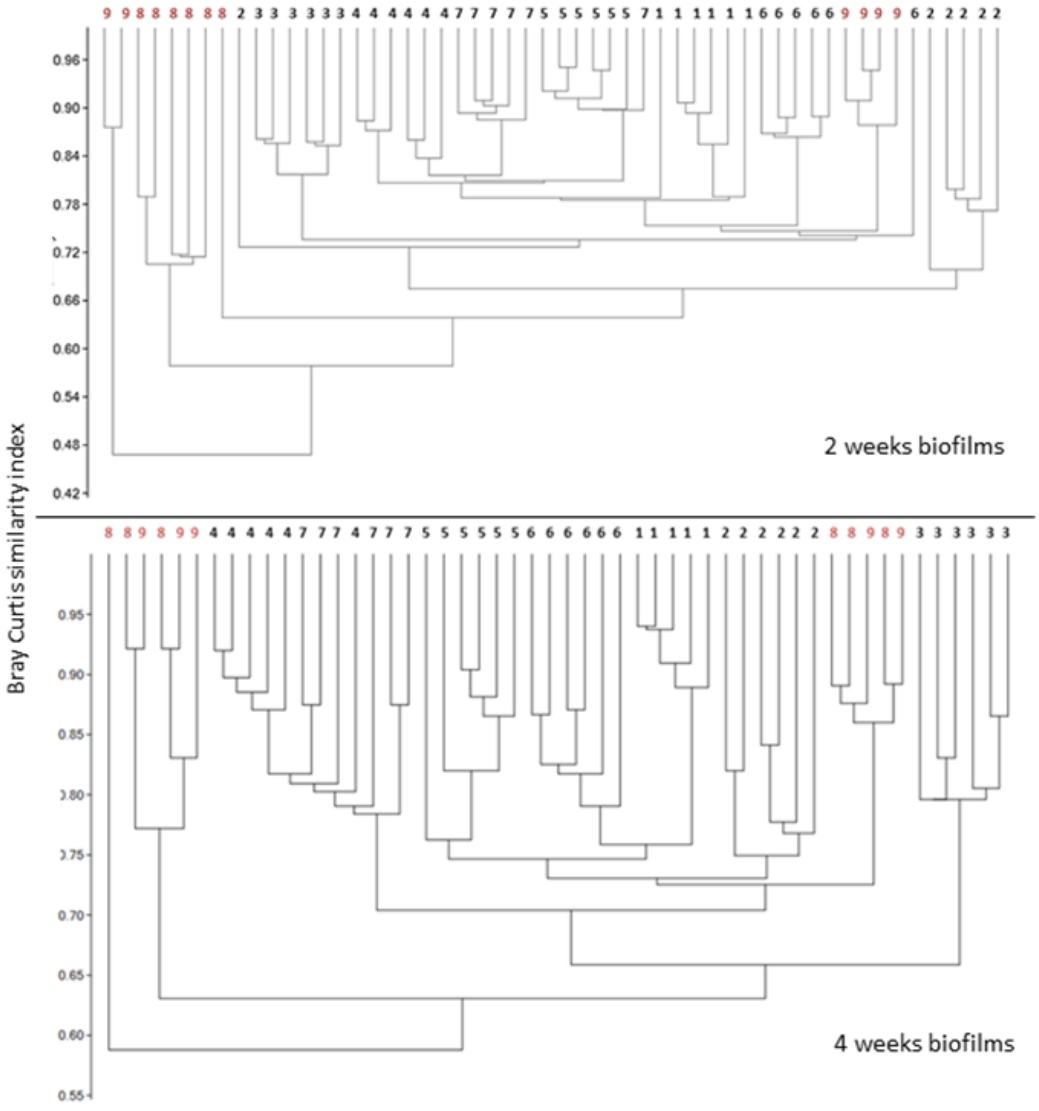


Figure 5.4 Relative abundance of major genera at 2 weeks (2w) and 4 weeks (4w) of microcosm biofilm growth (data shown represent median of triplicate biofilms) per individual donor.



Supplementary data. Figure.5.1 Hierarchical classical cluster analysis based on the Bray-Curtis similarity index of all biofilms replicates. Numbers represent the donor (black = Periodontitis derived-biofilms; red = health-derived biofilms).

Table 5.2 Reproducibility of duplicate experiments based on Bray Curtis similarity (BC) among biofilms. Data from experiment 1 (exp. 1) and experiment 2 (exp. 2) are expressed as the mean (SD, standard deviation) of 9 BC pair comparisons from 3 replicates (ǂ= two biofilms were lost). Differences among the replicates from exp. 1 and exp. 2 at 2w and 4w biofilms were not significant (p-value >0.05, paired sample T-test).

Donor	Bray Curtis similarity index					
	exp. 1 vs exp. 2				2w vs 4w*	
	2w		4w			
1	0.7	(0.12)	0.9 ǂ	(0.03)	0.7	(0.1)
2	0.6	(0.11)	0.7	(0.06)	0.6	(0.1)
3	0.8	(0.05)	0.8	(0.07)	0.6	(0.1)
4	0.8	(0.07)	0.7	(0.10)	0.7	(0.1)
5	0.9	(0.04)	0.8	(0.08)	0.4	(0.1)
6	0.8	(0.06)	0.8	(0.04)	0.7	(0.1)
7	0.8	(0.09)	0.7	(0.13)	0.7	(0.1)
8	0.6	(0.07)	0.6	(0.17)	0.5	(0.1)
9	0.5	(0.41)	0.6 ǂ	(0.14)	0.5	(0.2)

*Data represent 36 BC pair comparisons (ǂ 30 BC).

Table 5.3 Shannon diversity index of the inoculum and biofilms. Data are expressed as the mean of 6 replicates per age group biofilm; SD, standard deviation; (ǂ= two biofilms were lost).

		Shannon diversity index			
Donor	Inocula	2w		4w	
1	3.4	1.9	(0.2)	1.9 ǂ	(0.2)
2	2.7	2.1	(0.2)	1.9	(0.1)
3	3.1	2.4	(0.3)	2.4	(0.2)
4	2.4	2.0	(0.1)	2.1	(0.1)
5	2.9	2.0	(0.5)	1.9	(0.5)
6	2.9	2.2	(0.1)	2.1	(0.1)
7	1.9	1.8	(0.3)	1.8	(0.2)
8	3.1	1.8	(0.2)	1.9	(0.1)
9	3.7	1.6	(0.4)	1.5 ǂ	(0.5)

Supplementary data Table 5.1 Oral taxa targeted for 16SrRNA gene sequencing, identified using HOMD. Taxa that were at ≥0.2% of relative abundance of reads in the microcosm biofilms are presented.

OTU	Genera	Closest phylogenetic relative in HOMD (%sequence identity)	HOMD oral taxon	Strain / Clone
75	*Peptostreptococcus*	*Peptostreptococcus stomatis (100)*	112	Strain.A21H2
199	*Parvimonas*	*Parvimonas micra (100)*	111	Strain.ATCC33270
134	*Porphyromonas*	*Porphyromonas gingivalis (100)*	619	Strain.DSM20709
32	*Filifactor*	*Filifactor alocis (100)*	539	Strain.ATCC35896
233	*Veillonella*	*Veillonella dispar (100)*	160	Strain.DSM20735
142	*Prevotella*	*Prevotella oralis (100)*	705	Strain.ATCC33269
20	*Clostridiales Fam xiii*	*Eubacterium infirmum (100)*	105	Strain.A35MT
253	*Anaeroglobus*	*Anaeroglobus geminatus (98.9)*	121	Clone.BB166
21	*Fusobacterium*	*Fusobacterium nucleatum ss. Vincentii*	200	Clone.R002
207	*Phocaeicola*	*Bacteroidaceae sp. (100)*	272	Clone.X083
43	*Mogibacterium*	*Mogibacterium diversum (99.7)*	593	Strain.ATCC700923
81	*Oribacterium*	*Oribacterium sp. (100)*	102	Clone.FB046
152	*Dialister*	*Dialister invisus (100)*	118	Clone.BS095
23	*Moryella*	*Moryella sp. (93.8)*	373	Clone.MCE9.173
89	*Streptococcus*	*Streptococcus oralis (99.2)*	707	Strain.ATCC35037
89	*Streptococcus*	*Streptococcus sp. (99.2)*	71	Clone.P4PA.13
89	*Streptococcus*	*Streptococcus sp. (99.2)*	70	Strain.Hans7A
89	*Streptococcus*	*Streptococcus sp. (99.2)*	70	Strain.Hans12F
67	*Solobacterium*	*Solobacterium moorei (99.7)*	678	Strain.AHP13983
92	*Peptoniphilus*	*Peptoniphilus sp. (100)*	836	Strain.F0141
158	*Uncl. Prevotellaceae*	*Alloprevotella tannerae (99)*	466	Strain.ATCC51259
129	*Tannerella*	*Tannerella forsythia (100)*	613	Strain.92A2
126	*Uncl.*	*Enterococcus faecalis (100)*	604	Strain.not listed
261	*Clostridiales Fam xiii*	*Peptostreptococcaceae sp. (100)*	91	Clone.CK047
118	*Catonella*	*Catonella morbi (99.7)*	165	Strain.ATCC51271
79	*Campylobacter*	*Campylobacter showae (100)*	763	Strain.CCUG3054
87	*Actinomyces*	*Enterobacter cancerogenus (99.7)*	565	Strain.LMG2693
10	*Gemella*	*Gemella haemolysans (99.5)*	626	Strain.ATCC10379
10	*Gemella*	*Gemella morbillorum (99.5)*	46	Strain.ATCC27824

Biofilms were dominated by genus *Peptostreptococcus*. Genera *Fusobacterium, Parvimonas, Dialister, Solobacterium, Mogibacterium, Anaeroglobus*, unclassified *Enterococcaceae, Clostridiales* Fam. XIII IS, and *Veillonella* were present in all biofilms (Fig. 5.4). Genus *Prevotella* was present in all but one biofilm (donor 8). Individual biofilms per donor revealed that different genera, depending on the donor, accounted for the major dissimilarities (range 26%-57%) with *Porphyromonas*, *Peptostreptococcus*, *Parvimonas, Filifactor, Phocaeicola, Moryella* being the most different between 2w and 4w. Some genera increased in proportions during these weeks e.g. *Filifactor* and *Phocaeicola* whereas others like *Porphyromonas* decreased (Fig.5.3 and Fig.5.4).

In order to identify the most abundant genera at a strain level, we used the microbial nucleotide BLAST search. Taxa that were at ≥0.2% of relative abundance of reads in the biofilms (table 5.1 supplementary data) were identified as *Peptostreptococcus stomatis* (OTU 75, human oral taxon (HOT) 112 99.2% ID); *Fusobacterium nucleatum ss.vincentii* (OTU 21; HOT 200, 99.7% ID); *Parvimonas micra* (OTU 199; HOT 111, 100% ID); *Dialister invisus* (OTU 152; HOT 118, 100% ID), *Solobacterium moorei* (OTU 67; HOT 678, 99.7% ID), *Mogibacterium diversum* (OTU 43; HOT 593, 99,7%ID), *Anaeroglobus geminatus* (OTU 253; HOT121 , 98.4% ID) and *Prevotella oralis* (OTU 142; HOT 705, 99,7% ID) (supplementary data, table 5.1).

Genus *Porphyromonas* (OTU 134), identified as *Porphyromonas gingivalis* (HOT 619, 100% ID), was detected in all inocula except in donor 9. Also it was established in all biofilms but those from donor 9. Genus *Flifactor* OTU 32, identified as *Filifactor alocis* (HOT 539, 100% ID), was detected and established in all periodontitis biofilms, except those from donors 4 and 7. Genera *Tannerella*, identified as *Tannerella forsythia* (OTU 129; HOT 613, 100% ID), *Phocaeicola* and *Pseudoramibacter* were detected in all inocula from periodontitis patients and established in most periodontitis biofilms. Genus *Peptoniphilus* was present only in the inocula from donors 2 and 7, and both were found in their respective biofilms.

DISCUSSION

Our primary aim was to develop a biofilm model of subgingival bacterial biofilm communities. Here we have shown a reproducible growth of complex microbial communities including recently, with periodontal disease associated bacteria, using a relatively simple and robust *in vitro* model. Importantly, we demonstrated that using our model led to different biofilm composition depending on the inoculum source. Statistically significant differences in bacterial composition were consistently found in biofilms among donors clearly reflecting the large variability in the subgingival microbial composition *in vivo* among both periodontitis patients and healthy donors (Li et al., 2014, Griffen et al., 2012, Bizzarro et al., 2016, Camelo-Castillo et al., 2015a, Camelo-Castillo et al., 2015b).

In vitro biofilms models allow standardization for many parameters, increasing their reproducibility. However, they may not fully reproduce the complexity found *in vivo*. All existing *in vitro* biofilm models have limitations (Sissons, 1997). Various *in vitro* subgingival biofilm models have been developed to study biofilm formation, structure and antimicrobial

susceptibility in case of periodontitis. Most of these models have used pre-selected bacterial species as inocula (Eick et al., 2013, Guggenheim et al., 2009, Mouratidou et al., 2011, Peyyala et al., 2011, Soares et al., 2015, Belibasakis and Thurnheer, 2014). However, co-culture biofilms comprising a few selected species poorly reflect the complexity of hundreds of different bacteria present in the subgingival domain (Marsh and Devine, 2011, Wade, 2013). Having considered that bacteria may depend for growth on signal interaction and metabolic cooperation with other bacteria within the biofilm community that they inhabit (Marsh, 2005), in the present study no pre-selection of bacterial species was carried out but full subgingival dental plaque samples were used as inoculum source. A few previous studies have also taken up the challenge of using subgingival dental plaque derived from periodontitis pockets as inocula (Hope and Wilson, 2006, Shaddox et al., 2010, Thompson et al., 2015, Walker and Sedlacek, 2007). Although these studies have contributed greatly to our understanding of subgingival dental plaque formation, only two models have taken into account the importance of the donor source (Walker and Sedlacek, 2007, Thompson et al., 2015). Walker and Sedlacek (Walker and Sedlacek, 2007) included different donors. These authors reported that when subgingival dental plaque was combined with unstimulated saliva from the same donor, biofilms were established more easily (10- to 100-fold more colony forming units) than when adding specific strains of individual species to pooled saliva-treated discs. Unfortunately the inoculum source was not considered for final analysis and samples from all donors were pooled together. A recent study (Thompson et al., 2015) described the microcosm composition from three donors from which the interpersonal differences were limited and not further investigated.

Taking into consideration that recent clinical studies have found a great interpersonal variability in the composition of the subgingival microbiome of periodontitis patients (Bizzarro et al., 2016, Camelo-Castillo et al., 2015b, Camelo-Castillo et al., 2015a, Bizzarro et al., 2013), these findings endorse to studying microcosms relative to interpersonal variability. Interestingly, recent evidence derived from a cross-sectional study indicates that, despite the existence of interpersonal variability, increased inflammation (bleeding upon probing) at sites with periodontitis is associated with a more diverse subgingival microbiota and specific changes in the bacterial composition (Camelo-Castillo et al., 2015a). Patients with more severe levels of periodontal inflammation (BOP > 50% in sampled sites) showed a substantial decrease in bacterial load. These authors explained this particular finding as a possible dilution effect. Additionally, availability of nutrients derived from host-bacterial or inter-microbial interactions may have contributed to the bacterial composition. Arguably, the availability of nutrients at a niche is critical for bacterial growth. Therefore, at a site level it is conceivable that these growth conditions and nutrients availability within a periodontal pocket e.g. pH, Eh, bleeding probing, depth and geographical anatomic differences (e.g. furcations or angular bony defects) may play an important role in bacterial composition. This has been shown in earlier culture-based studies (Magnusson et al., 1984) and is beyond the scope of the current investigation.

The choice of growth conditions determines to a large extent the outcome of *in vitro* biofilm experiments (Guggenheim et al., 2009, ter Steeg and van der Hoeven, 1990, ter Steeg et al., 1987, Simon-Soro et al., 2013, Thompson et al., 2015, Vartoukian et al., 2016). It is known that environmental conditions have a great impact on the type of bacteria and the ability to survive and replicate within a mutualistic complex (Marsh and Devine, 2011). Therefore, the nutrients used in the medium deserve some debate. The gingival crevicular fluid is continuously available at the subgingival habitat and its amount produced fluctuates with the periodontal disease severity (Ozkavaf et al., 2000). A major component of gingival crevicular fluid is serum, an important nutritional source for bacteria. Depending on the serum the medium growth can be promoted or inhibited for some bacterial strains (Guggenheim et al., 2009). Interestingly, *in vitro*, an increase of serum concentration results in increased biomass (thicker biofilms) (Ammann et al., 2012).

It has also been reported that fastidious species such as *Treponema denticola* are difficult to grow and that growth in serum is likely to depend on many variables, among which bacterial interactions (ter Steeg and van der Hoeven, 1990). The recent successful isolation of a member of the phylum TM7 (He et al., 2015) has shown that the relationship between oral bacteria can be more intimate, with the demonstration that the TM7 organism isolated has an intracellular lifestyle, growing within the cells of another species. In the present study we did not succeed in growing *Treponema* species in the biofilm.

The peptone-based medium used in the current study (Thompson et al., 2015) included amino acids that are likely to be available in the periodontal pocket such as cysteine, arginine and lysine. In this habitat, amino acids can also be made available by degrading protein substrates by other bacteria for nutritional support (Eley and Cox, 2003). Importantly arginine degradation favors increase of the pH that in turn counteracts the acidic conditions generated from carbohydrate fermentation in a polymicrobial biofilm (Koopman et al., 2015). Thus, the use of arginine in the growth medium contributes to maintaining the pH 8 used in our biofilm model during the incubation period. Besides the composition of the medium, also the frequency of medium refreshment may influence bacterial growth. Some authors have chosen for subgingival biofilms a medium refreshment at 16.5 h and then 24 h (Guggenheim et al., 2009) and others between 24 and 48 h (Walker and Sedlacek, 2007, Thurnheer et al., 2016, Shaddox et al., 2010). Since no study compared different intervals of medium refreshments we have chosen a medium refreshment of 96 h based on the results from Thompson et al. (Thompson et al., 2015) because enabled the establishment of *in vitro* biofilms derived from subgingival plaque.

In vitro subgingival biofilms derived from a periodontal pocket have been shown to achieve a steady-state (climax) at 10 days of growth (Shaddox et al., 2010). *In vivo* studies have shown that subgingival climax in biofilm formation was observed at 2 weeks on titanium implants (Quirynen et al., 2006). Therefore in this study an initial 2-week time point was chosen because it was of interest to apply this model in the future in microcosms derived from peri-implantitis samples. A 4 week-time point was selected to evaluate whether slow growing bacteria might need more time to get established, which was not the case under the selected

growth conditions. *In vivo* it has been demonstrated that the recolonization of a subgingival microbiota in deep periodontal pockets, in the presence of supragingival plaque, occurs very fast, increasing the number of bacteria (spirochetes and motile rods) (Magnusson et al., 1984). In this study (Magnusson et al., 1984), this increase was observed at 2, 4 up to 8 weeks. Therefore we have chosen 2- and 4-week time points.

In the present study that involved complex subgingival communities, 126 OTUs established in multi-species biofilms, ranging from –'47 - 75 OTUs per biofilm depending on the inoculum source. 158 OTUs which were detected in the initial inocula, were not detected in the biofilms derived from them, among those were genera *Corynebacterium, Haemophilus, Aggregatibacter, Synergistaceae, Paludibacter, Treponema*, *Rothia, Neisseria, and Leptotrichia*. It should be noted that not all of these were present in all donors. For instance, genus *Aggregatibacter* (OTU 131) was found only in the inocula from donors 2 and 4 at a low relative abundance of 0.3%. The growth conditions used in this study favored the growth of certain taxa, which were in low abundance in the inocula e.g. *Peptostreptococcus*, *Porphyromonas, Parvimonas micra, Fusobacterium* and *Filifactor.*

When analyzing the microbial composition of the subgingival domain in periodontal pockets, studies have shown that in addition to the classical diseased associated bacteria (*Porphyromonas gingivalis, Tannerella forsythia* and *Treponema denticola*), other species such as *F. alocis, Selenomonas, Synergistes, Desulfobulbus, Phocaeicola, Anaeroglobus, Mogibacterium, TM7* were found to be associated with disease (Dewhirst et al., 2010, Griffen et al., 2012, Lourenco et al., 2014, Camelo-Castillo et al., 2015b). Adolescents who present aggressive periodontitis had high prevalence of *P. micra*, *F. alocis*, *A. actinomycetemcomitans*, and *Peptostreptococcus* (Fine et al., 2013). Shaddox et al. (Shaddox et al., 2012) reported that in addition to *A. actinomycetemcomitans*, the species *P. micra*, *Selenomonas moorei*, *T. forsythia, F. alocis* and *Capnocytophaga* sp. were more prevalent in localized aggressive periodontitis than in healthy children. In the present study, the inocula derived from 7 adult periodontitis patients resulted in a reproducible subgingival disease-associated multispecies biofilm model including the above-mentioned species.

Limitations

An important aspect to be considered is that the inocula from the donors were not treated with PMA. At the day of inoculum processing and inoculation of the model this was technically not possible. After the experiment, the biofilms were PMA treated and stored for DNA analyses. At that stage, the inocula were mistakenly omitted from this procedure and directly processed for DNA extraction together with the stored and PMA-treated biofilm samples. This means that all DNA from inocula was sequenced, including the DNA from dead or damaged bacterial cells. Therefore, a direct comparison in composition cannot be made between the inocula and the microcosm biofilms. For future experiments the inocula should be PMA-treated before the DNA processing. It may also be that viability of bacteria was lost during transport to the laboratory, or due to manipulation during the experiments (e.g. freezing and defrosting), or due to the selectivity of the growth conditions that were used.

When oral health status of the donor (health or disease) was taken into account, statistically significant difference (PERMANOVA p=0.0001, F=25.7) was observed between the periodontitis patients and the healthy subjects at 2w. It is important to realize that for standardization of the model, the biofilms derived from the inocula of periodontally healthy individuals (biofilms 8 and 9) where exposed to favorable growth conditions for periodontal pathogens. Consequently, after biofilm growth the composition may not be a true reflection of the healthy subgingival environment from their original source. It is also plausible that differences may also be a direct consequence of a different composition in the microbiota in healthy subjects.

Taking into consideration that biofilms formed by our model depend on the donors, arguably this model is reproducible within each donor however among different donors it is likely not reproducible and comparable.

Using this model will require careful inclusion criteria of the donors based on their oral health status. In this model only 4 deep periodontal pockets were sampled per donor resulting in 12 replicate biofilms. It is possible to increase the number of samples per donor if, in the case of a donor diagnosed with untreated severe generalized periodontitis the full mouth subgingival sampling is performed.

In order to be able to run multiple experiments under standard conditions it is required to collect samples beforehand. Although this might result some loss of species as, the model still shows a broad range of species that are able to attach and grow in the model. Nevertheless, the impact of sonication-based dispersal of bacterial and /or cryogenic storage of inoculum on fastidious organisms should be assessed in future investigations.

Directions for future research

The conventional approach of periodontitis treatment relies on the reduction of the bacterial load. Despite all efforts, some patients experience ongoing infection and inflammation. In such cases the alternative strategies can be beneficial. The *in vitro* model that is presented in this study can be used to compare and evaluate traditional antimicrobial therapeutics and novel ecology based strategies (Bizzarro et al., 2016).

However, since biofilms established in this model are depending on the donors, the number of donors should be increased in order to increase the reproducibility between different tests of antimicrobials. This high-throughput active attachment biofilm model is easy to handle and thus offers the opportunity to test different conditions in multiple replicates. Another application could be to associate a particular community with biofilm structures, e.g. endodontic infections (van der Waal et al., 2015).Given the high diversity among microorganisms and inter-microbial and microbiome-host interactions, the current model is likely to help in unraveling the role of individual organisms or group-members and their pathogenic properties in subgingival biofilms. Future work in this direction might lead to refinement of antimicrobial strategies in periodontal therapy (Fernandez y Mostajo et al., 2011).

In order to take host parameters into account, co-culture models have been developed in the past using relatively simple biofilms to trigger monolayers of epithelial cells (Guggenheim et al., 2009, Peyyala et al., 2011, Peyyala et al., 2013). A difficulty in these studies is that the host cells (e.g. epithelial cells) require an aerobic environment whereas the subgingival biofilms of dysbiotic communities (e.g. periodontitis), require an anaerobic environment. Therefore, a co-culture model to assess inter-microbial interactions and host-microbial interactions remains as an important future challenge for *in vitro* periodontal biofilm models. Relating the inflammatory potential of the subgingival biofilm (e.g. cytokine production by fibroblasts) may provide an indication of how these microcosm biofilms relate to disease-associated dysbiosis in the oral cavity.

CONCLUSION

The model developed in this study is donor-associated and allows reproducible production of complex subgingival microbial communities. Bacterial composition of the biofilm was greatly influenced by inoculum source.

Compliance with Ethical Standards

Conflict of Interest

The authors declare that they have no conflict of interest pertaining to the data presented in this article.

Funding source:

The study was financed by the Department of Preventive Dentistry from the Academic Centre for Dentistry Amsterdam (ACTA).

Ethical approval

Ethical approval of the protocol related to plaque collection and experimental research was confirmed by the Medical Ethical Committee from the VU University Medical Center Amsterdam (reference 2012/335).

Informed consent

All participants gave their informed consent prior to their inclusion in the study.

Acknowledgements

We are grateful to Prof. Dr. Bruno Loos for providing access to the periodontitis patients from the Department of Periodontology at the Academic Centre for Dentistry Amsterdam (ACTA).

Chapter 6

Focus

Does routine analysis of subgingival microbiota in periodontitis contribute to patient benefit?

This chapter has been published as:

Fernandez y Mostajo, M., Zaura, E., Crielaard, W. & Beertsen, W. (2011)

Eur J Oral Sci 119**,** 259-264. doi:10.1111/j.1600-0722.2011.00828.x.

License number: 3972011294457

ABSTRACT

In clinical periodontology it is common practice to sample subgingival plaque from periodontitis patients and to search for the presence of alleged periodontal pathogens by using routine laboratory techniques such as culturing, DNA-DNA hybridization or real time PCR. Usually, special attention is given to recognition of 'red complex' microorganisms and *Aggregatibacter actinomycetemcomitans.*

Recently, molecular open-ended techniques have been introduced which are distinct from the more 'classical' approaches in that they do not preselect for certain species. The question was addressed to what extent the outcome of these techniques has changed our insight into the composition of the subgingival microbiota and whether this has consequences to clinical decision making.

The open-ended approaches have shown that the composition of subgingival plaque is much more complex than previously thought. Next to the 'classical' putative periodontal pathogens several non-cultivable and fastidious species are now recognized as being associated with periodontitis, thus enlarging the group of suspected periodontal pathogens. We conclude that routine analyses of subgingival plaque in the clinic are not necessarily of benefit to the patient.

Key words: Biofilm, microbiome, molecular diagnostic techniques, periodontal diseases

INTRODUCTION

It is generally held that most chronic oral infections, such as gingivitis, periodontitis, caries, peri-implantitis, (denture) stomatitis and endodontic infections are caused by microorganisms residing in a polymicrobial biofilm (Costerton et al., 1995). Biofilms constitute a dynamic ecosystem (Kuboniwa and Lamont, 2010, Marsh et al., 2011), in which microorganisms influence each other by their products and signaling molecules as a result of which profound changes may occur in microbial physiology and behavior. The composition and properties of biofilms may change by environmental influences, such as diet (Baumgartner et al., 2009), oral hygiene (Magnusson et al., 1984) and smoking (Shchipkova et al., 2010).

Studies of cultivable subgingival microbiota in periodontal patients over the last few decades (reviewed by (Teles et al., 2006, Shaddox and Walker, 2009, van Winkelhoff and Winkel, 2009) have shown that several species are potentially harmful to the periodontium. This insight has brought microbiologists in several countries to promote routine microbial analysis of subgingival samples to help the clinician in identifying periodontal pathogens and even in the selection of a proper antimicrobial therapy.

Early studies (Listgarten, 1976, Loe et al., 1965, Page et al., 1997) have shown that the predominant bacteria from periodontally healthy sites belong to gram-positive facultative rods and cocci. In gingivitis and periodontitis these organisms decrease proportionally, while at the same time the proportions of gram-negative rods and spirochetes increase. As a major difference between health and disease, an increased proportion of members of the red complex (*Tannerella forsythia, Porphyromonas gingivalis,* and *Treponema denticola* is considered, (Haffajee et al., 2006, Ximenez-Fyvie et al., 2000).

It is important to distinguish between colonization (carrier state) and infection. The presence of a pathogen *per se* does not necessarily indicate that it causes disease. An ecological disturbance has to occur in order to let pathogens achieve relative numerical dominance and clinical significance (Marsh and Martin, 2009). Once symbiotic balance between the host and the microorganisms residing in the subgingival domain is disturbed for whatever reason, periodontal disease may occur, even in small laboratory animals which have only been exposed to their indigenous microbiota (Beertsen et al., 2008).

Microbial diagnostic techniques

Microbial diagnostic techniques are under continuous development. Whereas culturing has provided considerable knowledge of the microorganisms associated with diseased states of the periodontium (Moore and Moore, 1994) and may also be helpful in assessing antimicrobial sensitivity (van Winkelhoff et al., 2005), this 'classical' approach is limited by the fact that it focuses on cultivable microorganisms and thus tends to provide an incomplete picture. More recently, the search of putative pathogens in oral biofilms has extended beyond cultivable bacteria to non-cultivable organisms (Paster et al., 2001) and even viruses (Slots and Slots, 2011). Today, attention is increasingly being given to the microbiome: the ecological community of all commensal, symbiotic and pathogenic microorganisms that share our body

space (Lederberg, 2001, Wade, 2011). Recently it was shown that the human oral microbiome is highly diverse, consisting of approximately 19,000 phylotypes (Keijser et al., 2008). This huge number was detected by 454-pyrosequencing of saliva and plaque samples taken from 98 periodontally healthy adults. A major proportion of bacterial sequences of unrelated healthy individuals were identical (Zaura et al., 2009), supporting the concept of a 'core microbiome at health' (the set of phylotypes shared by healthy individuals). However, it is of interest that considerable variation was also found in supragingival and subgingival microbiota profiles from site to site in the same subject (Haffajee et al., 2009, Kamma et al., 1995, Teles et al., 2006, Zaura et al., 2009). Recent studies (reviewed by (Armitage, 2010) have shown that there are many other species than the currently recognized pathogens which are likely to be associated with periodontitis. At best these microorganisms can be considered as commensal opportunistic pathogens associated with periodontal infections (Armitage, 2010).

After decades of focusing on bacteria in pure culture and a few bacterial complexes (Socransky et al., 1998) it becomes clear that, in order to manage them to our benefit, we need to understand how the microbiome operates in the dynamic ecosystem that the oral cavity provides. The interplay between the oral microbiome, the immune defense system and the prevention/treatment strategy followed by the dentist, defines the outcome: health, transition to disease or recovery from infection and inflammation.

Aim

The World Workshop in Periodontics (1996) focused on a small group of periodonto-pathogenic species (1996), the ones that were detected by 'classical' detection methods like culturing and for which sufficient data had accumulated to consider them as potentially pathogenic. However, this focus on a small group has distracted our attention from other possible and perhaps equally important pathogens which are not readily isolated, cultured and identified (Armitage, 2010). In order to assess the extent to which the outcome of new molecular open-ended approaches change our insight into the composition of the subgingival microbiota or has consequences for clinical decision making we decided to undertake a review of current literature.

MATERIAL AND METHODS

Search strategy and outcome

We searched the US National Library of Medicine database (MEDLINE-PubMed) for papers that used a molecular open-ended approach for subgingival microbial detection (i.e. identification of species without pre-selection). Techniques that were considered: 454-pyrosequencing or cloning and sequencing of 16S rDNA using universal primers for PCR amplification.

The search terms were: ("Molecular Diagnostic Techniques"[Mesh]) OR "Sequence Analysis"[Mesh]) AND "Periodontal Diseases / microbiology"[Mesh]) OR "Aggressive Periodontitis / microbiology"[Mesh]) OR "Chronic Periodontitis / microbiology"[Mesh]) OR

"Gingiva / microbiology"[Mesh]), up to March 1 2011. The search and title screening were conducted by WC and MF. Abstracts were screened by WC and EZ. The reference list of available primary studies was reviewed to identify additional relevant articles in order to supplement the search.

The electronic search identified about 1,641 potential relevant titles. After screening of titles, 80 studies were selected for abstract screening. Of these 8 met the criterion of an open-ended approach. From the manual search two other studies were detected and included. Data heterogeneity prevented quantitative analysis. Table 1 summarizes the main features of these studies. Since the definition of species is controversial, particularly when only molecular sequence data are available, the term phylotype was used (19, see legend of Table 1). These studies, except two (Faveri et al., 2008, Shchipkova et al., 2010), lack a clear definition of clinical periodontal parameters and data were generated by a small number of research groups. The selected papers were heterogeneous with respect to clinical condition of the subjects, selection of sites analyzed and sampling procedures. All these studies included a relatively small number of subjects.

A landmark study (Paster et al., 2001) identified 2,522 bacterial clones in subgingival samples from 31 subjects and revealed many novel species. The authors reported that the 'classical' periodontal pathogens were typically a minor component of the subgingival microbiota and documented that human subgingival plaque harbors several hundreds of bacterial species or phylotypes, the majority of which are non-cultivable. Similarly, 30% of novel species were identified in subgingival plaque of 26 subjects with generalized aggressive periodontitis (Hutter et al., 2003). This is the only study with an open-ended-approach that found *P. gingivalis* relatively dominant in disease.

Gram-positive anaerobic species were more common in subjects with periodontitis than in healthy subjects (Kumar et al., 2005). Newly identified candidates outnumbered *P. gingivalis* and other species previously qualified as periodontopathogens. The diversity of the microbial community did not correlate with either periodontal health or disease, but the presence of *Aggregatibacter actinomycetemcomitans* was significantly associated with disease, as were *Treponema socranskii* and *Pseudomonas* spp. (Ledder et al., 2007). In an analysis of 10 patients suffering from generalized aggressive periodontitis, 57% of the clone (40 taxa) sequences represented phylotypes for which no cultivated isolates have been reported (Faveri et al., 2008). The most prevalent were *Selenomonas* and *Streptococcus* species. The classical putative periodontal pathogens, such as *A. Actinomycetemcomitans*, were below the limit of detection.

Table 6.1 Subgingival microbiota in studies using an open-ended approach (cloning and sequencing)

Reference	Subjects (n)	Recovered phylotypes* (n)
(Kroes et al., 1999)	Mild gingivitis (1)	Phylotypes (59)
(Paster et al., 2001)	Healthy (5), Gingivitis (HIV-associated (2), Acute necrotizing ulcerative (4), Periodontitis (9), Refractory periodontitis (11)	Phylotypes (347) 29 Associated with disease 40% newly detected
(Hutter et al., 2003)	Healthy (6), Generalized aggressive periodontitis (26)	Phylotypes (148) Associated with disease: *P. gingivalis, F. alocis, T. socranskii* *D. pneumosintes* 30% newly detected
(Kumar et al., 2005)	Healthy (15), Moderate to severe periodontitis (15)	Phylotypes (274) Associated with disease: *P. micra, F. alocis*, *Clostridia, Deferribacteres* 60% uncultivable
(Aas et al., 2005)	Healthy (5)	Phylotypes (47) 60% uncultivable
(Lillo et al., 2006)	Localized severe periodontitis (2)	Phylotypes (137) Associated with disease: 70% Firmicutes 38 newly detected phylotypes
(Ledder et al.,2007)	Healthy (18), Chronic periodontitis (29)	Associated with disease: *A. actinomycetemcomitans*, *T. socranskii*, *Pseudomonas*
(Aas et al., 2007)	HIV^+ subjects Gingivitis (5), Periodontitis (8), Localized gingival erythema (1)	Phylotypes (109) Associated with disease: *Gemella, Dialister, Streptococcus, Veillonella* 42% uncultivable
(Faveri et al., 2008)	Generalized aggressive periodontitis (10)	Phylotypes (110) Associated with disease: *Selenomonas*, *Streptococcus* 57% uncultivable
(Shchipkova et al., 2010)	Generalized chronic periodontitis Smokers (15) Never-smokers (15)	Phylotypes (176 in never smokers and 197 in smokers) Associated with disease: *Streptococcus, Selenomonas* (never smokers) *P. micra*, *C. gracilis* (smokers) 39 - 44% uncultivable

*Phylotype is a taxon-neutral term to describe sequences or their clusters at predetermined similarity level obtained by culture-independent molecular methods

The lack of consistent association with disease for the putative periodontal pathogens P. *gingivalis*, *T. forsythia* and *T. denticola* does of course not prove that these species are unimportant to periodontitis. Further, it remains to be established that newly identified microorganisms like *Filifactor alocis* (Dahlen and Leonhardt, 2006, Hutter et al., 2003, Kumar et al., 2003, Kumar et al., 2005, Schlafer et al., Shchipkova et al., Colombo et al., 2009, Tanner et al., 2006), *Dialister Pneumosintes* (Aas et al., 2007, Ghayoumi et al., 2002, Hutter et al., 2003, Doan et al., 2000, Contreras et al., 2000, Colombo et al., 2009) , *TM7* (Brinig et al., 2003, Kumar et al., 2003, Ouverney et al., 2003) *Archaea* (Kulik et al., 2001, Lepp et al., 2004, Li et al., 2009, Vianna et al., 2008, Yamabe et al., 2008) and *Campylobacter gracilis* (Shchipkova et al., 2010, Colombo et al., 2009) - organisms that are found in association with periodontitis - do really cause periodontitis. Further, it was noticed that there is considerable variety in bacterial composition in the subgingival domain among the studies that use an open-ended approach. A possible reason for this may be the use of varying PCR primer sets (de Lillo et al., 2006).

What becomes apparent from the studies summarized in Table 6.1 is that a substantial 40-60% of the subgingival microbiota had not been identified previously. These percentages are in agreement with DEWHIRST et al. (Dewhirst et al., 2010) who analyzed about 34,753 oral clones of 16S rRNA sequences in order to provide a set of reference 16S rRNA gene sequences to construct the Human Oral Microbiome Database (http://www.homd.org). So far, open-ended approaches tend to indicate that the more 'classical' approaches (e.g. culturing, real time PCR and DNA-DNA checkerboard analysis) are incomplete in the identification of potential periodontal pathogens.

Antibiotics

In order to treat bacterial infections, antibiotics are widely used in medicine. Also in periodontal patients prescription of antibiotics is nowadays more and more considered as a treatment option. However, many workers in the field (Rosenberg et al., 1993, Shaddox and Walker, 2009, Teles et al., 2006, van Winkelhoff and Winkel, 2005, van Winkelhoff and Winkel, 2009, Zambon, 1997, Slots and Ting, 2002, Walker and Karpinia, 2002, Herrera et al., 2008) have emphasized the necessity to limit the use of antibiotics, in order to prevent as much as possible the development of bacterial resistance. Generally speaking, antibiotics should be prescribed preferably on the basis of a microbiological diagnosis. In this respect some authors regard identification of alleged periodontal pathogens a valuable adjunct (Listgarten and Loomer, 2003, Sanz et al., 2004, Shaddox and Walker, 2009, Teles et al., 2006, van Winkelhoff and Winkel, 2009). Recently, it was concluded (Shaddox and Walker, 2009) that: "microbial testing may provide some guidance in choosing a specific antibiotic agent, mainly in periodontal cases that do not respond to initial periodontal therapy or to previously used antibiotics. However, most evidence that supports the use of these tests is based on case reports." We think that we should go one step further: based on current microbiological evidence, there is a multitude of potential pathogens in the subgingival domain that cannot be identified by routine analysis. Thus, if antibiotics are indeed to be used, a more realistic

approach would be to combat periodontal microbiota by using broad-spectrum antibiotics (Mombelli et al., 2011).

Implementation of microbiological screening has been suggested as preventive measure in order to reduce the risk of periodontitis in adolescents (e.g. those with a Moroccan background). However, as stated by MOMBELLI et al. (Mombelli et al., 2010), "until an interventional study identifies a superior therapeutic decision other than amoxicillin and metronidazole in *A. Actinomycetemcomitans* (JP2-positive cases), the utility of such a test remains hypothetical. The same principle holds true for any other member of the subgingival microbiota and any other form of periodontitis". Laboratory testing is in fact only meaningful when the acquired information helps to direct therapeutic measures and when it assists in providing optimal therapy. As it was also underlined before (Friedrich, 2008) we should not think in terms of 'pathogens' but rather in terms of 'a disturbed microbial community'.

We would like to emphasize that 8 y after Listgarten & Loomer (2003), the selection of discriminatory 'biomarker' species and their clinical diagnostic significance in order to guide therapeutic measures is still questionable, since it appears that different forms of periodontal diseases are not yet characterized by specific microbiological profiles (Armitage, 2010, Listgarten and Loomer, 2003, Mombelli et al., 2002, Riep et al., 2009, Shaddox and Walker, 2009). Yet, the fact that a small proportion of the total microbiota is routinely recognized does not by itself discredit the use of bacterial surrogate markers for research purposes.

In the light of above, microbiological information seems to have no consistent value for providing optimal antibiotic treatment of periodontal patients. It may even distract the clinician from a straightforward clinical diagnosis and the use of other more rational tools (e.g. the use of plaque index, probing pocket depth, bleeding on probing, clinical attachment level) to follow the outcome of therapeutic measures. It is obvious that periodontal treatment is thought to be most effective in a periodontal specialist practice, where main focus is to guide the patient to carefully and consistently improve their oral hygiene (van der Weijden and Slot, 2011) and their lifestyle habits (Warnakulasuriya et al., 2010). Antibiotics can be useful in the treatment of aggressive cases (Griffiths et al., 2011, Guerrero et al., 2005) but should not take away the attention of the clinician from preventive measures, initial therapy and adequate maintenance care.

The most common and successful combined antibiotic regimen reported to date is the combination of metronidazole + amoxicillin, as a result of their synergistic effects (Pavicic et al., 1992). However, there are no strictly controlled clinical trials in which different treatment strategies are tested in comparable patient groups. Recent molecular studies, using cloning and sequencing have identified several non-cultivable and fastidious Gram-positive and Gram-negative species that are associated with periodontitis (Kuboniwa et al., 2010, Armitage, 2010, Colombo et al., 2009, Dewhirst et al., 2010, Kumar et al., 2005, Paster et al., 2001, Shchipkova et al., 2010, Faveri et al., 2008, Hutter et al., 2003), in addition to the classical putative periodontopathogens. Given the highly diverse composition of the subgingival microbiota, it is clear that treatment with of amoxicillin + metronidazole, although quite effective in many

cases (Cionca et al., 2009, Flemmig et al., 1998, Griffiths et al., 2011, Guerrero et al., 2005, Matarazzo et al., 2008, Mestnik et al., Pavicic et al., 1994, Winkel et al., 2001), is based on empiricism. The rationale to choose only one of these two antibiotics seems questionable. Given the danger of increasing bacterial resistance, we underline the notion that the use of antibiotics should be restricted (Deas and Mealey, 2010, Griffiths et al., 2011).

CONCLUSION

Our perspective of the state of the art in periodontal microbiology is that neither routine analyses such as culturing or real time PCR, nor the use of an open-ended approach (e.g. 454-pyrosequencing) currently represent convincing and cost-effective tools to support and guide periodontal therapy in the individual patient level in the general dental practice. Advances in periodontal microbiology have provided us with comprehensive insight into the composition and structure of oral biofilms but still have not brought us to a level where we can, with certainty, identify the pathogens that are responsible for periodontitis in an individual patient.

Chapter 7

A multispecies subgingival biofilm model from periodontitis and peri-implantitis patients.

A pilot study

Authors:

Fernandez y Mostajo,M., Exterkate, R. A. M., Buijs, M. J., Zaura, E. & Crielaard, W.

ABSTRACT

Objectives

To compare the growth of microcosm biofilms derived from subgingival plaque of periodontitis and peri-implantitis patients on 2 types of surfaces – titanium (Ti) and glass (G). Additionally, to determine the treatment effect of chlorhexidine (PA) and an oxygenating agent Ardox –X® (AX) on biofilms.

Materials and methods

Subgingival plaque samples were collected from pockets (PPD≥6mm) of 3 patients diagnosed with periodontitis (P) and 3 patients diagnosed with peri-implantitis (PI). An active attachment biofilm model and specific medium (Thompson) supplemented with 30% serum was used. Ti and G discs were used as substratum (triplicate). After 2 weeks of growth, biofilms were treated with water (W, control), chlorhexidine (PA) or AX and harvested. DNA of dead cells was blocked for amplification by propidium monoazide (PMA) treatment. Composition was analyzed using 16S rRNA gene amplicon pyrosequencing. Similarities between the biofilms were assessed by non-metric Multidimensional scaling (n-MDS) using Bray Curtis (BC) similarity index. Inoculum source, surface type and treatments were compared using PERMANOVA.

Results

The most abundant taxa in the biofilms were *Peptostreptococcus* (P=16%, PI=2%), *Streptococcus* (P=21%, PI=56%), *Parvimonas* (P=27%, PI=17%), *Peptoniphilus* (P=10%, PI=1%), *Anaeroglobus* (P=1%, PI=3%); *Prevotella* (P=2%, PI=1%); *Filifactor* (P=3%, PI= 0%), *Veillonella* (P=1%, PI=5%) among the other 80 genera. P and PI derived biofilms were significantly different in composition ($p<0.001$). No statistical difference was found between biofilms grown on Ti or G derived from P (p-value=0.242) or Pi (p=0.748). Exposure to PA or AX did not result in significant changes in microbial composition as compared to W control (p= 0.136; p= 1).

Conclusions

The growth of complex multispecies biofilms derived from subgingival plaque in pockets with periodontitis or peri-implantitis was donor-associated and was not significantly affected by the surface type. Neither PA nor AX significantly affected the biofilm composition after a single exposure to these agents.

Key words: periodontitis, peri-implantitis, biofilm, microcosm, pyrosequencing

INTRODUCTION

Osseointegrated dental implants are currently a well-established treatment option in restorative dentistry. Titanium is considered the "gold standard" for dental implants due to its corrosion resistance and biocompatibility. Dental implants are screw-shaped devices made of titanium alloy that provide the support for a replacement tooth. As soon as the healing abutment on the implant is exposed to the environment, a so called 'pristine pocket" is formed and immediately colonized by microorganisms attached to the surface, resulting in a polymicrobial biofilm. A climax community is reached within 2 weeks (Quirynen et al., 2006). At health, this biofilm is in a symbiotic relation with the host. When this harmony is lost, a dysbiotic biofilm triggers a pro-inflammatory host response (Hajishengallis and Lamont, 2012).

The use of chemotherapeutics such as mouthwashes sprays or gels, in combination with the daily mechanical oral hygiene of an implant can be considered as an adjunctive to mechanical plaque control (Jepsen et al., 2015) to promote the resolution of inflammation and bacterial symbiosis However evidence derived from clinical studies on the efficacy of the patient-administered component in the management of peri-implant mucositis is limited (Louropoulou et al., 2014).

Oral microbes contribute to the healthy homeostasis within the oral cavity (Marsh, 2012). Therefore, besides addressing a clinical efficacy of a mouthwash, it is important to investigate its effects on the oral microbiome (ten Cate and Zaura, 2012). For this purpose, the use of a model which allows reproducible production of complex subgingival microbial communities is an essential approach to investigate the impact of new potential chemotherapeutics or techniques at an early stage of product development (Eick et al., 2013, Ntrouka et al., 2011) before more elaborated clinical studies are performed.

In order to evaluate the '*in vitro'* efficacy of chemotherapeutic agents, various biofilm models have been developed. Many of these are models available using a preselection of a few bacterial species (consortium) (Eick et al., 2013, Ntrouka et al., 2011, Thurnheer and Belibasakis, 2014). Given that the peri-implant biofilms are complex, such models may not represent the true multispecies nature of the peri-implant microbiome. In order to assess the efficacy of chemotherapeutics in a multispecies model, other researchers have used a model in which titanium discs were inoculated with saliva derived from periodontally healthy volunteers having a natural dentition (Ntrouka et al., 2011) or a model in which the discs were incubated in the oral cavity of healthy volunteers (Tastepe et al., 2013, Gosau et al., 2010, Schwarz et al., 2007). Since the peri-implant microbiome in health or disease greatly differs from the microbiome from a natural tooth surface (in health or disease) (Dabdoub et al., 2013), using saliva derived from healthy donors as an inoculum for *in vitro* biofilms most likely will not result in a representative microbiome as found at the peri-implant sites.

Recently we developed a reproducible biofilm model for subgingival microbial communities (Fernandez et al 2016, manuscript submitted to JPR), where subgingival plaque is used as inoculum. This model can be used to assess the treatment effects of antimicrobials. Advantages of this model are that it allows the assessment of multispecies biofilms using

samples from different donors as inoculum and it is relatively simple to use. However, there are currently no data available evaluating the growth of a multispecies biofilm derived from peri-implantitis lesions.

The aims of this pilot study were to compare the growth of multispecies biofilms derived from periodontitis and peri-implantitis donors. In addition, we assessed the treatment effect of chlorhexidine and a mouthrinse product containing an oxygenating agent Ardox –X® on periodontits and peri-implantitis derived biofilms grown on titanium.

MATERIALS AND METHODS

Selection of donors

Patients referred to the Academic Centre for Dentistry Amsterdam (ACTA) for periodontitis or peri-implantitis treatment were asked to participate as donors of subgingival plaque. The inclusion criteria were: patients diagnosed with periodontitis with at least 4 sites with probing pocket depth ≥6 mm. Patients diagnosed with peri-implantitis (Zitzmann and Berglundh, 2008), confirmed with a periapical radiograph with a probing pocket depth of ≥5 mm. Partially dentate and edentulous patients were included. All donors were in good general health and had not been exposed to antibiotics, professional dental prophylaxis and antimicrobial mouthwash within the previous 3 months.

Ethical approval was provided by the Medical Ethical Committee from the Vrije Universiteit Medical Center Amsterdam (ref 2012/335). All participants had to sign an informed consent prior to the study.

Sampling

In order to perform proper initial non-surgical periodontal or peri-implant therapy from deep pockets routine local anesthetics are placed. To collect inocula from deep pockets without causing discomfort to the patient, samples were taken after the anesthetics were placed. Selected sites were isolated with cotton rolls and supragingival plaque was carefully removed with sterile universal curette (Hu-Friedy, Chicago, USA) or KerrHawe implant (KerrHawe, Lugano, Switzerland) and discarded. Subgingival plaque samples were collected using a new sterile curette (for periodontitis sites) or KerrHawe implant (for peri-implantitis sites).

The sampling from each donor (having periodontitis or peri-implantitis) was done and kept separately. Samples from the different sites of the same donor were pooled and placed into a vial containing 500 µl reduced transport fluid (RTF), put on ice and immediately transported to the laboratory. To protect the bacterial cells from cryodamage, 500 µl of sterile 60% glycerol was added. Then, samples were briefly sonicated with 3 pulsations (5 s) and vortexed for 10 s (Vibra Cell; Sonics & Materials Inc, Newtown, CT). Subsequently the samples were divided into 2 vials: one vial containing 900 µl serving as inoculum and one vial with 100 µl serving to determine the composition of the inoculum. Samples were stored at –80°C until use.

Biofilm model and production of the biofilms

An active attachment biofilm model was used as previously described [11]. In brief, 24-well polystyrene culture plates (Greiner Bio- One, Alphen a/d. Rijn, the Netherlands) were used. The lid of the plate was replaced by a custom-made stainless-steel lid, onto which 24 nylon clamps were fixed allowing for various substrata to be inserted. The clamps were positioned in such a way that the inserted substrata would fit into the wells of the culture plate. Since the substrata were positioned vertically, bacterial attachment plays a key role in this model. By transferring the lid with the substrata to a new plate, the medium could be refreshed. In this study, glass cover slips (12 mm; Menzel, Braunschweig, Germany) and sandblasted and acid etched titanium discs (10 mm; Ningbo Cibei Medical Treatment Appliance Co.,China) were used as a substratum. The titanium discs were sandblasted with large grid sand and thereafter acid etched with H_2SO_4 and HCl.

The biofilms were grown using a peptone based medium described by Thompson et al (Thompson et al., 2015) and modified by Fernandez et al. (manuscript submitted to JPR). Immediately after defrosting the inocula, the inoculation medium was prepared using subgingival dental plaque in a ratio of 1:27 (Fernandez et al, manuscript in preparation). The models were subsequently incubated anaerobically (10% CO_2, 10% H_2 and 80% N_2) for 24 h at 37°C. After this initial inoculation period, the lids were transferred to new plates containing sterile fresh medium. Medium was refreshed after every 3.5 days, with a total biofilm growth period of 2 weeks.

Treatment of the biofilms

After 2 weeks of growth, the biofilms were treated by transferring them to a new plate containing the treatment solution (1.8 ml/well).

The biofilms were treated for 10 min at room temperature with one of the following compounds:

1. Perioaid® (Dentaid Benelux, Houten, the Netherlands) containing 0.12% chlorhexidine (PA).
2. O7-active mouthrinse (Oral Company International B.V., Weesp, The Netherlands), containing Ardox-X-technology®, (NGen Oral Pharma N.V., Curacao) (AX).
3. Sterile demineralized water as a negative control (W).

Biofilms were then rinsed with phosphate-buffered saline (PBS) (1.9 ml/well) moving the lid 10 times up and down in the plate (Exterkate et al., 2010). This rinsing procedure was repeated 3 times with fresh PBS to remove excess treatment solution.

Harvesting

The discs with biofilms were removed from the lid and transferred into 2 ml PBS. All biofilms were kept on ice, dispersed by sonication (Vibra Cell; Sonics & Materials Inc, Newtown, CT) for 2 min at 1 s pulsations at the amplitude of 40 W and vortexed for 30 s.

PMA treatment of dispersed biofilms

To assure that only DNA from the cells with intact membranes (undamaged cells) was processed for sequencing, the dispersed biofilms were treated with propidium monoazide (PMA) (Exterkate et al., 2014). A total of 1 mg PMA (Biotum Inc., Hayward, Calif., USA) was dissolved in 100 µl 20% dimethyl sulfoxide. Approximately 2.5 µl PMA was added to 500 µl of suspended biofilm, incubated in the dark for 5 min and then exposed to intense light for 2 min using a 650 W halogen lamp placed 25 cm from the samples. During this procedure the samples were kept on ice (Nocker et al., 2006).

The inocula from the donors were not treated with PMA, which implies that all 16S rDNA was sequenced including DNA from dead or damaged bacterial cells.

Assessment of microbial composition

DNA extraction, amplicon preparation and sequencing data analysis.

DNA was extracted from the inocula and PMA treated biofilms (Crielaard et al., 2011). Barcoded amplicon libraries of the small subunit ribosomal RNA gene hypervariable region V5–V7 were generated for each of the individual samples, pooled and sequenced using Genome Sequencer FLX Titanium system (Roche, Basel, Switzerland) (Koopman et al., 2015). The sequencing data was processed using Quantitative Insights Into Microbial Ecology (QIIME) (Caporaso et al., 2010) version 1.5.0 as described previously (Koopman et al., 2015).

To allow comparisons among different samples with the inocula and between the groups, the dataset containing Operational Taxonomic Units (OTUs) was randomly subsampled at an equal depth. Hierarchical clustering based on the Bray Curtis similarities index (BC) was used to assess the similarities among the biofilms per group. The Shannon diversity index was calculated, which takes into account the abundance of each OTU, as well as the number of OTUs. To visualize microbial profile data, non-metric multidimensional plots (n-MDS) based on the BC were used (nMDS) (Clarke, 1993). All calculations were performed using PAST software version 3.0 (Hammer Ø, 2001).

Taxonomy of the representative sequences of the most abundant OTUs was identified by microbial nucleotide BLAST search against Human Oral Microbiome Database (HOMD) reference set (version 13.2). When the highest percentage of identification matched with

more than one sequence, the possible alternatives for the OTU identification were then provided. This was the case for OTU 35; *Peptostreptococcus stomatis* sp (99.2 % sequence identity), OTU 122; *Actinomyces* sp. (100 % sequence identity) and for OTU 733*; Porphyromonas* (98.4 % sequence identity) (see table 7.1 at the supplementary data).

Statistical analysis

The non-metric multidimensional plots (n-MDS) based on the three-dimensional Bray Curtis coefficient similarities (BC) were used to visualize similarities between inocula type, substratum and among treatments. Stress <0.2 (Kruskal's stress formula 1) was used as an acceptable threshold (Clarke and Ainsworth, 1993). Similarity percentage (SIMPER) was used to identify the OTUs with the highest contribution to dissimilarity between substrata and inocula source.

One-way permutational multivariate analysis of variance (PERMANOVA) was used to assess differences in bacterial composition between substratum, inoculum type (periodontitis or peri-implantitis) individual donors and treatment group. Calculations were performed using PAST software version 3.0 (Hammer Ø, 2001). When assessing differences among treatment, p-values were corrected for multiple comparisons using Bonferroni correction. $P<0.05$ were considered statistically significant. The software package IBM SPSS Statistics version 20.0 (2011, IBM Corp., Armonk, NY, USA) was used to perform the statistical analyses for comparisons of the Shannon diversity index among the groups (Paired sample T-test).

RESULTS

Six peri-implantitis patients (not having periodontitis) donated subgingival plaque for this study. However, only three of these inocula were able to grow as a biofilm, while the remaining samples did not visually form a biofilm and were discarded. To allow a comparison with periodontitis derived biofilms, three subgingival plaque donors diagnosed with periodontitis were included in this study.

Inocula composition

The relative abundance of bacterial genera in the inocula differed to a large extent by donor (Fig. 7.1). In brief, bacterial composition of the inocula derived from periodontitis patients was dominated by genus *Fusobacterium* (20% of the reads), *Streptococcus* (16%), *Treponema* (7%), *Paludibacter* (4%), *Tannerella* (4%), *Parvimonas* (4%), *Prevotella* (4%), *Corynebacterium* (4%), *unclasified Clostridiales IS* (6%), *Actinomyces* (3%) and *Porphyromonas* (3%). The inocula derived from peri-implantitis patients were dominated by genus *Prevotella* (9%), *Fusobacterium* (8%), *Porphyromonas* (8%), *Streptococcus* (6%), *Treponema* (5%), *Actinomyces* (5%), *Tannerella* (4%), *Peptostreptococcus* (4%), *Capnocytophaga* (3%) and *unclassified Clostridiales* (3%).

The mean Shannon diversity index of the inocula from periodontitis patients was 4.0 for all donors (SD 0), whereas the diversity for the inocula from the peri-implantitis patients was numerically lower (mean 3.2, SD 0.2) (Table 7.1) but not statistically significant different (paired sample T-test p-value >0.05).

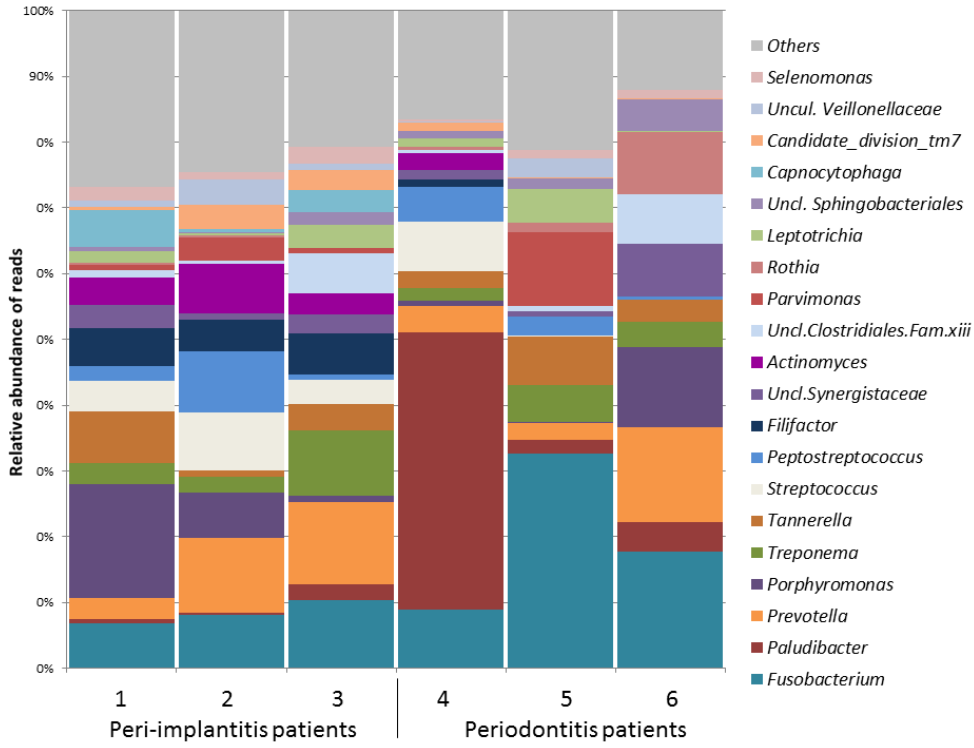


Figure 7.1 Relative abundance of major genera in the inocula per donor.

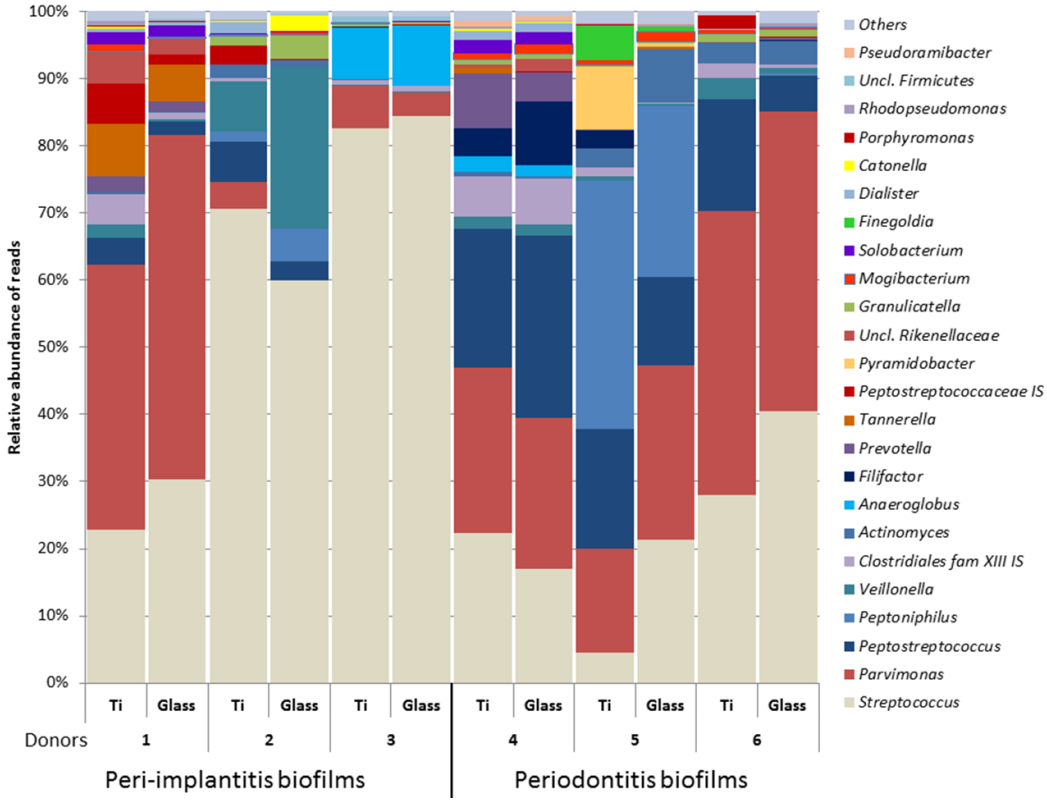


Figure 7.2 Relative abundance of major genera on biofilms per donor and substratum. Data shown is median of quadruplicate biofilms.

Biofilm bacterial composition and the effect of substratum and inoculum source

Figure 7.2 shows the major genera that were able to grow in the model. Peri-implantitis-derived biofilms (PI) grown on titanium surfaces were dominated by genera *Streptococcus* (53% of the reads), *Parvimonas* (17%)*, Veillonella* (6%), *Anaeroglobus* (3%), *Peptostreptocuccus* (3%)*, uncl. Peptostreptococcaceae IS (3%), Tannerella* (3%) and *unclassified Clostridiales fam xiii incertae sedis* (2%), with 37 remaining genera contributing to 9% of the reads. On glass, the main genera were *Streptococcus* (56% of the reads), *Parvimonas* (17%)*, Veillonella* (6%), *Anaeroglobus* (4%), *Peptostreptococcus* (2%)*, uncl. Peptostreptococcaceae incertae sedis (1%), Tannerella* (2%) and *Granulicatella* (2%), with 36 remaining genera contributing to 9% of the reads (Fig. 7.2).

Microbial profiles of peri-implantitis biofilms grown on glass surfaces did not differ statistically from the profiles of the biofilms grown on titanium (Ti) (Fig. 7.3) (p-value=0.748, F=0.375, PERMANOVA). At the individual OTU-level, *Peptostreptococcaceae IS* (OTU 695 and OTU 35), *Tannerella* (OTU 986) and *Parvimonas* (OTU 93) were more abundant on Ti than in glass (similarity percentage, SIMPER). While the most abundant on G as compared to the Ti were *Streptococcus* (OTU 698), *Anaeroglobus* (OTU 883) *Granulicatella* (OTU 722) and *Peptoniphilus* (OTU 667).

Periodontitis-derived biofilms (P) grown on titanium surfaces were dominated by *Parvimonas (28%)*, *Peptostreptococcus* (18%), *Streptococcus* (17%)*, Peptoniphilus* (13%)*, Pyramidobacter* (3%)*, unclasified Clostridiales family xiii incertae sedis* (3%), *Prevotella* (3%), *Filifactor* (2%), *Finegoldia* (2%), *Actinomyces* (2%), *Veillonella* (2%) and other 31 genera (7%). Biofilms grown on glass surfaces were dominated by *Parvimonas (30%)*, *Streptococcus* (25%), *Peptostreptococcus* (14%), *Peptoniphilus* (9%)*, Actinomyces* (5%)*, Filifactor* (3%), *unclassified Clostridiales family xiii incertae sedis* (2%) and 37 other genera (12 %).

Microbial profiles of periodontitis-derived biofilms grown on glass surfaces did not differ statistically from the profiles of the biofilms grown on titanium (Fig. 7.2) (p-value=0.242, F= 1.404, PERMANOVA) (Fig. 7.3). *Streptococcus* (OTU 863) was more abundant in biofilms grown on glass from donors 4, 5 and 6 and more abundant on biofilms grown on Ti from donors 2 and 3 (SIMPER), while *Pyramidibacter* (OTU 678) was more abundant on Ti discs than on glass discs from donors 5 and 6.

Streptococcus (OTU 698) established at a higher abundance on all biofilms grown on glass discs and it was identified in HOMD as *Streptococcus constellatus* (HOT 576; 100% blast ID). Whereas *Streptococcus* (OTU 863), classified as *Streptococcus anginosus* (HOT 543; 99.2% blast ID), was established on both substrata. This genus was the most abundant in the peri-implantitis derived biofilms than in the periodontitis-derived biofilms (Fig 7.2).

Regarding the inoculum source, the compositional difference between biofilms derived from two types of inoculum source, PI and P, was statistically significant (p=0.0001, F=15,86 PERMANOVA; Fig.7.3). Also the profiles from different donors were significantly different (Fig 7.3). SIMPER was used to identified the most abundant OTUs (Table 7.2). As an example, a

clear difference can be observed (Fig. 7.2) on the biofilms grown on titanium from donor 5, in which *Pyramidobacter* (OTU 678), *Finegoldia* (OTU 329), and *Peptoniphilus* (OTU 498) were at a higher abundance on the Ti than on the glass substrata.

The diversity index of the biofilms grown on glass ranged from 0.8 to 2.2 and from the biofilms grown on Ti ranged from 1.2 to 2.2 (table 7.1). This difference was not statistically significant (paired sample T-test, p=0.137).

Table 7.1 Shannon diversity index

Donor	Inoculum	Glass		Water		AX		PA	
		Mean	(SD)	Mean	(SD)	Mean	(SD)	Mean	(SD)
1	4,001	1,745	(0,232)	2,117	(0,124)	1,938	(0,113)	2,111	(0,201)
2	4,026	1,584	(0,364)	1,470	(0,310)	1,279	(0,326)	0,973	(0,805)
3	4,033	0,815	(0,252)	1,178	(0,473)	0,425	(0,039)	1,547	(0,159)
4	3,006	2,173	(0,078)	2,214	(0,068)	1,975	(0,159)	2,173	(0,082)
5	3,310	1,940	(0,109)	1,984	(0,056)	1,911	(0,223)	2,227	(0,221)
6	3,239	1,751	(0,212)	1,855	(0,137)	2,121	(0,918)	2,196	(0,351)

Paired sample T-test, Glass vs. Water p = 0,137; Water vs PA p = 0,702; Water vs AX p= 0,163

Effect of antimicrobial treatment on biofilms grown on titanium surfaces

After 10-min exposure to an antimicrobial rinse (AX = oxygenating agent Ardox-X, PA = chlorhexidine containing rinse Perio-aid or W = water control), two-week old biofilms that were exposed to AX clustered together with the water-treated biofilms, whereas the PA-treated biofilms clearly clustered separately from the water- and AX-treated biofilms (Fig. 7.4). However compared to W this difference was not statistically significant for PA (p=0.136; F=2,33), nor for Ardox-X, (p=1; F=0.46). The OTUs that showed most decrease in abundance in PA comparison with water were *Streptococcus* (OTU 698), *Parvimonas* (OTU 93) and *Peptostreptococcus* (OTU 35), while in comparison with AX a small decrease in abundance was found for *Peptostreptococcus* (OTU 35), *Anaeroglobus* (OTU 883), *Clostridiales IS* (OTU 394 and OTU 695), *Prevotella* (OTU 22) (SIMPER). In terms of diversity, none of the tested compounds significantly affected the diversity of biofilms (PA: p=0.702 and AX: p=0.163) (Table 7.1).

Table 7.2 Most abundant OTUs identified with SIMPER on periodontitis and peri-implantits derived biofilms

OTU	Genera	*Closest phylogenetic relative in HOMD (% sequence identity)*	HOMD oral taxon no.	Strain / Clone
22	*Prevotella*	*Prevotella intermedia* (98.6)	643	Strain.ATCC
35	*Peptostreptococcus*	*Peptostreptococcus stomatis*	112	Strain.A21H2
35	*Peptostreptococcus*	*Peptostreptococcus stomatis*	112	Clone.CK035
79	*Prevotella*	*Prevotella buccae* (98.9)	560	Strain.ATCC
87	*Actinomyces*	*Actinomyces cardiffensis* (100)	850	Strain.F0333
93	*Parvimonas*	*Parvimonas micra* (100)	111	Strain.ATCC
96	*Dialister*	*Dialister invisus* (100)	118	Clone.BS095
12	*Actinomyces*	*Actinomyces odontolyticus*	701	Strain.NCTC
12	*Actinomyces*	*Actinomyces sp.* (100)	180	Strain.C3M24/
12	*Prevotella*	*Prevotella oralis* (99.7)	705	Strain.ATCC
15	*Succiniclasticum*	*Megasphaera sp.* (90.6)	123	Clone.CS025
25	*Rhodopseudomonas*	*Afipia sp. genomospecies 4*	652	Strain.G9018
29	*Parvimonas*	*Streptococcus constellatus*	576	
32	*Finegoldia*	*Peptostreptococcaceae [XIII][G-*	790	Clone.EX153
35	*Pseudoramibacter*	*Pseudoramibacter alactolyticus*	538	Clone.CK057
38	*Phocaeicola*	*Bacteroidaceae [G-1] sp.* (100)	272	Clone.X083
39	*Uncl. Clostridiales.*	*Eubacterium [XI][G-1] infirmum*	105	Strain.A35MT
39	*Candidate_division_tm7*	*TM7 [G-4] sp.* (99.4)	355	Clone.F061
41	*Mogibacterium*	*Mogibacterium timidum* (98.4)	042	Strain.ATCC
47	*Prevotella*	*Prevotella pallens* (100)	714	Strain.9423
49	*Peptoniphilus*	*Peptoniphilus sp.* (99.7)	386	Clone.P4PA.156
51	*Solobacterium*	*Solobacterium moorei* (100)	678	Strain.AHP
52	*Uncl. Erysipelotrichaceae*	*Lactobacillus [XVII]*	569	Strain.ATCC
58	*Filifactor*	*Filifactor alocis* (100)	539	Strain.ATCC
60	*Prevotella*	*Prevotella melaninogenica*	469	Strain.ATCC
62	*Uncl. Rikenellaceae. Rc9*	*Bacteroidetes [G-3] sp.* (99.5)	365	
66	*Peptoniphilus*	*Peptoniphilus sp.* (99.4)	836	Strain.F0141
67	*Pyramidobacter*	*Pyramidobacter piscolens* (100)	357	Clone.BA121
69	*Uncl.*	*Peptostreptococcaceae [XI][G-*	106	Strain.A03MT
69	*Streptococcus*	*Streptococcus constellatus*	576	

OTU	Genera	*Closest phylogenetic relative in HOMD (% sequence identity)*	HOMD oral taxon no.	Strain / Clone
722	*Granulicatella*	*Granulicatella adiacens* (100)	534	Strain.TKT1
726	*Prevotella*	*Alloprevotella sp.* (99.7)	914	Clone.DL303
733	*Porphyromonas*	*Porphyromonas uenonis* (98.4)	785	Strain.F0120
733	*Porphyromonas*	*Porphyromonas asaccharolytica*	547	Strain.ATCC 25260
747	*Fusobacterium*	*Fusobacterium nucleatum ss*	200	Clone.R002
751	*Prevotella*	*Prevotella sp.* (99.7)	299	Clone.BI027
763	*Veillonella*	*Veillonella dispar* (99.7)	160	Strain.DSM 20735
776	*Porphyromonas*	*Porphyromonas gingivalis* (99.7)	619	Strain.DSM 20709
843	*Actinomyces*	*Actinomyces naeslundii* (99.7)	176	Strain.NCTC 10301
863	*Streptococcus*	*Streptococcus anginosus* (99.2)	543	Strain.ATCC 33397
883	*Anaeroglobus*	*Anaeroglobus geminatus* (99.7)	121	Clone.BB166
953	*Porphyromonas*	*Porphyromonas sp.* (100)	278	Clone.BS045
953	*Porphyromonas*	*Porphyromonas sp.* (99.7)	279	Clone.CW034
955	*Gemella*	*Gemella haemolysans* (100)	626	Strain.ATCC 10379
955	*Gemella*	*Gemella morbillorum* (100)	046	Strain.ATCC 27824
986	*Tannerella*	*Tannerella forsythia* (99.7)	613	Strain.FDC 338

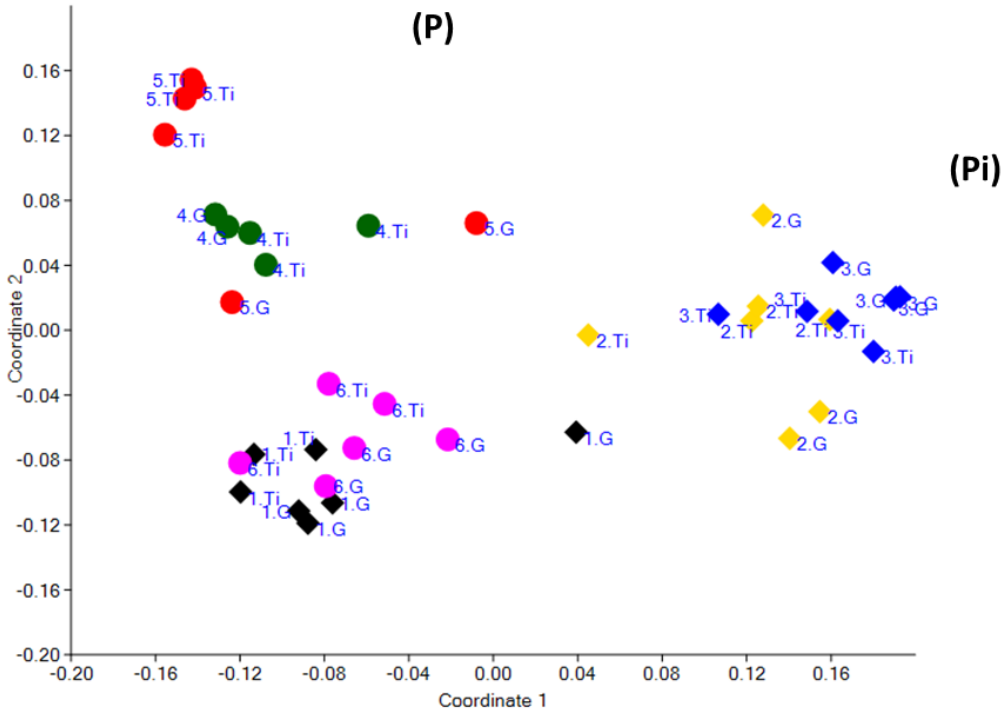


Figure 7.3 Spatial distribution of biofilms by clinical diagnosis (dots: periodontitis (P), diamonds: peri-implantitis (Pi) and the inoculum donor. Each donor is represented by a different color. The number that indicates the donor is the same as in figures 7.1 and 7.2. Biofilms grown on different substratum are presented by Ti - titanium or G - glass. Non-metric multidimensional scaling (n-MDS) based on three-dimensional Bray-Curtis similarity index was performed (stress=0.07). Comparisons between the inoculum source (P and Pi) were done using PERMANOVA and corrected for multiple comparisons (Bonferroni): p-value=0.0001, F=15.86 (PERMANOVA). Comparisons between the substratum type G or Ti were done according to the inoculum source using PERMANOVA: Pi: p=0.748, F=0.375 and P: p=0.242, F= 1.404. Comparisons between Individual biofilms were included in the data analyses.

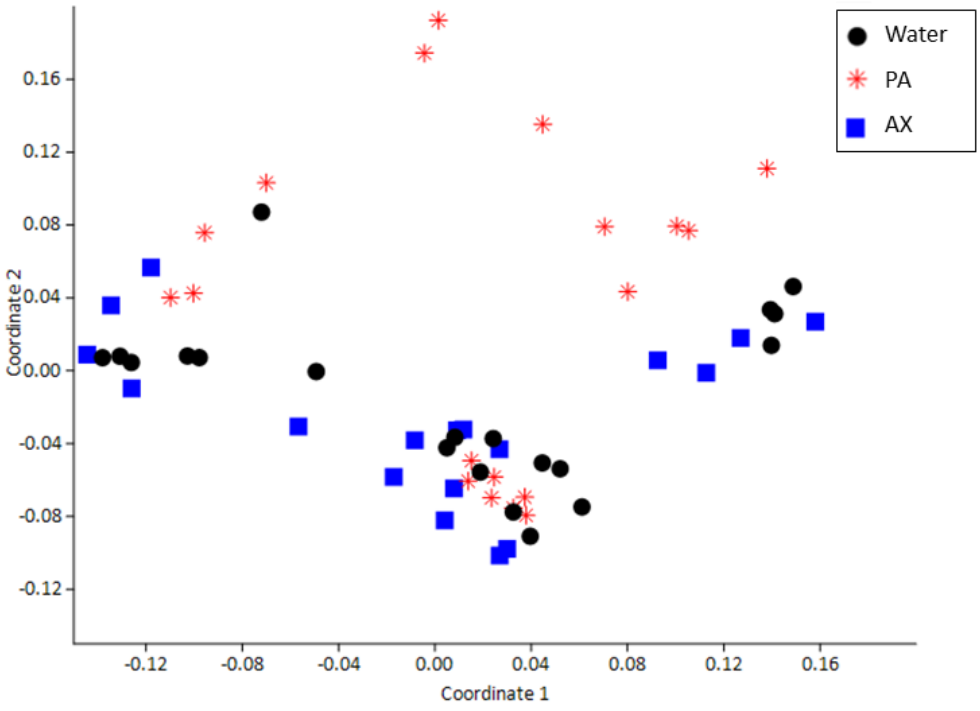


Figure 7.4 Spatial distribution of biofilms by treatment using non-metric multidimensional scaling (n-MDS) based on three-dimensional Bray-Curtis similarity index (p=0.063; F=1.82; stress=0.081). Colors and shape indicate different treatment (black dot: water; red star: chlorhexidine (PA); blue square: Ardox-X). Comparisons between groups were done using PERMANOVA and corrected for multiple comparisons (Bonferroni). There was no statistically significant difference for PA (p=0.136; F=2,33), nor for Ardox-X (p=1; F=0.46) as compared to water. Individual biofilms grown on titanium were included in the data analyses.

DISCUSSION

The results obtained in this pilot study demonstrated that the growth of complex multispecies biofilms derived from periodontitis and peri-implantitis pockets was donor-associated and was not significantly affected by the surface type. In addition, we aimed to assess the effect of exposure to mouthrinse products containing chlorhexidine or an oxygenating agent Ardox–X® on biofilms grown on titanium. In this study, single exposure to PA resulted in changes in microbial composition as compared to water and Ardox-X, while AX did not significantly affect the biofilm composition.

Different models have been developed to study peri-implantitis biofilms (Ntrouka et al., 2011, Schwarz et al., 2014, Tastepe et al., 2013). Preselected bacterial strains and/or *Candida* species have been used as inoculum (Burgers et al., 2012, Cavalcanti et al., 2016, Eick et al., 2013, Belibasakis and Thurnheer, 2014). Other authors have preferred intraorally contaminated titanium discs (Schwarz et al., 2014, Tastepe et al., 2013). However, in these studies the volunteers who have kept these devices in their mouth were healthy individuals with a natural dentition, having no implants. Therefore, this method produces biofilms derived from the supragingival environment and saliva used as inoculum. Other authors have chosen to use stimulated or whole saliva as inoculum source (Ntrouka et al., 2011, Sousa et al., 2016). Considering that the complexity and diversity in bacterial composition from peri-implant diseases (peri-implant mucositis and peri-implantitis) are more similar to each other than the microbiome found at dental sites (Dabdoub et al., 2013, Kumar et al., 2012, Robitaille et al., 2016), it is obvious that microcosms derived from saliva or a few pre-selected bacteria will not resemble the complexity of the *in vivo* microbiome. Therefore, in this study no pre-selection of bacterial species was done. Instead, the inoculum consisted of microbes derived from deep peri-implant pockets (>6 mm) from patients diagnosed with peri-implantitis.

Recent clinical studies using high throughput sequencing technologies have shown that the predominant taxa in peri-implant communities, belonged to *Butyrivibrio, Campylobacter, Eubacterium, Prevotella, Selenomonas, Streptococcus, Actinomyces, Leptotrichia, Propionibacterium, Peptococcus, Lactococcus, and Treponema* (Robitaille et al., 2016). Clinical studies have shown that often some microorganisms, such as *Staphylococcus aureus, Staphylococcus epidermidis, Enterobacter aerogenes, Enterobacter cloace, Escherichia coli, Helicobacter pylori, Parvimonas micra, Pseudomonas* spp., *or Candida* spp., not known to be common in periodontitis patients, have been detected in peri-implant pockets (Robitaille et al., 2016). We have taken these clinical observation into consideration and therefore have included two different inocula sources: patients having periodontitis or peri-implantitis, from which biofilms were grown and analyzed separately.

In a previous study we have shown that this model is suitable for the development of subgingival biofilms derived from periodontitis patients (Fernandez et al 2016, manuscript submitted to JPR). A problem that we encountered in the present pilot study was that not all inocula derived from the peri-implantitis niche were able to grow as a biofilm. Three sets of biofilms derived from three donors did not grow and were discarded. This was not the case for

subgingival biofilms derived from deep pockets of periodontitis patients. One could speculate that the inoculum source did not contain enough microbial material, since no visual biofilm formation was observed. Another reason might be that in this *in vitro* study, peri-implantitis-derived biofilms were dominated by aerobes and it is conceivable that more appropriate growth conditions would have been more succesfull. Another explanation might be related to the fact that two of these samples were derived from fully edentulous peri-implantitis patients. These limitations in the subgingival peri-implantitis-derived biofilm needs to be further clarified in future investigations.

It is well recognized that bacterial colonization depends both on inter-microbial as well as host-bacterial interactions. Therefore, immune characteristics of the peri-implant tissues (Berglundh et al., 2011) will play a role in selecting for a microbial community that is distinct from the periodontal microbiome (Robitaille et al., 2016). Since the dynamics of living tissues cannot be reproduced in a simple *in vitro* model, it is conceivable that the lack of this dynamics is a major limitation and could have influenced the results of bacterial composition found in our model. *In vivo*, within the niche of a periodontal pocket, nutrients derived from the dentinal channels and from the gingival crevicular fluid are important to bacterial growth (Love and Jenkinson, 2002). Whereas within the niche of a peri-implant site the host cells continuously interact with the titanium surfaces. Recent clinical studies suggested that the mere geographical proximity between a periodontal pocket and an osseointegrated implant was not sufficient to have a similar microbiome composition (Dabdoub et al., 2013). These findings further suggested that titanium implants might dictate the composition of a unique ecosystem that forces microbial adaptation and selection (Dabdoub et al., 2013).

In this study we have compared the microcosms derived from the same inocula but grown on two different substrata, titanium and glass. Intriguingly, despite the fact that these two abiotic surfaces structurally differed from each other, no significant difference was found between these two substrata in microbial composition of the 2-week old biofilms. Thus, Ti discs per se did not favor the growth of different bacterial taxa as compared to the biofilm grown on glass discs. This finding suggests that *in vivo*, the host-surface interactions might play a larger role than the surface alone in selecting for bacterial growth. This pilot finding needs to be further corroborated in more elaborate *in vitro* studies.

Biofilms grown in the oral cavity are known to reduce the sensitivity to antimicrobial agents as compared to planktonic cells. The age of the biofilm can influence the sensitivity of the cells to an antimicrobial agent, such as chlorhexidine. These can be due to limited penetration of the antimicrobial indicating a superficial bactericidal effect on biofilm vitality. With increasing plaque age, the more resistant the biofilm is to an antimicrobial, e.g. CHX (Zaura-Arite et al., 2001). In the present pilot study biofilms that were allowed to grow for two weeks, were highly resistant to antimicrobials, even to CHX which is considered the 'golden standard' in antimicrobial mouthrinses (Van Strydonck et al., 2012).

Regarding the treatment of peri-implant diseases, although different therapies to treat peri-implantitis have resulted in various degrees of clinical success, the treatment outcomes are

still not predictable (Esposito et al., 2014). According to the meta-analysis performed at the consensus of the European workshop on Periodontology, adjunctive measures to mechanical oral hygiene measures (antiseptics, local and systemic antibiotics, air abrasive devices) were not found to improve the efficacy of professionally- and patient-administered mechanical plaque control in reducing clinical signs of inflammation.

In this study, a compound AX was tested and its antimicrobial effect on multispecies biofilms was compared to the 'gold standard' chlorhexidine (PA). The results of this study revealed that a single incubation with AX had no significant effect on well-established subgingival biofilms derived from subgingival plaque of periodontitis and peri-implantitis patients. Neither PA nor AX significantly affected the biofilm composition after a single exposure to these agents. Our results regarding the antimicrobial effect of AX are in line with the study from Ntrouka et al (2011). In that study a 48h multispecies biofilm (saliva derived) grown on titanium surfaces was used to test the antimicrobial effect of chemotherapeutic agents, among those AX and PA. They have shown that AX treatment had no significant effect (reduction of CFUs) as compared to PA, while a significant effect was observed as compared to the water treatment.

Currently, there is insufficient evidence for the use of chemotherapeutic agents, however new compounds are commercially available. Therefore, this model is promising in the screening and assessment of new potential antimicrobial agents in the treatment and prevention of peri-implant mucositis.

Limitation

This biofilm model is that does not allow for multiple antimicrobial treatments or recovery of the biofilm after a treatment. This is because biofilms need to be sacrificed for the harvesting and assessment of bacterial composition.

We have recently shown that when multiple donors are included, microbial profiles of biofilms after a single exposure to an antimicrobial, clustered per inoculum donor (Fernandez et al., 2016). It is interesting to note that in this study biofilms derived from some donors were more sensitive to PA, whereas others were not.

CONCLUSIONS

We conclude that within the limitations of this pilot study, the growth of complex multispecies biofilms derived from subgingival plaque of periodontitis and peri-implantitis patients was donor-associated and was not significantly affected by the surface type. Neither PA or AX nor the W control significantly affected the biofilm composition after a single exposure to these agents. The growth of peri-implantitis derived biofilms is complex and needs to be further investigated.

Compliance with Ethical Standards

Conflict of Interest

The authors declare that they have no conflict of interest pertaining to the data presented in this article.

Funding source

The study was financed with a commission from the Academic Centre for Dentistry Amsterdam (ACTA) Dental Research BV (grant number A10-0886). The Department of Preventive Dentistry of ACTA received financial support from NGen Oral Pharma N.V., Curacao. This company had no influence on the design, content, results and publication of this study.

Ethical approval

Ethical approval of the protocol related to plaque-saliva collection and experimental research was confirmed by the Medical Ethical Committee from the VU University Medical Center Amsterdam (reference 2011/236).

Informed consent

All participants gave their informed consent prior to their inclusion in the study.

Acknowledgements

We are very grateful, to Prof. Dr. Wouter Beertsen and Prof. Dr. Fridus van der Weijden for their valuable scientific contribution, to Ms. Ceylin Tastepe and Joyce van der Horst for their technical support and to all donors.

Chapter 8

Summary, general conclusions and future directions

Summary, general discussion and future directions

In **chapter 2** we described how the advances in the field of molecular microbial ecology have opened a new era of studying microbial communities in relation to oral health. Our understanding of periodontal microbial communities in health and disease is in continuous development. Microorganisms are by nature present in and on the human body and have beneficial functions. In the mouth they maintain the healthy balance between biofilms and host. Therefore, from an ecological perspective, preserving symbiotic biofilms should be a keystone in the prevention of oral diseases. Oral biofilms are daily exposed to challenges such as diet and lifestyle factors (i.e. smoking). The resilience to those disturbances is critical for each individual. In addition to these challenges, people use antimicrobial agents for different purposes (e.g. to improve mouth odor or for whitening teeth). Optimizing the use of antimicrobial agents and reducing their side effects, while at the same time taking care that the oral microbiota are kept in balance with oral health, has initiated interest in the assessment of chemotherapeutical agents.

Worldwide, there are numerous over the counter available mouthwashes, many without support of scientific evidence where it concerns their effectiveness in plaque control or reduction of gingival inflammation. Anti-plaque agents should not eradicate the oral microbiota. Instead, they should maintain the microbiota of the mouth at the level and composition that is compatible with oral health, in this way preserving the beneficial functions of resident microbes (Marsh, 2012, ten Cate and Zaura, 2012). This requirement is not met by so-called broad spectrum antimicrobial agents such as chlorhexidine (CHX). Interestingly, oxygenating mouthwashes containing peroxoborate are able to reduce the dental plaque amount and retard the colonization and growth of anaerobes (Binney et al., 1992, Moran et al., 1995, Wennstrom and Lindhe, 1979) and Gram-negative bacteria (Hernandez et al., 2013). Gram-negative anaerobes are generally associated with oral infections (e.g. periodontitis, peri-implantitis, endodontic infections). Therefore, a mouthwash containing the oxygenating agent Ardox-x® technology (AX), commercially known as O7-active (NGen Oral Pharma N.V., Curacao), raised our interest.

Today, AX is a component of numerous products for oral hygiene, including toothpastes,

mouth rinse (O7 active; http://www.o7active.com/) and gels (Implaclean http://www.implaclean.com/nl/).

The understanding that microbial cells growing in a biofilm are physiologically distinct from planktonic bacteria (single cells that float or swim in a liquid medium) it is rational to study *in vitro* biofilm models that allow us to study the properties of antimicrobial agents outside the patient under more controlled conditions than the *in vivo* situation. Biofilms are structurally complex and dynamic systems that provide a protected local environment to micro-organisms enabling them to survive in otherwise hostile locations and from there to colonize new niches by dispersion. The oral cavity is a complex ecosystem. There are continuous interactions of the host cells and microbes - a symbiotic relationship. Although *in vitro* studies have their

limitations (Sissons, 1997), they can be strictly controlled, which is virtually impossible in the *in vivo* situation. It is a challenge to mimic the *in vivo* complexity in an *in vitro* biofilm model.

In vitro biofilm models are suitable to analyze the biofilm interaction with antimicrobial agents. In this way, we demonstrated (**chapter 3)** that using the ACTA active attachment biofilm model (Exterkate et al., 2010), exposure to a mouthwash resulted in different patterns in terms of bacterial composition and metabolic activity of the microcosm biofilm, depending on the chemotherapeutic agent used. We found that biofilms treated with AX presented with the largest changes in bacterial composition, metabolism and viability as compared to two other mouthwashes - Meridol® (GABA International, Basel, Switzerland; an amine fluoride/stannous fluoride (AmF/SnF2)- containing mouthwash) and Perioaid® (Dentaid Benelux, Houten, the Netherlands; containing 0.12% chlorhexidine) mouthwashes.

The most striking effect of the AX containing solution was on bacterial metabolism. We measured a high amount of lactate along with a reduction of the colony forming units (CFUs) up to 40% of the water control. CFUs represent the relative amount of viable bacteria. In the remaining treated biofilms genus *Veillonella* was significantly lower accompanied with the higher relative proportion of other genera mainly *Streptococcus*. The interaction of these genera can affect the metabolic activity reflected in lactate utilization by biofilms. Most likely *Veillonella* was unable to use lactate as energy source, which can potentially affect microbial composition. Low pH due to lactate is considered to have antimicrobial potential against bacteria associated with periodontal diseases (Teughels et al., 2011). In this way, AX could contribute to the resilience of the subgingival microbiome towards disease. However, this study did not evaluate the recovery of the biofilms after the exposure to AX.

Although the results achieved in **chapter 3** with AX are promising, *in vivo* studies are needed to assess this potentially beneficial effect. Therefore, a clinical study was performed with focus on this oxygenating agent (AX). The results of this study are presented in **chapter 4a**. The aims of this study were first, to determine the antimicrobial effect of AX on selected oral bacteria *in vitro*; second, to evaluate *in vivo* the effect of AX (twice-daily exposure) on the composition of undisturbed plaque accumulation in a one-week non-brushing model in 19 healthy volunteers.

The preliminary results of the *in vitro* part of this study revealed that AX has the potential for selective inhibition of oral bacteria. Further, the clinical part of this study showed a high stability in the supragingival microbiome composition during 1 month without any intervention. As expected, the plaque amount increased during the experimental period. The composition of the sampled plaque also changed impressively. The microbial composition showed a shift toward compositionally less diverse plaque dominated by primary colonizers *Streptococcus* and *Veillonella* compared to *Corynebacterium*-dominated plaque at baseline. Interestingly, genus *Veillonella* increased significantly. This could be attributed to the 'pioneering' function of these bacteria. Veillonellae are secondary fermenters – they consume lactic acid produced during glucose fermentation by primary fermenters such as streptococci (Keller and Surette, 2006, Periasamy and Kolenbrander, 2010) and produce other, weaker acids, such as acetic and propionic acid. By doing so, the environmental conditions are created

that promote growth of both of these genera (Bradshaw et al., 1989). The most likely explanation of the increase of genus *Veillonella* during the experimental period could be related to this ecologically beneficial relationship with streptococci. These genera are found at higher abundance in healthy subgingival sites than in periodontal pockets (Griffen et al., 2011, Shi et al., 2015). Therefore, the use of an AX product showed selective inhibition of oral bacteria which may lead to a healthier ecosystem. The results indicate that AX containing mouthwash may contribute to this demanding balance and deserves further investigation. The lack of proper controls of this study was a major limitation which could have potential bias on the interpretation of the study outcomes. Nevertheless, we conclude that this pilot clinical study indicated a rationale for more elaborate studies with a randomized clinical trial (RCT) protocol including both a positive control such as CHX, and a negative control without any antimicrobial effects.

In the dental practice the assessment of dental plaque is important in order to improve preventive measures such us oral hygiene instructions. To visualize this dental plaque, a disclosing solution is frequently used. Plaque may fluoresce red when excited with visible violet light (405 nm) (Thomas et al., 2008, Volgenant et al., 2013). This property was used to visualize plaque and to measure coverage of the dental surfaces by using a camera sensitive to light induced fluorescence (QLF). This fluorescence is an intrinsic characteristic of biofilms. Therefore it is referred to as auto-fluorescence and it can be visualized and recorded using clinical photography. In **chapter 4b**, we compared red fluorescing plaque (RFP) with disclosed plaque. The aim of this cross-sectional study was to assess the relation between dental plaque scores determined by the measurement of red auto-fluorescence and plaque scores established by visualization with a two-tone solution (Mira-2-Ton; Hager & Werken, Duisburg, Germany). For this study, overnight plaque from the anterior teeth of 48 participants was assessed for red fluorescence on photographs using a modified Quigley & Hein index (Paraskevas et al., 2007).

Our results revealed a strong correlation between the total disclosed plaque as scored on photographs and the clinical scores. This means that standard photographs (white-light photographs) can be used to obtain an indication of the oral hygiene of the anterior teeth. The assessments can be performed by multiple examiners at a convenient moment. The correlation between red fluorescent plaque (RFP) and total plaque, as assessed on the photographs, was moderate to strong and significant. Interestingly, what is believed to be mature plaque (blue tone) correlated weakly with RFP. Based on the results of this study, we can neither confirm nor reject the use of a fluorescence device to screen for mature dental plaque. A recent study at our department showed that RFP has the potential to be used to identify subjects at risk for developing gingival inflammation (van der Veen et al., 2016). These are promising results, which need to be investigated further for instance whether this observation it related to the fluorescence or can also be found relative to disclosed dental plaque as assessed on white-light photographs. A limitation of this technique is that only anterior teeth (from cuspid to cuspid) can be photographed and not the premolars and

molars. Future research should investigate and improve the methods of taking these clinical photographs in a standardized manner.

Further in this thesis we focused on the development of a subgingival biofilm model (**chapter 5**). Currently many of such models are limited to 10 – 35 pre-selected laboratory species (Belibasakis and Thurnheer, 2014, Eick et al., 2016, Thurnheer and Belibasakis, 2016) lacking the hundreds of species recently identified under dysbiotic condition of the subgingival ecosystem. In order to overcome this limitation, some investigators have used saliva as inoculum source (Ntrouka et al., 2011), also because it is relatively easy to obtain. Recently other investigators have shown that saliva poorly reflects the microbial composition of dental biofilms (Simon-Soro et al., 2013). Although the use of polymicrobial biofilms derived from the subgingival niche have been studied in the past using *in vitro* studies (Walker and Sedlacek, 2007, Shaddox et al., 2010), they have employed inocula pooled (mixed) from different donors.

Therefore, in **chapter 5** we have used the AAA- model (Exterkate et al., 2010) and as inocula subgingival biofilms derived from deep pockets from periodontitis patients, an important characteristic of this model. As a result, the subgingival-derived biofilms included bacteria known to be associated with periodontal disease such as *Porphyromonas gingivalis, Parvimonas micra, Peptostreptococcus, Fusobacterium, Filifactor, Phocaeicola, Anaeroglobus,* and *Mogibacterium.* An important finding was that statistically significant differences in bacterial composition were consistently found in biofilms derived from different inoculum donors. This finding reflects the large variability in the subgingival microbial composition *in vivo* among periodontitis patients and healthy donors (Bizzarro et al., 2016, Griffen et al., 2012, Shi et al., 2015, Camelo-Castillo et al., 2015a, Camelo-Castillo et al., 2015b). Our study supports the notion that pooling samples and averaging the data can mask the individual variability observed *in vivo*, and in this way provides a more realistic output of the response to treatments. Although the model is reproducible among biofilms derived from the same donor, it is not reproducible among different donors. This means that higher numbers of donors are needed for testing new compounds.

Efforts were made in **chapter 7** to further develop the subgingival biofilm model for peri-implantitis biofilms. In this initial study, the same growth conditions were used for both inoculum types. Additionally we included two different substrata for biofilm growth, glass and titanium. The challenge of this study was to grow microcosm biofilms derived from deep peri-implantitis pockets. Three sets of peri-implantitis biofilms derived from three different donors did not grow and were discarded. This was not the case for subgingival biofilms derived from deep pockets of periodontitis patients. The periodontitis biofilms (P) and peri-implantitis biofilms (PI) were significantly different in composition ($p<0.001$). These biofilms were dominated by *Peptostreptococcus* (P=16%, PI=2%), *Streptococcus* (P=21%, PI=56%), *Parvimonas* (P=27%, PI=17%), *Peptoniphilus* (P=10%, PI=1%), *Anaeroglobus* (P=1%, PI=3%), *Prevotella* (P=2%, PI=1%), *Filifactor* (P=3%, PI= 0%), *Veillonella* (P=1%, PI=5%) among the other 80 genera. The growth of complex multispecies biofilms derived from subgingival plaque in pockets with periodontitis or peri-implantitis was donor-associated.

Further, we tested the effect of an AX and chlorhexidine on microcosms derived from deep pockets from patients having periodontitis and peri-implantitis. These two week old biofilms were exposed to Perio-aid (containing 0.12% chlorhexidine) or AX treatment and did not result in significant changes in microbial composition as compared to water control. Most likely these results are underpowered, due to the loss of half of the biofilms in the peri-implantitis group. Moreover, this initial study needs to be repeated including more donors.

Regarding the treatment effect of an AX containing product and chlorhexidine, one should realize that a comparison between the studies presented in **chapter 3** and **chapter 7** is not possible because of the different inocula used (supragingival and subgingival dental plaque) and the difference in age of the biofilms. It has been reported in the literature that older biofilms are more resistant to antimicrobials than younger biofilms (Zaura-Arite et al., 2001). Since two weeks old biofilms **(chapter 5 and 7**) are more mature than 96 h old biofilms (**chapter 3**), we conclude that older biofilms are more resistant to the AX product than the 96 h biofilms.

The complexity and dynamics of living tissues cannot be reproduced in a simple *in vitro* model. The subgingival niche in disease, the periodontal pocket, has a three-dimensional architecture which is affected by the host-bacterial and inter-bacterial interactions that occur in the oral biofilms. In order to resemble such a complex environment, multispecies biofilm models in combination with oral cells have been developed.

Guggenheim and collaborators (Guggenheim et al., 2009) developed a multispecies biofilm model (9 species) using hydoxyapatite discs as substratum. The grown biofilm was used to trigger response of primary human gingival epithelial cells (HGEC) which allowed the determination of cytokines levels (interleukin (IL)-1ß, IL-6, IL-8) from oral epithelial cells (monolayer). Peyyala et al. (2013) developed a different multispecies (3-6 species) biofilm model, in which a rigid gas-permeable contact lens (RGPL) was incorporated as novel substratum. The biofilm grown on this RGPL was used to stimulate profiles of cytokines/chemokines from oral epithelial cells. These complex multispecies biofilms (Guggenheim et al., 2009, Peyyala et al., 2013) were dominated by a very limited number of species and used a monolayer of epithelial cells which do not reflect the characteristics of the periodontal pockets in an advanced lesion.

Recently, a novel host-biofilm interaction gingival organotypic model, consisting of gingival epithelial cells, fibroblasts and monocytes has been developed (Bao et al., 2015). This model aims to resemble the periodontal pocket using a dynamic perfusion bioreactor in order to develop a gingival epithelial-fibroblast-monocyte co-culture on collagen sponges. An 11 species subgingival biofilm was used to challenge the generated tissue in the bioreactor for a period of 24 h. This model allows for the determination of multiplex immunoassay analysis of cytokine secretion levels (IL-1 β, IL-2, IL-4, and tumor necrosis factor - α). Although this model includes human cells and continuous sheer forces (thus more closely represents the periodontal pocket in periodontitis than the previously discussed models) (Guggenheim et al., 2009, Peyyala et al., 2013), it does not resemble the complexity of the dysbiotic microbiome

since only 11 pre-selected bacterial species were included. *In vitro* biofilm studies are dependent on the growth conditions provided, and we have shown (this thesis) that the results are also affected by the variability among the donors. The above mentioned studies (Bao et al., 2015, Guggenheim et al., 2009, Peyyala et al., 2013) have greatly enhanced our knowledge on the complex host- bacterial interactions *in vitro*. Our model (**chapter 5 and 7**) lacks the flow and the host component. However, it allows simultaneous formation of a number of biofilms from the same donor. Moreover, it allows controlled exposure to various treatments and it is possible to vary substrata for biofilm growth.

To address the diagnostic value of the outcome of microbial analysis commonly used at the dental practice in the Netherlands, we wrote **chapter 6**. We raised the question: does routine analysis of subgingival microbiota in periodontitis contribute to patient benefit? Here we highlighted the importance of distinguishing between colonization (carrier state) and dysbiosis. In periodontal diseases, the presence of a pathogen *per se* does not necessarily indicate that it causes disease. An ecological disturbance has to occur in order to allow pathogens to cause disease.

In this chapter, the value of the NGS techniques regarding periodontal diseases has been addressed. We concluded that at the time of writing this review there was no specific value in bacteriological testing, whatever laboratory testing method is used. Considering that this review was published in 2011, we would like to reconsider our conclusion based on available data derived from two recent longitudinal clinical studies (Bizzarro et al., 2016, Shi et al., 2015) using NGS techniques. These two studies have followed the bacterial shifts in subgingival pockets after initial periodontal treatment (on average after 2 months) (Shi et al., 2015) and with or without the adjunctive use of antibiotics (up to 1 year) (Bizzarro et al., 2016). The results of these studies are in line with the concept that periodontal disease is associated with alterations of the complex microbial community rather than dominance of a single pathogenic species. The predictive value of specific subgingival bacterial profiles for the decision to prescribe antibiotics in the treatment of periodontitis has been proposed. Authors also highlighted the need for alternative therapies based on ecological approaches. Given the concerns of increasing bacterial resistance, the use of antibiotics should be restricted. Instead of aiming to kill “pathogens”, the search to restore the symbiotic biofilm by other means than using antibiotics are of interest.

Future directions

The potential effect of an AX mouthwash solution products on reductions in both the clinical manifestations of gingivitis and the inhibition of or reduction of plaque or plaque pathogenicity still needs to be demonstrated. For that purpose a 21 days experimental gingivitis model could be used or alternatively a 6 month trial as proposed by the American Dental Association in their Acceptance Guidelines of Chemotherapeutic Products for Control of Gingivitis (ADA, Acceptance Guidelines 2016).

Properly designed clinical trials (including an adequate sample size per group), usually results in an expensive and time-consuming study. Therefore we underline that before running expensive clinical trials, a compound should prove to have the potential to be effective. This can be accomplished with *in vitro* studies.

A disadvantage of the ACTA active attachment model is that it requires that biofilms are discarded for assessment of their composition. Future in vitro studies could be performed using a biofilm model that allows for harvesting without contamination while allowing the biofilms to recover from a treatment. This may provide insights about the resilience of biofilms *in vitro* against an antimicrobial treatment.

It is recognized that bacterial colonization depends both on inter-microbial as well as host-microbial interactions. Immune characteristics of the peri-implant tissues (Berglundh et al., 2011) and the microbial community is distinct from the periodontal microbiome (Robitaille et al., 2016). Therefore, It is conceivable that different growth conditions are required for these two different inoculum types. Growth conditions for the peri-implantitis biofilms need to be further investigated and optimized.

The model presented in **chapter 5** and **7** could be improved by including human cells (e.g. gingival organotypic model) in an attempt to mimic the host. Finally, inter-bacterial dependence on nutrition input and utilization of end-products of the bacterial metabolism needs to be further investigated when studying the growth of subgingival biofilms (Huang et al., 2011).

Samenvatting

Samenvatting

Er is wereldwijd een verscheidenheid aan mondspoelingen verkrijgbaar, echter veelal zonder een wetenschappelijke onderbouwing van het effect op plak of gingivitis Het is te prefereren dat deze zogenaamde antiplakmiddelen niet alle micro-organismen in de mond uitroeien, maar de microbioom houdt op een niveau en een samenstelling die in overeenstemming is met mondgezondheid. Aan dit aspect wordt voorbij gegaan door zogenaamde breedspectrum antimicrobiële middelen zoals chloorhexidine (CHX). Oxiderende mondspoelingen zouden echter juist van nut kunnen zijn. Zo zijn er spoelmiddelen die peroxoboraat bevatten en in staat zijn om de kolonisatie en groei van anaeroben (Binney et al.,1992, Moran et al.,1995, Wennstrom en Lindhe, 1979) en Gram-negatieve bacteriën te reduceren (Hernandez et al.,2013). Gram-negatieve anaerobe zijn micro-organismen die vaak worden geassocieerd met orale infecties (bijv. parodontitis, peri-implantitis, wortelkanaalontstekingen). Vandaar dat de mondspoeling, met het oxiderend agens Ardox-x (AX) - commercieel bekend als O7-active (NGen Oral Pharma N.V., Curaçao) - onze interesse heeft gewekt en een centraal thema is geworden van dit proefschrift. AX wordt in verschillende mondverzorgingsproducten toegepast, inclusief tandpasta, mondspoelingen (O7-active; www.o7active.com) en gels (Implacean www.implaclean.com/nl).

In hoofdstuk 2 beschrijven we hoe de ontwikkelingen in het veld van de moleculaire microbiologische ecologie hebben geleid tot een nieuwe fase in het onderzoek van microbiële gemeenschappen in relatie tot mondgezondheid. In de mond spelen vele soorten een rol bij het in stand houden van een gezonde balans tussen microbioom en gastheer. Vandaar dat, vanuit ecologisch perspectief, het behoud van symbiotische biofilms als een belangrijke factor wordt gezien in de preventie van orale aandoeningen.

Orale biofilms worden dagelijks blootgesteld aan uitdagingen voortkomend uit dieet of lifestyle (bijv. roken). Naast deze uitdagingen, gebruikt men soms antimicrobiële producten (bijv. om slechte adem te verbeteren of voor het bleken van tanden). Het optimaliseren van het gebruik van antimicrobiële producten en ervoor te zorgen dat de orale ecosysteem in balans is met mondgezondheid, heeft geleid tot de interesse in dit proefschrift waarbij diverse chemotherapeutische middelen worden beoordeeld.

Omdat micro-organismen die in een biofilm groeien fysiologisch verschillen van planktonische bacteriën (losse cellen die drijven of zwemmen in een vloeibaar medium), is het aanbevelenswaardig om in vitro biofilmmodellen te gebruiken in plaats van losse bacterien ter bestudering van de werking van antimicrobiële middelen in een gecontroleerde setting; dus buiten de patiënt om. Hoewel in vitro studies beperkingen kennen (Sissons, 1997), kunnen zij in het algemeen goed gecontroleerd worden, wat niet zo goed mogelijk is in de in vivo situatie.

Op deze manier konden wij vaststellen (hoofdstuk 3), met gebruikmaking van de 'ACTA-active-attachment-biofilmmodel' (AAA; Exterkate et al., 2010), dat blootstelling aan verschillende mondspoelingen kan resulteren in verschillende patronen van bacteriële samenstelling en metabole activiteit in gekweekte biofilms afkomstig uit supragingivale plak. Met het spoelmiddel dat ondermeer AX bevatte vonden wij de grootste veranderingen in bacteriële samenstelling, metabolisme en levensvatbaarheid, vergeleken met twee andere mondspoelmiddelen (Meridol en Perio-aid).

Het meest opmerkelijke resultaat van het AX mondspoelmiddel betrof het bacteriële metabolisme, met name het hoge gehalte aan lactaat (zuurrest van melkzuur). In deze biofilms was de soort *Veillonella* significant lager, terwijl er een hogere relatieve proportie van andere soorten werd waargenomen, voornamelijk *Streptokokken*. De interactie tussen deze soorten kan de metabole activiteit, zichtbaar in het melkzuurgebruik door de biofilms, aantasten. Melkzuur in de mond wordt beschouwd als een antimicrobiële factor voor bacteriën die geassocieerd worden met parodontale aandoeningen (Teughels et al., 2011). Op deze manier zou het AX spoelmiddel wellicht een bijdrage kunnen leveren aan de weerstand tegen tandvleesontsteking.

Alhoewel de resultaten in hoofdstuk 3 met het AX product veelbelovend lijken, blijven in vivo studies noodzakelijk. Om die reden werd er een klinische studie opgezet. De resultaten zijn gepresenteerd in hoofdstuk 4a. Het doel van deze studie was om niet alleen het antimicrobiële effect van het AX product op bepaalde mondbacteriën in vitro te bepalen maar vooral ook om het in vivo effect van AX (tweemaal per dag gebruikt) te meten op de samenstelling van tandplak in 19 gezonde vrijwilligers die een week niet hadden gepoetst.

De resultaten van het in vitro gedeelte van deze studie lieten zien dat een mondspoelmiddel met AX de potentie heeft tot selectieve remming van orale bacteriën.

Het klinische deel van de studie liet een hoge stabiliteit in de samenstelling van het supragingivale microbioom zien gedurende een maand voor de experimentele periode zonder enige vorm van interventie. Zoals verwacht nam de plak score toe tijdens de periode van niet poetsen en alleen spoelen met het AX product (experimentele periode). De samenstelling van de tandplak veranderde ook: een shift naar een minder diverse microbiologische samenstelling, gedomineerd door de primaire kolonisatoren *Streptokokkus* en *Veillonella*, in vergelijking met een *Corynebacterium* gedomineerde plak op baseline. Opvallend was dat de Veillonella stam significant toenam.

Zo toont het gebruik van een AX-product een selectieve remming van bepaalde mondbacteriën die mogelijk kan leiden tot een gezonder ecosysteem, een meer gezonde balans. Deze mogelijkheid behoeft echter verder onderzoek. Er ontbrak helaas een goede controlegroep in deze studie. Een belangrijke beperking waardoor er vertekening zou kunnenontstaan in een correcte interpretatie van de resultaten. Het is dus van belang deze

klinische pilotstudie te vervolgen met een gerandomiseerde klinische trial van substantiële omvang (met een positieve en een negatieve controlegroep: bijv. CHX versus geen antimicrobiële werking).

In de tandheelkundige praktijk is het beoordelen van tandplak van belang, bijvoorbeeld ter verbetering van mondhygiëne door de patiënt. Ter visualisatie wordt dan vaak een 'twee-kleuren' plakverklikker gebruikt.

Plak fluoresceert rood wanneer het wordt blootgesteld aan violet licht (405 nm) (Thomas et al., 2008, Volgenant et al., 2013). Deze eigenschap werd in Hoofdstuk 4 gebruikt ter visualisatie van plak en ter meting van de bedekking van het tandoppervlak door gebruik te maken van een camera die gevoelig is voor door licht geïnduceerde fluorescentie (QLF). Deze fluorescentie is een intrinsieke karakteristiek van biofilms; vandaar dat het ook wel auto-fluorescentie wordt genoemd, die zichtbaar kan worden gemaakt en gedocumenteerd via fotografie. In dit hoofdstuk vergeleken we rood fluorescerende plak (RFP) met plak gekleurd via een 2-kleuren plakverklikker. Het doel van deze cross-sectionele studie was om te bepalen of er een relatie bestaat tussen tandplakscores bepaald door het meten van auto-fluorescentie en plakscores bepaald door visualisatie met een 'twee-kleuren' plakverklikker (Mira-2-ton; Hagar & Werken, Duisburg, Duitsland). Voor deze studie is de 'overnight' plak van de voortanden van 48 deelnemers beoordeeld voor rode fluorescentie op foto's gescoord met behulp van een aangepaste Quigley & Hein index (Paraskevas et al., 2007).

Onze resultaten toonden een hoge correlatie tussen de plak op foto's en de klinische score. Dit betekent dat de standaard foto's (wit-licht fotografie) gebruikt kunnen worden ter verkrijging van een indicatie van de mondhygiëne van de voortanden. Vooral van belang in epidemiologisch onderzoek, omdat de beoordelingen achteraf kunnen worden uitgevoerd door meerdere beoordelaars (Van der Veen et al., 2016). De correlatie tussen de RFP en de totale hoeveelheid plak, die gescoord werd op de foto's, was middelmatig tot sterk en significant. 'Volwassen plak' (blauwe toon) correleerde zwak met de RFP. Een beperking van deze techniek is echter wel dat de voortanden (van hoektand tot hoektand) gestandaardiseerd kunnen worden gefotografeerd, maar de kiezen niet.

In het vervolg van deze thesis hebben we ons geconcentreerd op de ontwikkeling van een subgingivaal biofilmmodel (hoofdstuk 5). Vele van de in de literatuur beschreven modellen beperken zich tot 10-35 voorgeselecteerde laboratorium bacteriesoorten (Belibasakis en Thurnheer, 2014, Eick et al., 2016, Thurnheer en Belibasakis, 2016), maar er ontbreken dus honderden soorten die recentelijk zijn geïdentificeerd in dysbiotische condities van het subgingivale ecosysteem. Om deze beperking te vermijden hebben sommige onderzoekers speeksel als inoculum gebruikt (Ntrouka et al., 2011), omdat dit gemakkelijk bij patiënten af te nemen is. Recent hebben andere onderzoekers aangetoond dat speeksel de microbiële

compositie van dentale biofilms slecht weergeeft (Simon-Soro et al., 2013). Alhoewel polymicrobiële biofilms verkregen uit subgingivale niches in het verleden wel bestudeerd zijn in vitro (Walker en Sedlacek, 2007, Shaddox et al., 2010), zijn deze studies uitgevoerd met een gemengd inoculum, afkomstig van verschillende donoren.

In dit hoofdstuk hebben wij wederom het AAA-model (Exterkate et al., 2010) gebruikt met als inoculum subgingivale biofilms verkregen uit diepe pockets van parodontitis patiënten. Als resultaat verkregen wij van elke bemonsterde pocket biofilms met bacteriën die bekend staan om hun associatie met parodontale ziekten zoals *Porphyromonas gingivalis, Parvimonas micra, Peptostreptococcus, Fusobacterium, Filifactor, Phocaeicola, Anaerogobus* en *Mogibacterium*. Een belangrijke bevinding was dat statistisch significante verschillen in bacteriële samenstelling consistent waren tussen biofilms verkregen uit de verschillende inoculum donoren, terwijl de samenstelling van gekweekte films juist goed overeen kwamen met de subgingivale plaquesamenstelling van de diverse proefpersonen. Deze bevinding onderschrijft de grote variatie in subgingivale microbiële samenstelling onder parodontitis patiënten en gezonde donoren (Bizzarro et al., 2016, Griffen et al.,2012, Shi et al.,2015, Camelo-Castillo et al., 2015a, Camelo-Castillo et al., 2015b).

In hoofdstuk 7 werd een poging ondernomen tot het verder ontwikkelen van het subgingivale biofilmmodel, maar nu voor biofilms afkomstig van patiënten met peri-implantitis of parodontitis. Tevens gebruikten wij twee verschillende substraten voor biofilmgroei, namelijk glas en titanium. De parodontitis en peri-implantitis biofilms waren significant verschillend in samenstelling ($p<0.001$).

Verder hebben we het effect van AX en CHX mondspoelmiddel getest op de microcosmos verkregen uit diepe pockets van patiënten met parodontitis en peri-implantitis. Twee weken oude biofilms werden blootgesteld aan Perio-aid (0.12% CHX) of het AX product en toonden geen significant verschil in hun microbiële samenstelling vergeleken met de water controlegroep. De uitkomst van deze tests moet met de nodige zorg geïnterpreteerd worden de helft van de biofilms in de peri-implantitis groep verloren is gegaan.

Wat betreft het effect van het AX bevattend product en CHX, moet men realiseren dat een vergelijking tussen de studies gepresenteerd in hoofdstuk 3 en hoofdstuk 7 niet goed mogelijk is vanwege de verschillende inocula die gebruikt zijn (supragingivale versus subgingivale tandplak), en het verschil in leeftijd van de biofilms (96 uur en 2 weken).

Ter bespreking van de diagnostische waarde van de uitkomst van microbiële analyse die vaak wordt gebruikt in de Nederlandse tandartspraktijken (identificatie van enkele indicator bacteriën in gekweekte plaquemosters), schreven wij hoofdstuk 6. Wij stelden ons de vraag in hoeverre stelselmatige analyse van enkele subgingivale microbiota, wezenlijk bijdraagt tot het

bestrijden van parodontale infecties. In dit review hebben we het belang van het verschil tussen kolonisators (carrier state) en dysbiose benadrukt.

Bij parodontale aandoeningen staat de aanwezigheid van een zogenaamde paropathogeen niet garant voor een indicatie van ziekte. Er moet een ecologische verstoring ontstaan alvorens de pathogeen tot ziekte kan leiden. In dit hoofdstuk is het belang van de nieuwe generatie sequencing - (NGS)-technieken, omtrent parodontale ziekten aangekaart. Wij concludeerden op het moment van het schrijven van dit review dat er geen duidelijk toegevoegde waarde in bacteriologische tests kan worden gevonden, ongeacht het gebruikte testmodel. Denkend aan de publicatie van dit review in 2011, voelen wij ons verder gesteund door data verkregen uit twee recente longitudinale klinische studies (Bizzarro et al.,2016, Shi et al., 2015) met gebruik van NGS-technieken. De voorspellende waarde van specifieke subgingivale bacteriële profielen voor het bepalen voor het uitschrijven van een antibioticakuur voor parodontitis achten wij onvoldoende onderbouwd. Wat betreft zorgen omtrent antimicrobiële resistentie zou het antibioticagebruik moeten worden beperkt. In plaats van zich te richten op het doden van “pathogenen” is het herstellen van de symbiotische biofilm via andere methodes dan de gebruikelijke antibiotica het ultieme doel.

Toekomstig onderzoek naar de werking van AX

Het potentiële effect van een AX mondspoelmiddel product op het verminderen van zowel de klinische manifestatie van gingivitis, als de inhibitie of reductie van plak of plakpathogeniciteit moet nog bewezen worden. Goed opgezette klinische trials (inclusief een adequate studiepopulatiegrootte per groep), leiden vaak tot dure en tijd consumerende studies. Om kosten te besparen zou het door ons toegepaste biofilmmodel ingezet kunnen worden voor het verrichten van in vitro voorstudies met de bedoeling wijze van toedienen van AX, duur van contact met AX en daarmee geassocieerde verschuivingen in samenstelling van de flora vast te stellen. Uiteindelijk zal met een ‘in vivo’ studie het klinische bewijs voor het effectiviteit en het nut van een product vastgesteld moeten worden.

Algemene conclusies:

- Vanuit ecologisch perspectief, is het behoud van symbiotische biofilms een belangrijke factor in de preventie van orale aandoeningen.

- Antiplakmiddelen zouden de orale micro-organismen op een niveau en samenstelling moeten houden die compatibel is met de mondgezondheid, zodat de nuttige factoren van endogene microben behouden blijven.

- De resultaten van zowel in vitro als in vivo studies laten zien dat het gebruik van het AX product een selectieve inhibitie van orale bacteriën kan teweegbrengen. Dit kan mogelijk bijdragen aan een gezonder ecosysteem in de mond. Deze mogelijkheid behoeft echter nog zorgvuldig vervolgonderzoek.

- De ontwikkeling van het subgingivaal biofilm model dat in deze thesis is gebruikt leidt tot de groei van biofilms welke bekend staan om hun associatie met parodontale ziekten zoals *Porphyromonas gingivalis, Parvimonas micra, Peptostreptococcus, Fusobacterium, Filifactor, Phocaeicola, Anaerogobus* en *Mogibacterium*. Deze bacteriën komen in het model tot verdere ontwikkeling en bieden de mogelijkheid tot verder onderzoek naar hun onderlinge interacties. Een belangrijke bevinding was dat statistisch significante verschillen in bacteriële samenstelling consistent waren tussen biofilms verkregen uit de verschillende inoculum donoren. Deze bevinding reflecteert op de grote variatie in subgingivale microbiële samenstelling gevonden bij parodontitis patiënten.

- De ontwikkeling van een peri-implantitis biofilm model lijkt mogelijk en kan wellicht dienen voor verder onderzoek naar de pathogene factoren die mogelijk een bijdrage leveren aan het verlies van steunweefsel rond aangedane implantaten.

- Parodontale aandoeningen zijn geassocieerd met veranderingen in de complexe bacteriële gemeenschap in plaats van dominantie van slechts één, of een paar pathogene stammen. De voorspellende waarde van specifieke subgingivale bacteriële profielen voor het bepalen van het uitschrijven van een antibioticakuur voor parodontitis is onvoldoende onderbouwd. In dit proefschrift wordt benadrukt dat een ecologische benadering te prefereren is boven het gebruik van antibiotica.

References

(1996) Proceedings of the 1996 World Workshop in Periodontics. Lansdowne, Virginia, July 13-17, 1996. *Ann Periodontol* **1,** 1-947.

Aas, J. A., Barbuto, S. M., Alpagot, T., Olsen, I., Dewhirst, F. E. & Paster, B. J. (2007) Subgingival plaque microbiota in HIV positive patients. *J Clin Periodontol* **34,** 189-195.

Aas, J. A., Griffen, A. L., Dardis, S. R., Lee, A. M., Olsen, I., Dewhirst, F. E., Leys, E. J. & Paster, B. J. (2008) Bacteria of dental caries in primary and permanent teeth in children and young adults. *J Clin Microbiol* **46**. doi:10.1128/jcm.01410-07.

Aas, J. A., Paster, B. J., Stokes, L. N., Olsen, I. & Dewhirst, F. E. (2005) Defining the normal bacterial flora of the oral cavity. *J Clin Microbiol* **43,** 5721-5732. doi:10.1128/JCM.43.11.5721-5732.2005.

Abusleme, L., Dupuy, A. K., Dutzan, N., Silva, N., Burleson, J. A., Strausbaugh, L. D., Gamonal, J. & Diaz, P. I. (2013) The subgingival microbiome in health and periodontitis and its relationship with community biomass and inflammation. *ISME J* **7,** 1016-1025. doi:10.1038/ismej.2012.174.

Addy, M. (1986) Chlorhexidine compared with other locally delivered antimicrobials. A short review. *J Clin Periodontol* **13,** 957-964.

Addy, M. & Moran, J. (1985) Extrinsic tooth discoloration by metals and chlorhexidine. II. Clinical staining produced by chlorhexidine, iron and tea. *Br Dent J* **159,** 331-334. doi:10.1038/sj.bdj.4805722.

Ammann, T. W., Gmur, R. & Thurnheer, T. (2012) Advancement of the 10-species subgingival Zurich biofilm model by examining different nutritional conditions and defining the structure of the in vitro biofilms. *BMC Microbiol* **12,** 227. doi:10.1186/1471-2180-12-227.

Armitage, G. C. (2010) Comparison of the microbiological features of chronic and aggressive periodontitis. *Periodontol 2000* **53,** 70-88.

Arweiler, N. B., Netuschil, L. & Reich, E. (2001) Alcohol-free mouthrinse solutions to reduce supragingival plaque regrowth and vitality. A controlled clinical study. *J Clin Periodontol* **28,** 168-174.

Axelsson, P. & Lindhe, J. (1978) Effect of controlled oral hygiene procedures on caries and periodontal disease in adults. *J Clin Periodontol* **5,** 133-151.

Bao, K., Papadimitropoulos, A., Akgul, B., Belibasakis, G. N. & Bostanci, N. (2015) Establishment of an oral infection model resembling the periodontal pocket in a perfusion bioreactor system. *Virulence* **6,** 265-273. doi:10.4161/21505594.2014.978721.

Baumgartner, S., Imfeld, T., Schicht, O., Rath, C., Persson, R. E. & Persson, G. R. (2009) The impact of the stone age diet on gingival conditions in the absence of oral hygiene. *J Periodontol* **80,** 759-768.

Becker, M. R., Paster, B. J., Leys, E. J., Moeschberger, M. L., Kenyon, S. G., Galvin, J. L., Boches, S. K., Dewhirst, F. E. & Griffen, A. L. (2002) Molecular analysis of bacterial species associated with childhood caries. *J Clin Microbiol* **40,** 1001-1009.

Beertsen, W., Willenborg, M., Everts, V., Zirogianni, A., Podschun, R., Schroder, B., Eskelinen, E. L. & Saftig, P. (2008) Impaired phagosomal maturation in neutrophils leads to periodontitis in lysosomal-associated membrane protein-2 knockout mice. *J Immunol* **180,** 475-482.

Belda-Ferre, P., Alcaraz, L. D., Cabrera-Rubio, R., Romero, H., Simon-Soro, A., Pignatelli, M. & Mira, A. (2012) The oral metagenome in health and disease. *ISME J* **6,** 46-56. doi:10.1038/ismej.2011.85.

Belibasakis, G. N. & Thurnheer, T. (2014) Validation of antibiotic efficacy on *in vitro* subgingival biofilms. *J Periodontol* **85,** 343-348. doi:10.1902/jop.2013.130167.

Bentley, C. D. & Disney, J. A. (1995) A comparison of partial and full mouth scoring of plaque and gingivitis in oral hygiene studies. *J Clin Periodontol* **22,** 131-135.

Berglundh, T., Zitzmann, N. U. & Donati, M. (2011) Are peri-implantitis lesions different from periodontitis lesions? *J Clin Periodontol* **38 Suppl 11,** 188-202. doi:10.1111/j.1600-051X.2010.01672.x.

Bik, E. M., Long, C. D., Armitage, G. C., Loomer, P., Emerson, J., Mongodin, E. F., Nelson, K. E., Gill, S. R., Fraser-Liggett, C. M. & Relman, D. A. (2010) Bacterial diversity in the oral cavity of 10 healthy individuals. *ISME J* **4,** 962-974. doi:10.1038/ismej.2010.30.

Binney, A., Addy, M. & Newcombe, R. G. (1992) The effect of a number of commercial mouthrinses compared with toothpaste on plaque regrowth. *J Periodontol* **63,** 839-842. doi:10.1902/jop.1992.63.10.839.

Bizzarro, S., Laine, M. L., Buijs, M. J., Brandt, B. W., Crielaard, W., Loos, B. G. & Zaura, E. (2016) Microbial profiles at baseline and not the use of antibiotics determine the clinical outcome of the treatment of chronic periodontitis. *Sci Rep* **6,** 20205. doi:10.1038/srep20205.

Bizzarro, S., Loos, B. G., Laine, M. L., Crielaard, W. & Zaura, E. (2013) Subgingival microbiome in smokers and non-smokers in periodontitis: an exploratory study using traditional targeted techniques and a next-generation sequencing. *J Clin Periodontol* **40,** 483-492. doi:10.1111/jcpe.12087.

Bjurshammar, N., Johannsen, Buhlin K., Tranaeaus S., Ostman C. (2012) On the red fluorescence emission of Aggregatibacter actinomycetemcomitans. *Open Journal of Stomatology* **2,** 299-306.

Block, P. L., Lobene, R. R. & Derdivanis, J. P. (1972) A two-tone dye test for dental plaque. *J Periodontol* **43,** 423-426. doi:10.1902/jop.1972.43.7.423.

Bradshaw, D. J., McKee, A. S. & Marsh, P. D. (1989) Effects of carbohydrate pulses and pH on population shifts within oral microbial communities in vitro. *J Dent Res* **68,** 1298-1302.

Brailsford, S. R., Shah, B., Simons, D., Gilbert, S., Clark, D., Ines, I., Adams, S. E., Allison, C. & Beighton, D. (2001) The predominant aciduric microflora of root-caries lesions. *J Dent Res* **80,** 1828-1833.

Brailsford, S. R., Sheehy, E. C., Gilbert, S. C., Clark, D. T., Kidd, E. A., Zoitopoulos, L., Adams, S. E., Visser, J. M. & Beighton, D. (2005) The microflora of the erupting first permanent molar. *Caries Res* **39,** 78-84. doi:10.1159/000081661.

Brinig, M. M., Lepp, P. W., Ouverney, C. C., Armitage, G. C. & Relman, D. A. (2003) Prevalence of bacteria of division TM7 in human subgingival plaque and their association with disease. *Appl Environ Microbiol* **69,** 1687-1694.

Burgers, R., Witecy, C., Hahnel, S. & Gosau, M. (2012) The effect of various topical peri-implantitis antiseptics on *Staphylococcus epidermidis*, *Candida albicans*, and *Streptococcus sanguinis*. *Arch Oral Biol* **57,** 940-947. doi:10.1016/j.archoralbio.2012.01.015.

Camelo-Castillo, A., Novoa, L., Balsa-Castro, C., Blanco, J., Mira, A. & Tomas, I. (2015a) Relationship between periodontitis-associated subgingival microbiota and clinical inflammation by 16S pyrosequencing. *J Clin Periodontol*. doi:10.1111/jcpe.12470.

Camelo-Castillo, A. J., Mira, A., Pico, A., Nibali, L., Henderson, B., Donos, N. & Tomas, I. (2015b) Subgingival microbiota in health compared to periodontitis and the influence of smoking. *Front Microbiol* **6,** 119. doi:10.3389/fmicb.2015.00119.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., Pena, A. G., Goodrich, J. K., Gordon, J. I., Huttley, G. A., Kelley, S. T., Knights, D., Koenig, J. E., Ley, R. E., Lozupone, C. A., McDonald, D., Muegge, B. D., Pirrung, M., Reeder, J., Sevinsky, J. R., Turnbaugh, P. J., Walters, W. A., Widmann, J., Yatsunenko, T., Zaneveld, J. & Knight, R. (2010a) QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* **7,** 335-336. doi:10.1038/nmeth.f.303.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., Pena, A. G., Goodrich, J. K., Gordon, J. I., Huttley, G. A., Kelley, S. T., Knights, D., Koenig, J. E., Ley, R. E., Lozupone, C. A., McDonald, D., Muegge, B. D., Pirrung, M., Reeder, J., Sevinsky, J. R., Turnbaugh, P. J., Walters, W. A., Widmann, J., Yatsunenko, T., Zaneveld, J. & Knight, R. (2010b) *QIIME allows analysis of high-throughput community sequencing data*. Nat Methods. 2010 May;7(5):335-6. doi: 10.1038/nmeth.f.303. Epub 2010 Apr 11.

Carrilho, M. R., Carvalho, R. M., Sousa, E. N., Nicolau, J., Breschi, L., Mazzoni, A., Tjaderhane, L., Tay, F. R., Agee, K. & Pashley, D. H. (2010) Substantivity of chlorhexidine to human dentin. *Dent Mater* **26,** 779-785. doi:10.1016/j.dental.2010.04.002.

Caufield, P. W., Dasanayake, A. P., Li, Y., Pan, Y., Hsu, J. & Hardin, J. M. (2000) Natural history of *Streptococcus sanguinis* in the oral cavity of infants: evidence for a discrete window of infectivity. *Infect Immun* **68,** 4018-4023.

Cavalcanti, Y. W., Wilson, M., Lewis, M., Del-Bel-Cury, A. A., da Silva, W. J. & Williams, D. W. (2016) Modulation of *Candida albicans* virulence by bacterial biofilms on titanium surfaces. *Biofouling* **32,** 123-134. doi:10.1080/08927014.2015.1125472.

Cephas, K. D., Kim, J., Mathai, R. A., Barry, K. A., Dowd, S. E., Meline, B. S. & Swanson, K. S. (2011) Comparative analysis of salivary bacterial microbiome diversity in edentulous infants and their mothers or primary care givers using pyrosequencing. *PLoS One* **6,** e23503. doi:10.1371/journal.pone.0023503.

Chapple, I. L., Van der Weijden, F., Doerfer, C., Herrera, D., Shapira, L., Polak, D., Madianos, P., Louropoulou, A., Machtei, E., Donos, N., Greenwell, H., Van Winkelhoff, A. J., Bahar, E. K., Arweiler, N., Teughels, W., Aimetti, M., Molina, A., Montero, E. & Graziani, F. (2015) Primary prevention of periodontitis: managing gingivitis. *J Clin Periodontol*. doi:10.1111/jcpe.12366.

Chen, T., Yu, W. H., Izard, J., Baranova, O. V., Lakshmanan, A. & Dewhirst, F. E. (2010) The Human Oral Microbiome Database: a web accessible resource for investigating oral microbe taxonomic and genomic information. *Database (Oxford)* **2010**. doi:10.1093/database/baq013.

Cho, I. & Blaser, M. J. (2012) The human microbiome: at the interface of health and disease. *Nat Rev Genet* **13,** 260-270. doi:10.1038/nrg3182.

Cionca, N., Giannopoulou, C., Ugolotti, G. & Mombelli, A. (2009) Amoxicillin and metronidazole as an adjunct to full-mouth scaling and root planing of chronic periodontitis. *J Periodontol* **80,** 364-371.

Clarke, K. R. (1993) Nonparametric Multivariate Analyses of Changes in Community Structure. *Australian Journal of Ecology* **18,** 117-143. doi:DOI 10.1111/j.1442-9993.1993.tb00438.x.

Clarke, K. R. & Ainsworth, M. (1993) A Method of Linking Multivariate Community Structure to Environmental Variables. *Marine Ecology Progress Series* **92,** 205-219. doi:Doi 10.3354/Meps092205.

Cole, J. R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R. J., Kulam-Syed-Mohideen, A. S., McGarrell, D. M., Marsh, T., Garrity, G. M. & Tiedje, J. M. (2009) The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. *Nucleic Acids Res* **37,** D141-145. doi:10.1093/nar/gkn879.

Colombo, A. P., Boches, S. K., Cotton, S. L., Goodson, J. M., Kent, R., Haffajee, A. D., Socransky, S. S., Hasturk, H., Van Dyke, T. E., Dewhirst, F. & Paster, B. J. (2009) Comparisons of subgingival microbial profiles of refractory periodontitis, severe periodontitis, and periodontal health using the human oral microbe identification microarray. *J Periodontol* **80,** 1421-1432. doi:10.1902/jop.2009.090185.

Contreras, A., Doan, N., Chen, C., Rusitanonta, T., Flynn, M. J. & Slots, J. (2000) Importance of Dialister pneumosintes in human periodontitis. *Oral Microbiol Immunol* **15,** 269-272.

Corby, P. M., Lyons-Weiler, J., Bretz, W. A., Hart, T. C., Aas, J. A., Boumenna, T., Goss, J., Corby, A. L., Junior, H. M., Weyant, R. J. & Paster, B. J. (2005) Microbial risk indicators of early childhood caries. *J Clin Microbiol* **43,** 5753-5759. doi:10.1128/JCM.43.11.5753-5759.2005.

Cortellini, P., Labriola, A., Zambelli, R., Prato, G. P., Nieri, M. & Tonetti, M. S. (2008) Chlorhexidine with an anti discoloration system after periodontal flap surgery: a cross-over, randomized, triple-blind clinical trial. *J Clin Periodontol* **35,** 614-620. doi:10.1111/j.1600-051X.2008.01238.x.

Costerton, J. W., Lewandowski, Z., Caldwell, D. E., Korber, D. R. & Lappin-Scott, H. M. (1995) Microbial biofilms. *Annu Rev Microbiol* **49,** 711-745.

Coulthwaite, L., Pretty, I. A., Smith, P. W., Higham, S. M. & Verran, J. (2006) The microbiological origin of fluorescence observed in plaque on dentures during QLF analysis. *Caries Res* **40,** 112-116. doi:10.1159/000091056.

Coulthwaite, L., Pretty, I. A., Smith, P. W., Higham, S. M. & Verran, J. (2009) QLF is not readily suitable for *in vivo* denture plaque assessment. *J Dent* **37,** 898-901. doi:10.1016/j.jdent.2009.07.002.

Crielaard, W., Zaura, E., Schuller, A. A., Huse, S. M., Montijn, R. C. & Keijser, B. J. (2011) Exploring the oral microbiota of children at various developmental stages of their dentition in the relation to their oral health. *BMC Med Genomics* **4,** 22. doi:10.1186/1755-8794-4-22.

Curtis, M. A., Zenobia, C. & Darveau, R. P. (2011) The relationship of the oral microbiotia to periodontal health and disease. *Cell Host Microbe* **10,** 302-306. doi:10.1016/j.chom.2011.09.008.

Dabdoub, S. M., Tsigarida, A. A. & Kumar, P. S. (2013) Patient-specific analysis of periodontal and peri-implant microbiomes. *J Dent Res* **92,** 168s-175s. doi:10.1177/0022034513504950.

Dahan, M., Timmerman, M. F., Van Winkelhoff, A. J. & Van der Velden, U. (2004) The effect of periodontal treatment on the salivary bacterial load and early plaque formation. *J Clin Periodontol* **31,** 972-977. doi:10.1111/j.1600-051X.2004.00595.x.

Dahlen, G. & Leonhardt, A. (2006) A new checkerboard panel for testing bacterial markers in periodontal disease. *Oral Microbiol Immunol* **21,** 6-11. doi:OMI243 [pii] 10.1111/j.1399-302X.2005.00243.x.

Dancey, C. R. (2004) Statistics without Maths for Psychology: using SPSS for Windows. London: Prentice Hall.

de Lillo, A., Ashley, F. P., Palmer, R. M., Munson, M. A., Kyriacou, L., Weightman, A. J. & Wade, W. G. (2006) Novel subgingival bacterial phylotypes detected using multiple universal polymerase chain reaction primer sets. *Oral Microbiol Immunol* **21,** 61-68.

Deas, D. E. & Mealey, B. L. (2010) Response of chronic and aggressive periodontitis to treatment. *Periodontol 2000* **53,** 154-166.

Deng, D. M., Hoogenkamp, M. A., Exterkate, R. A., Jiang, L. M., van der Sluis, L. W., ten Cate, J. M. & Crielaard, W. (2009) Influence of *Streptococcus mutans* on Enterococcus faecalis biofilm formation. *J Endod* **35,** 1249-1252. doi:10.1016/j.joen.2009.05.038.

Derks, J. & Tomasi, C. (2015) Peri-implant health and disease. A systematic review of current epidemiology. J Clin Periodontol 42 Suppl 16, S158-171. doi:10.1111/jcpe.12334.

Dewhirst, F. E., Chen, T., Izard, J., Paster, B. J., Tanner, A. C., Yu, W. H., Lakshmanan, A. & Wade, W. G. (2010) The human oral microbiome. *J Bacteriol* **192,** 5002-5017. doi:10.1128/jb.00542-10.

Diaz, P. I., Chalmers, N. I., Rickard, A. H., Kong, C., Milburn, C. L., Palmer, R. J., Jr. & Kolenbrander, P. E. (2006) Molecular characterization of subject-specific oral microflora during initial colonization of enamel. *Appl Environ Microbiol* **72,** 2837-2848. doi:10.1128/AEM.72.4.2837-2848.2006.

Doan, N., Contreras, A., Flynn, J., Slots, J. & Chen, C. (2000) Molecular identification of Dialister pneumosintes in subgingival plaque of humans. *J Clin Microbiol* **38,** 3043-3047.

Dominguez-Bello, M. G., Costello, E. K., Contreras, M., Magris, M., Hidalgo, G., Fierer, N. & Knight, R. (2010) Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. *Proc Natl Acad Sci U S A* **107,** 11971-11975. doi:10.1073/pnas.1002601107.

Dridi, B., Raoult, D. & Drancourt, M. (2011) Archaea as emerging organisms in complex human microbiomes. *Anaerobe* **17,** 56-63. doi:10.1016/j.anaerobe.2011.03.001.

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C. & Knight, R. (2011) UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* **27,** 2194-2200. doi:10.1093/bioinformatics/btr381.

Eick, S., Gloor, N., Puls, C., Zumbrunn, J. & Sculean, A. (2016) In vitro activity of taurolidine gel on bacteria associated with periodontitis. *Clin Oral Investig* **20,** 597-606. doi:10.1007/s00784-015-1549-6.

Eick, S., Markauskaite, G., Nietzsche, S., Laugisch, O., Salvi, G. E. & Sculean, A. (2013) Effect of photoactivated disinfection with a light-emitting diode on bacterial species and biofilms associated with periodontitis and peri-implantitis. *Photodiagnosis Photodyn Ther* **10,** 156-167. doi:10.1016/j.pdpdt.2012.12.001.

Eick, S., Radakovic, S., Pfister, W., Nietzsche, S. & Sculean, A. (2012) Efficacy of taurolidine against periodontopathic species--an in vitro study. *Clin Oral Investig* **16,** 735-744. doi:10.1007/s00784-011-0567-2.

Eley, B. M. & Cox, S. W. (2003) Proteolytic and hydrolytic enzymes from putative periodontal pathogens: characterization, molecular genetics, effects on host defenses and tissues and detection in gingival crevice fluid. *Periodontol 2000* **31,** 105-124.

Esposito, M., Ardebili, Y. & Worthington, H. V. (2014) Interventions for replacing missing teeth: different types of dental implants. *Cochrane Database Syst Rev* **7,** Cd003815. doi:10.1002/14651858.CD003815.pub4.

Exterkate, R. A., Crielaard, W. & ten Cate, J. M. (2010) Different response to amine fluoride by *Streptococcus mutans* and polymicrobial biofilms in a novel high-throughput active attachment model. *Caries Res* **44,** 372-379. doi:10.1159/000316541.

Exterkate, R. A., Zaura, E., Buijs, M. J., Koopman, J., Crielaard, W. & ten Cate, J. M. (2014) The effects of propidium monoazide treatment on the measured composition of polymicrobial biofilms after treatment with chlorhexidine. *Caries Res* **48,** 291-298. doi:10.1159/000356869.

Faveri, M., Mayer, M. P., Feres, M., de Figueiredo, L. C., Dewhirst, F. E. & Paster, B. J. (2008) Microbiological diversity of generalized aggressive periodontitis by 16S rRNA clonal analysis. *Oral Microbiol Immunol* **23,** 112-118. doi:OMI397 [pii] 10.1111/j.1399-302X.2007.00397.x.

Feiz, A., Barekatain, B., Khalesi, S., Khalighinejad, N., Badrian, H. & Swift, E. J. (2014) Effect of several bleaching agents on teeth stained with a resin-based sealer. *Int Endod J* **47,** 3-9. doi:10.1111/iej.12116.

Fernandez y Mostajo, M., van der Reijden, W. A., Buijs, M. J., Beertsen, W., van der Weijden, F., Crielaard, W. & Zaura, E. (2014) Effect of an oxygenating agent on oral bacteria in vitro and on dental plaque composition in healthy young adults. *Front Cell Infect Microbiol* **4,** 95. doi:10.3389/fcimb.2014.00095.

Fernandez y Mostajo, M., Zaura, E., Crielaard, W. & Beertsen, W. (2011) Does routine analysis of subgingival microbiota in periodontitis contribute to patient benefit? *Eur J Oral Sci* **119,** 259-264. doi:10.1111/j.1600-0722.2011.00828.x.

Fernandez, Y. Mostajo, M., Exterkate, R. A., Buijs, M. J., Crielaard, W. & Zaura, E. (2016) Effect of mouthwashes on the composition and metabolic activity of oral biofilms grown in vitro. *Clin Oral Investig*. doi:10.1007/s00784-016-1876-2.

Filoche, S. K., Soma, D., van Bekkum, M. & Sissons, C. H. (2008) Plaques from different individuals yield different microbiota responses to oral-antiseptic treatment. *FEMS Immunol Med Microbiol* **54,** 27-36. doi:10.1111/j.1574-695X.2008.00443.x.

Fine, D. H., Markowitz, K., Fairlie, K., Tischio-Bereski, D., Ferrendiz, J., Furgang, D., Paster, B. J. & Dewhirst, F. E. (2013) A consortium of *Aggregatibacter actinomycetemcomitans, Streptococcus parasanguinis*, and *Filifactor alocis is* present in sites prior to bone loss in a longitudinal study of localized aggressive periodontitis. *J Clin Microbiol* **51,** 2850-2861. doi:10.1128/jcm.00729-13.

Flemmig, T. F., Milian, E., Karch, H. & Klaiber, B. (1998) Differential clinical treatment outcome after systemic metronidazole and amoxicillin in patients harboring Actinobacillus actinomycetemcomitans and/or *Porphyromonas gingivalis*. *J Clin Periodontol* **25,** 380-387.

Fransson, C., Mooney, J., Kinane, D. F. & Berglundh, T. (1999) Differences in the inflammatory response in young and old human subjects during the course of experimental gingivitis. *J Clin Periodontol* **26,** 453-460.

Friedrich, M. J. (2008) Microbiome project seeks to understand human body's microscopic residents. *JAMA* **300,** 777-778.

Gallagher, I. H. & Cutress, T. W. (1977) Clinical trial in mentally retarded of chlorhexidine dental gel: bacteriology. *Community Dent Oral Epidemiol* **5,** 1-6.

Ge, Y., Caufield, P. W., Fisch, G. S. & Li, Y. (2008) *Streptococcus mutans* and *Streptococcus sanguinis* colonization correlated with caries experience in children. *Caries Res* **42,** 444-448. doi:10.1159/000159608.

Gerardu, V. A., Buijs, M. J., ten Cate, J. M. & van Loveren, C. (2003) The effect of a single application of 40% chlorhexidine varnish on the numbers of salivary mutans streptococci and acidogenicity of dental plaque. *Caries Res* **37,** 369-373. doi:72170.

Ghannoum, M. A., Jurevic, R. J., Mukherjee, P. K., Cui, F., Sikaroodi, M., Naqvi, A. & Gillevet, P. M. (2010) Characterization of the oral fungal microbiome (mycobiome) in healthy individuals. *PLoS Pathog* **6,** e1000713. doi:10.1371/journal.ppat.1000713.

Ghayoumi, N., Chen, C. & Slots, J. (2002) Dialister pneumosintes, a new putative periodontal pathogen. *J Periodontal Res* **37,** 75-78.

Gosau, M., Hahnel, S., Schwarz, F., Gerlach, T., Reichert, T. E. & Burgers, R. (2010) Effect of six different peri-implantitis disinfection methods on *in vivo* human oral biofilm. *Clin Oral Implants Res* **21,** 866-872. doi:10.1111/j.1600-0501.2009.01908.x.

Griffen, A. L., Beall, C. J., Campbell, J. H., Firestone, N. D., Kumar, P. S., Yang, Z. K., Podar, M. & Leys, E. J. (2012) Distinct and complex bacterial profiles in human periodontitis and health revealed by 16S pyrosequencing. *ISME J* **6,** 1176-1185. doi:10.1038/ismej.2011.191.

Griffen, A. L., Beall, C. J., Firestone, N. D., Gross, E. L., Difranco, J. M., Hardman, J. H., Vriesendorp, B., Faust, R. A., Janies, D. A. & Leys, E. J. (2011) CORE: a phylogenetically-

curated 16S rDNA database of the core oral microbiome. *PLoS One* **6**. doi:10.1371/journal.pone.0019051.

Griffiths, G. S., Ayob, R., Guerrero, A., Nibali, L., Suvan, J., Moles, D. R. & Tonetti, M. S. (2011) Amoxicillin and metronidazole as an adjunctive treatment in generalized aggressive periodontitis at initial therapy or re-treatment: a randomized controlled clinical trial. *J Clin Periodontol* **38,** 43-49. doi:10.1111/j.1600-051X.2010.01632.x.

Gross, E. L., Leys, E. J., Gasparovich, S. R., Firestone, N. D., Schwartzbaum, J. A., Janies, D. A., Asnani, K. & Griffen, A. L. (2010) Bacterial 16S sequence analysis of severe caries in young permanent teeth. *J Clin Microbiol* **48**. doi:10.1128/jcm.01232-10.

Grundemann, L. J., Timmerman, M. F., IJzerman, Y. & van der Weijden, G. A. (2000) Stain, plaque and gingivitis reduction by combining chlorhexidine and peroxyborate. *J Clin Periodontol* **27,** 9-15.

Guerrero, A., Griffiths, G. S., Nibali, L., Suvan, J., Moles, D. R., Laurell, L. & Tonetti, M. S. (2005) Adjunctive benefits of systemic amoxicillin and metronidazole in non-surgical treatment of generalized aggressive periodontitis: a randomized placebo-controlled clinical trial. *J Clin Periodontol* **32,** 1096-1107.

Guggenheim, B., Gmur, R., Galicia, J. C., Stathopoulou, P. G., Benakanakere, M. R., Meier, A., Thurnheer, T. & Kinane, D. F. (2009) In vitro modeling of host-parasite interactions: the 'subgingival' biofilm challenge of primary human epithelial cells. *BMC Microbiol* **9,** 280. doi:10.1186/1471-2180-9-280.

Gurgan, C. A., Zaim, E., Bakirsoy, I. & Soykan, E. (2006) Short-term side effects of 0.2% alcohol-free chlorhexidine mouthrinse used as an adjunct to non-surgical periodontal treatment: a double-blind clinical study. *J Periodontol* **77,** 370-384. doi:10.1902/jop.2006.050141.

Haffajee, A. D., Teles, R. P., Patel, M. R., Song, X., Yaskell, T. & Socransky, S. S. (2009) Factors affecting human supragingival biofilm composition. II. Tooth position. *J Periodontal Res* **44,** 520-528.

Haffajee, A. D., Teles, R. P. & Socransky, S. S. (2006) The effect of periodontal therapy on the composition of the subgingival microbiota. *Periodontol 2000* **42,** 219-258.

Hajishengallis, G. (2015) Periodontitis: from microbial immune subversion to systemic inflammation. *Nat Rev Immunol* **15,** 30-44. doi:10.1038/nri3785.

Hajishengallis, G. & Lamont, R. J. (2012) Beyond the red complex and into more complexity: the polymicrobial synergy and dysbiosis (PSD) model of periodontal disease etiology. *Mol Oral Microbiol* **27,** 409-419. doi:10.1111/j.2041-1014.2012.00663.x.

Hall-Stoodley, L., Costerton, J. W. & Stoodley, P. (2004) Bacterial biofilms: from the natural environment to infectious diseases. *Nat Rev Microbiol* **2,** 95-108. doi:10.1038/nrmicro821.

Hammer, Ø., Harper,D.A.T., & Ryan,P.D. (2001b) PAST: paleontological statistics software package for education and data analysis. Palaeontologia Electron. **4**, 1–9.

Hart, T. C., Corby, P. M., Hauskrecht, M., Hee Ryu, O., Pelikan, R., Valko, M., Oliveira, M. B., Hoehn, G. T. & Bretz, W. A. (2011) Identification of microbial and proteomic

biomarkers in early childhood caries. *Int J Dent* 2011**,** 196721. doi:10.1155/2011/196721.

He, X., McLean, J. S., Edlund, A., Yooseph, S., Hall, A. P., Liu, S. Y., Dorrestein, P. C., Esquenazi, E., Hunter, R. C., Cheng, G., Nelson, K. E., Lux, R. & Shi, W. (2015) Cultivation of a human-associated TM7 phylotype reveals a reduced genome and epibiotic parasitic lifestyle. *Proc Natl Acad Sci U S A* **112,** 244-249. doi:10.1073/pnas.1419038112.

Hecht, D. W. (2007) *Methods for Antimicobial Susceptibility Testing of Anaerobic Bacteria: Approved Standard*. Clinical & Laboratory Standards Institute.

Heijnsbroek, M., Gerardu, V. A., Buijs, M. J., van Loveren, C., ten Cate, J. M., Timmerman, M. F. & van der Weijden, G. A. (2006) Increased salivary fluoride concentrations after post-brush fluoride rinsing not reflected in dental plaque. *Caries Res* **40,** 444-448. doi:10.1159/000094292.

Heinrich-Weltzien, R., Kuhnisch, J., van der Veen, M., de Josselin de Jong, E. & Stosser, L. (2003) Quantitative light-induced fluorescence (QLF)--a potential method for the dental practitioner. *Quintessence Int* **34,** 181-188.

Hernandez, V., Crepin, T., Palencia, A., Cusack, S., Akama, T., Baker, S. J., Bu, W., Feng, L., Freund, Y. R., Liu, L., Meewan, M., Mohan, M., Mao, W., Rock, F. L., Sexton, H., Sheoran, A., Zhang, Y., Zhang, Y. K., Zhou, Y., Nieman, J. A., Anugula, M. R., Keramane el, M., Savariraj, K., Reddy, D. S., Sharma, R., Subedi, R., Singh, R., O'Leary, A., Simon, N. L., De Marsh, P. L., Mushtaq, S., Warner, M., Livermore, D. M., Alley, M. R. & Plattner, J. J. (2013a) Discovery of a novel class of boron-based antibacterials with activity against gram-negative bacteria. *Antimicrob Agents Chemother* **57,** 1394-1403. doi:10.1128/aac.02058-12.

Hernandez, V., Crepin, T., Palencia, A., Cusack, S., Akama, T., Baker, S. J., Bu, W., Feng, L., Freund, Y. R., Liu, L., Meewan, M., Mohan, M., Mao, W., Rock, F. L., Sexton, H., Sheoran, A., Zhang, Y., Zhang, Y. K., Zhou, Y., Nieman, J. A., Anugula, M. R., Keramane, E. M., Savariraj, K., Reddy, D. S., Sharma, R., Subedi, R., Singh, R., O'Leary, A., Simon, N. L., De Marsh, P. L., Mushtaq, S., Warner, M., Livermore, D. M., Alley, M. R. & Plattner, J. J. (2013b) Discovery of a Novel Class of Boron-based Antibacterials with Activity against Gram-negative Bacteria. *Antimicrob Agents Chemother* 1394 – 1403. doi:10.1128/AAC.02058-12.

Herrera, D., Alonso, B., Leon, R., Roldan, S. & Sanz, M. (2008) Antimicrobial therapy in periodontitis: the use of systemic antimicrobials against the subgingival biofilm. *J Clin Periodontol* **35,** 45-66.

Hezel, M. & Weitzberg, E. (2013) The oral microbiome and nitric oxide homoeostasis. *Oral Dis* **28,** 12157.

Hillam, D. G. & Hull, P. S. (1977) The influence of experimental gingivitis on plaque formation. *J Clin Periodontol* **4,** 56-61.

Hioe, K. P. & van der Weijden, G. A. (2005) The effectiveness of self-performed mechanical plaque control with triclosan containing dentifrices. *Int J Dent Hyg* **3,** 192-204. doi:10.1111/j.1601-5037.2005.00150.x.

Hope, C. K. & Wilson, M. (2006) Biofilm structure and cell vitality in a laboratory model of subgingival plaque. *J Microbiol Methods* **66,** 390-398. doi:10.1016/j.mimet.2006.01.003.

Horz, H. P. & Conrads, G. (2010) The discussion goes on: What is the role of Euryarchaeota in humans? *Archaea* **2010,** 967271. doi:10.1155/2010/967271.

Hossainian, N., Slot, D. E., Afennich, F. & Van der Weijden, G. A. (2011) The effects of hydrogen peroxide mouthwashes on the prevention of plaque and gingival inflammation: a systematic review. *Int J Dent Hyg* **9,** 171-181. doi:10.1111/j.1601-5037.2010.00492.x.

Huang, R., Li, M. & Gregory, R. L. (2011a) Bacterial interactions in dental biofilm. *Virulence* **2,** 435-444. doi:10.4161/viru.2.5.16140.

Huang, S., Li, R., Zeng, X., He, T., Zhao, H., Chang, A., Bo, C., Chen, J., Yang, F., Knight, R., Liu, J., Davis, C. & Xu, J. (2014) Predictive modeling of gingivitis severity and susceptibility via oral microbiota. *ISME J* **8,** 1768-1780. doi:10.1038/ismej.2014.32.

Huang, S., Yang, F., Zeng, X., Chen, J., Li, R., Wen, T., Li, C., Wei, W., Liu, J., Chen, L., Davis, C. & Xu, J. (2011b) Preliminary characterization of the oral microbiota of Chinese adults with and without gingivitis. *BMC Oral Health* **11,** 1472-6831.

Hujoel, P. P., White, B. A., Garcia, R. I. & Listgarten, M. A. (2001) The dentogingival epithelial surface area revisited. *J Periodontal Res* **36,** 48-55.

Hutter, G., Schlagenhauf, U., Valenza, G., Horn, M., Burgemeister, S., Claus, H. & Vogel, U. (2003) Molecular analysis of bacteria in periodontitis: evaluation of clone libraries, novel phylotypes and putative pathogens. *Microbiology* **149,** 67-75.

Iniesta, M., Herrera, D., Montero, E., Zurbriggen, M., Matos, A. R., Marin, M. J., Sanchez-Beltran, M. C., Llama-Palacio, A. & Sanz, M. (2012) Probiotic effects of orally administered Lactobacillus reuteri-containing tablets on the subgingival and salivary microbiota in patients with gingivitis. A randomized clinical trial. *J Clin Periodontol* **39,** 736-744. doi:10.1111/j.1600-051X.2012.01914.x.

Iwano, Y., Sugano, N., Matsumoto, K., Nishihara, R., Iizuka, T., Yoshinuma, N. & Ito, K. (2010) Salivary microbial levels in relation to periodontal status and caries development. *J Periodontal Res* **45,** 165-169. doi:10.1111/j.1600-0765.2009.01213.x.

Jepsen, S., Berglundh, T., Genco, R., Aass, A. M., Demirel, K., Derks, J., Figuero, E., Giovannoli, J. L., Goldstein, M., Lambert, F., Ortiz-Vigon, A., Polyzois, I., Salvi, G. E., Schwarz, F., Serino, G., Tomasi, C. & Zitzmann, N. U. (2015) Primary prevention of peri-implantitis: managing peri-implant mucositis. *J Clin Periodontol* **42** Suppl 16**,** S152-157. doi:10.1111/jcpe.12369.

John, G. H. (1984) *Bergey's Manual of Systematic Bacteriology*. Baltimore, MD: Williams & Wilkins.

Jones, C. G. (1997) Chlorhexidine: is it still the gold standard? *Periodontol 2000* **15,** 55-62.

Kamma, J. J., Nakou, M. & Manti, F. A. (1995) Predominant microflora of severe, moderate and minimal periodontal lesions in young adults with rapidly progressive periodontitis. *J Periodontal Res* **30,** 66-72.

Katayama, T., Suzuki, T. & Okada, S. (1975) Clinical observation of dental plaque maturation. Application of oxidation-reduction indicator dyes. *J Periodontol* **46,** 610-613. doi:10.1902/jop.1975.46.10.610.

Keijser, B. J., Zaura, E., Huse, S. M., van der Vossen, J. M., Schuren, F. H., Montijn, R. C., ten Cate, J. M. & Crielaard, W. (2008) Pyrosequencing analysis of the oral microflora of healthy adults. *J Dent Res* **87,** 1016-1020.

Keijser, J. A., Verkade, H., Timmerman, M. F. & Van der Weijden, F. A. (2003) Comparison of 2 commercially available chlorhexidine mouthrinses. *J Periodontol* **74,** 214-218. doi:10.1902/jop.2003.74.2.214.

Keller, L. & Surette, M. G. (2006) Communication in bacteria: an ecological and evolutionary perspective. *Nat Rev Microbiol* **4,** 249-258. doi:10.1038/nrmicro1383.

Keukenmeester, R., Slot, D., Rosema, N., Van Loveren, C. & Van der Weijden, G. (2014) Effects of sugar-free chewing gum sweetened with xylitol or maltitol on the development of gingivitis and plaque: a randomized clinical trial. *Int J Dent Hyg*. **12**, 238-44. doi:10.1111/idh.12071.

Kim, Y. S., Lee, E. S., Kwon, H. K. & Kim, B. I. (2014) Monitoring the maturation process of a dental microcosm biofilm using the Quantitative Light-induced Fluorescence-Digital (QLF-D). *J Dent* **42,** 691-696. doi:10.1016/j.jdent.2014.03.006.

Kistler, J. O., Booth, V., Bradshaw, D. J. & Wade, W. G. (2013) Bacterial community development in experimental gingivitis. *PLoS One* **8,** e71227. doi:10.1371/journal.pone.0071227.

Kistler, J. O., Pesaro, M. & Wade, W. G. (2015) Development and pyrosequencing analysis of an in-vitro oral biofilm model. *BMC Microbiol* **15,** 24. doi:10.1186/s12866-015-0364-1.

Knight, E. T., Liu, J., Seymour, G. J., Faggion, C. M., Jr. & Cullinan, M. P. (2016) Risk factors that may modify the innate and adaptive immune responses in periodontal diseases. *Periodontol 2000* **71,** 22-51. doi:10.1111/prd.12110.

Kolenbrander, P. E., Palmer, R. J., Jr., Periasamy, S. & Jakubovics, N. S. (2010) Oral multispecies biofilm development and the key role of cell-cell distance. *Nat Rev Microbiol* **8,** 471-480. doi:10.1038/nrmicro2381.

Koll-Klais, P., Mandar, R., Leibur, E., Marcotte, H., Hammarstrom, L. & Mikelsaar, M. (2005a) Oral lactobacilli in chronic periodontitis and periodontal health: species composition and antimicrobial activity. *Oral Microbiol Immunol* **20,** 354-361. doi:10.1111/j.1399-302X.2005.00239.x.

Koll-Klais, P., Mandar, R., Leibur, E. & Mikelsaar, M. (2005b) Oral microbial ecology in chronic periodontitis and periodontal health. *Microbial Ecology in Health and Disease* **17,** 146-155.

Kononen, E. (2000) Development of oral bacterial flora in young children. *Ann Med* **32,** 107-112.

Konopka, A. (2009) What is microbial community ecology? *ISME J* **3,** 1223-1230. doi:10.1038/ismej.2009.88.

Koopman, J. E., Roling, W. F., Buijs, M. J., Sissons, C. H., Ten Cate, J. M., Keijser, B. J., Crielaard, W. & Zaura, E. (2015) Stability and Resilience of Oral Microcosms Toward Acidification and Candida Outgrowth by Arginine Supplementation. *Microb Ecol*. **69**, 422-433. doi:10.1007/s00248-014-0535-x

Kraneveld, E. A., Buijs, M. J., Bonder, M. J., Visser, M., Keijser, B. J., Crielaard, W. & Zaura, E. (2012) The relation between oral Candida load and bacterial microbiome profiles in Dutch older adults. *PLoS One* **7,** 10.

Kroes, I., Lepp, P. W. & Relman, D. A. (1999) Bacterial diversity within the human subgingival crevice. *Proc Natl Acad Sci U S A* **96,** 14547-14552.

Kuboniwa, M., Inaba, H. & Amano, A. (2010) Genotyping to distinguish microbial pathogenicity in periodontitis. *Periodontol 2000* **54,** 136-159.

Kuboniwa, M. & Lamont, R. J. (2010) Subgingival biofilm formation. *Periodontol 2000* **52,** 38-52.

Kulik, E. M., Sandmeier, H., Hinni, K. & Meyer, J. (2001) Identification of archaeal rDNA from subgingival dental plaque by PCR amplification and sequence analysis. *FEMS Microbiol Lett* **196,** 129-133.

Kumar, P. S., Griffen, A. L., Barton, J. A., Paster, B. J., Moeschberger, M. L. & Leys, E. J. (2003) New bacterial species associated with chronic periodontitis. *J Dent Res* **82,** 338-344.

Kumar, P. S., Griffen, A. L., Moeschberger, M. L. & Leys, E. J. (2005) Identification of candidate periodontal pathogens and beneficial species by quantitative 16S clonal analysis. *J Clin Microbiol* **43,** 3944-3955.

Kumar, P. S., Mason, M. R., Brooker, M. R. & O'Brien, K. (2012) Pyrosequencing reveals unique microbial signatures associated with healthy and failing dental implants. *J Clin Periodontol* **39,** 425-433. doi:10.1111/j.1600-051X.2012.01856.x.

Lamont, R. J. & Hajishengallis, G. (2015) Polymicrobial synergy and dysbiosis in inflammatory disease. *Trends Mol Med*. **21**, 172 - 83. doi:10.1016/j.molmed.2014.11.004.

Lang, N. P., Schatzle, M. A. & Loe, H. (2009) Gingivitis as a risk factor in periodontal disease. *J Clin Periodontol* **36 Suppl 10,** 3-8. doi:10.1111/j.1600-051X.2009.01415.x.

Lang, N. P., Suvan, J. E. & Tonetti, M. S. (2015) Risk factor assessment tools for the prevention of periodontitis progression a systematic review. *J Clin Periodontol* **42** Suppl 16**,** 59-70. doi:10.1111/jcpe.12350.

Lazarevic, V., Whiteson, K., Hernandez, D., Francois, P. & Schrenzel, J. (2010) Study of inter- and intra-individual variations in the salivary microbiota. *BMC Genomics* **11,** 523. doi:10.1186/1471-2164-11-523.

Ledder, R. G., Gilbert, P., Huws, S. A., Aarons, L., Ashley, M. P., Hull, P. S. & McBain, A. J. (2007) Molecular analysis of the subgingival microbiota in health and disease. *Appl Environ Microbiol* **73,** 516-523.

Ledder, R. G., Gilbert, P., Pluen, A., Sreenivasan, P. K., De Vizio, W. & McBain, A. J. (2006) Individual microflora beget unique oral microcosms. *J Appl Microbiol* **100,** 1123-1131. doi:10.1111/j.1365-2672.2006.02847.x.

Ledder, R. G., Latimer, J., Humphreys, G. J., Sreenivasan, P. K. & McBain, A. J. (2014) Bacteriological effects of dentifrices with and without active ingredients of natural origin. *Appl Environ Microbiol* **80,** 6490-6498. doi:10.1128/aem.02315-14.

Lederberg, J.& Mccray, A. T. (2001) 'Ome sweet 'omics—a genealogical treasury of words *Scientist* **15,** 8-10.

Lee, A., Ghaname, C. B., Braun, T. M., Sugai, J. V., Teles, R. P., Loesche, W. J., Kornman, K. S., Giannobile, W. V. & Kinney, J. S. (2012) Bacterial and salivary biomarkers predict the gingival inflammatory profile. *J Periodontol* **83,** 79-89. doi:10.1902/jop.2011.110060.

Lee, E. S., Kang, S. M., Ko, H. Y., Kwon, H. K. & Kim, B. I. (2013) Association between the cariogenicity of a dental microcosm biofilm and its red fluorescence detected by Quantitative Light-induced Fluorescence-Digital (QLF-D). *J Dent* **41,** 1264-1270. doi:10.1016/j.jdent.2013.08.021.

Lennon, A. M., Buchalla, W., Rassner, B., Becker, K. & Attin, T. (2006a) Efficiency of 4 caries excavation methods compared. *Oper Dent* **31,** 551-555. doi:10.2341/05-92.

Lepp, P. W., Brinig, M. M., Ouverney, C. C., Palm, K., Armitage, G. C. & Relman, D. A. (2004) Methanogenic Archaea and human periodontal disease. *Proc Natl Acad Sci U S A* **101,** 6176-6181. doi:10.1073/pnas.0308766101 [pii].

Leys, E. J., Lyons, S. R., Moeschberger, M. L., Rumpf, R. W. & Griffen, A. L. (2002) Association of Bacteroides forsythus and a novel Bacteroides phylotype with periodontitis. *J Clin Microbiol* **40,** 821-825.

Li, C. L., Liu, D. L., Jiang, Y. T., Zhou, Y. B., Zhang, M. Z., Jiang, W., Liu, B. & Liang, J. P. (2009) Prevalence and molecular diversity of Archaea in subgingival pockets of periodontitis patients. *Oral Microbiol Immunol* **24,** 343-346.

Li, J., Helmerhorst, E. J., Leone, C. W., Troxler, R. F., Yaskell, T., Haffajee, A. D., Socransky, S. S. & Oppenheim, F. G. (2004) Identification of early microbial colonizers in human dental biofilm. *J Appl Microbiol* **97,** 1311-1318. doi:10.1111/j.1365-2672.2004.02420.x.

Li, Y., He, J., He, Z., Zhou, Y., Yuan, M., Xu, X., Sun, F., Liu, C., Li, J., Xie, W., Deng, Y., Qin, Y., VanNostrand, J. D., Xiao, L., Wu, L., Zhou, J., Shi, W. & Zhou, X. (2014) Phylogenetic and functional gene structure shifts of the oral microbiomes in periodontitis patients. *ISME J* **8,** 1879-1891. doi:10.1038/ismej.2014.28.

Lie, M. A., Danser, M. M., van der Weijden, G. A., Timmerman, M. F., de Graaff, J. & van der Velden, U. (1995) Oral microbiota in subjects with a weak or strong response in experimental gingivitis. *J Clin Periodontol* **22,** 642-647.

Lie, M. A., Timmerman, M. F., van der Velden, U. & van der Weijden, G. A. (1998) Evaluation of 2 methods to assess gingival bleeding in smokers and non-smokers in natural and experimental gingivitis. *J Clin Periodontol* **25,** 695-700.

Lillo, A. d., Ashley, F. P., Palmer, R. M., Munson, M. A., Kyriacou, L., Weightman, A. J. & Wade, W. G. (2006) Novel subgingival bacterial phylotypes detected using multiple universal polymerase chain reaction primer sets. *Oral Microbiol Immunol* **21,** 61-68. doi:10.1111/j.1399-302X.2005.00255.x.

Lillo, A. d., Booth, V., Kyriacou, L., Weightman, A. J. & Wade, W. G. (2004) Culture-independent identification of periodontitis-associated *Porphyromonas* and *Tannerella* populations by targeted molecular analysis. *J Clin Microbiol* **42,** 5523-5527. doi:10.1128/JCM.42.12.5523-5527.2004.

Listgarten, M. A. (1976) Structure of the microbial flora associated with periodontal health and disease in man. A light and electron microscopic study. *J Periodontol* **47,** 1-18.

Listgarten, M. A. & Loomer, P. M. (2003) Microbial identification in the management of periodontal diseases. A systematic review. *Ann Periodontol* **8,** 182-192.

Lobene, R. R., Soparkar, P. M. & Newman, M. B. (1982) Use of dental floss. Effect on plaque and gingivitis. *Clin Prev Dent* **4,** 5-8.

Loe, H., Theilade, E. & Jensen, S. B. (1965) Experimental gingivitis in man. *J Periodontol* **36,** 177-187. doi:10.1902/jop.1965.36.3.177.

Lourenco, T. G., Heller, D., Silva-Boghossian, C. M., Cotton, S. L., Paster, B. J. & Colombo, A. P. (2014) Microbial signature profiles of periodontally healthy and diseased patients. *J Clin Periodontol* **41,** 1027-1036. doi:10.1111/jcpe.12302.

Louropoulou, A., Slot, D. E. & Van der Weijden, F. (2014) Mechanical self-performed oral hygiene of implant supported restorations: a systematic review. *J Evid Based Dent Pract* 14 Suppl**,** 60-69.e61. doi:10.1016/j.jebdp.2014.03.008.

Love, R. M. & Jenkinson, H. F. (2002) Invasion of dentinal tubules by oral bacteria. *Crit Rev Oral Biol Med* **13,** 171-183.

Macuch, P. J. & Tanner, A. C. (2000) Campylobacter species in health, gingivitis, and periodontitis. *J Dent Res* **79,** 785-792.

Magnusson, I., Lindhe, J., Yoneyama, T. & Liljenberg, B. (1984) Recolonization of a subgingival microbiota following scaling in deep pockets. *J Clin Periodontol* **11,** 193-207.

Mantilla Gomez, S., Danser, M. M., Sipos, P. M., Rowshani, B., van der Velden, U. & van der Weijden, G. A. (2001) Tongue coating and salivary bacterial counts in healthy/gingivitis subjects and periodontitis patients. *J Clin Periodontol* **28,** 970-978.

Marchant, S., Brailsford, S. R., Twomey, A. C., Roberts, G. J. & Beighton, D. (2001) The predominant microflora of nursing caries lesions. *Caries Res* **35,** 397-406.

Marcy, Y., Ouverney, C., Bik, E. M., Losekann, T., Ivanova, N., Martin, H. G., Szeto, E., Platt, D., Hugenholtz, P., Relman, D. A. & Quake, S. R. (2007) Dissecting biological "dark matter" with single-cell genetic analysis of rare and uncultivated TM7 microbes from the human mouth. *Proc Natl Acad Sci USA* **104**. doi:10.1073/pnas.0704662104.

Marsh, P. (2004) Dental plaque as a microbial biofilm. *Caries Res* **38**. doi:10.1159/000077756.

Marsh, P. & Martin, M. (2009) *Oral microbiology*. Edinburgh ; New York: Churchill Livingstone/Elsevier, pp. ix, 222 p.

Marsh, P. D. (1994) Microbial ecology of dental plaque and its significance in health and disease. *Adv Dent Res* **8,** 263-271.

Marsh, P. D. (2005) Dental plaque: biological significance of a biofilm and community life-style. *J Clin Periodontol* 32 Suppl 6**,** 7-15. doi:10.1111/j.1600-051X.2005.00790.x.

Marsh, P. D. (2012) Contemporary perspective on plaque control. *Br Dent J* **212,** 601-606. doi:10.1038/sj.bdj.2012.524.

Marsh, P. D. & Devine, D. A. (2011) How is the development of dental biofilms influenced by the host? *J Clin Periodontol* 38 Suppl 11**,** 28-35. doi:10.1111/j.1600-051X.2010.01673.x.

Marsh, P. D., Moter, A. & Devine, D. A. (2011b) Dental plaque biofilms: communities, conflict and control. *Periodontol 2000* **55**. doi:10.1111/j.1600-0757.2009.00339.x.

Matarazzo, F., Figueiredo, L. C., Cruz, S. E., Faveri, M. & Feres, M. (2008) Clinical and microbiological benefits of systemic metronidazole and amoxicillin in the treatment of smokers with chronic periodontitis: a randomized placebo-controlled study. *J Clin Periodontol* **35,** 885-896.

Matarazzo, F., Ribeiro, A. C., Feres, M., Faveri, M. & Mayer, M. P. (2011) Diversity and quantitative analysis of Archaea in aggressive periodontitis and periodontally healthy subjects. *J Clin Periodontol* **38,** 621-627. doi:10.1111/j.1600-051X.2011.01734.x.

McBain, A. J., Bartolo, R. G., Catrenich, C. E., Charbonneau, D., Ledder, R. G. & Gilbert, P. (2003) Growth and molecular characterization of dental plaque microcosms. *J Appl Microbiol* **94,** 655-664.

McBain, A. J., Sissons, C., Ledder, R. G., Sreenivasan, P. K., De Vizio, W. & Gilbert, P. (2005) Development and characterization of a simple perfused oral microcosm. *J Appl Microbiol* **98,** 624-634. doi:10.1111/j.1365-2672.2004.02483.x.

Mestnik, M. J., Feres, M., Figueiredo, L. C., Duarte, P. M., Lira, E. A. & Faveri, M. (2010) Short-term benefits of the adjunctive use of metronidazole plus amoxicillin in the microbial profile and in the clinical parameters of subjects with generalized aggressive periodontitis. *J Clin Periodontol* **37,** 353-365.

Meyle, J. & Chapple, I. (2015) Molecular aspects of the pathogenesis of periodontitis. *Periodontol 2000* **69,** 7-17. doi:10.1111/prd.12104.

Mombelli, A., Casagni, F. & Madianos, P. N. (2002) Can presence or absence of periodontal pathogens distinguish between subjects with chronic and aggressive periodontitis? A systematic review. *J Clin Periodontol* **29** Suppl 3**,** 10-21; discussion 37-18.

Mombelli, A., Cionca, N. & Almaghlouth, A. (2011) Does adjunctive antimicrobial therapy reduce the perceived need for periodontal surgery? *Periodontol 2000* **55,** 205-216.

Mombelli, A., McNabb, H. & Lang, N. P. (1993) Distribution patterns of black-pigmenting gram-negative bacteria in periodontitis patients. *FEMS Immunol Med Microbiol* **6,** 199.

Moore, W. E. & Moore, L. V. (1994) The bacteria of periodontal diseases. *Periodontol 2000* **5,** 66-77.

Moorer, W. R., Ten Cate, J. M. & Buijs, J. F. (1993) Calcification of a cariogenic *Streptococcus* and of *Corynebacterium* (Bacterionema) matruchotii. *J Dent Res* **72,** 1021-1026.

Moran, J., Addy, M., Wade, W., Milson, S., McAndrew, R. & Newcombe, R. G. (1995) The effect of oxidising mouthrinses compared with chlorhexidine on salivary bacterial counts and plaque regrowth. *J Clin Periodontol* **22,** 750-755.

Mouratidou, A., Karbach, J., d'Hoedt, B. & Al-Nawas, B. (2011) Antibiotic susceptibility of cocultures in polymicrobial infections such as peri-implantitis or periodontitis: an *in vitro* model. *J Periodontol* **82,** 1360-1366. doi:10.1902/jop.2011.100657.

Nasidze, I., Li, J., Quinque, D., Tang, K. & Stoneking, M. (2009) Global diversity in the human salivary microbiome. *Genome Res* **19**. doi:10.1101/gr.084616.108.

Nasidze, I., Li, J., Schroeder, R., Creasey, J. L., Li, M. & Stoneking, M. (2011) High diversity of the saliva microbiome in Batwa Pygmies. *PLoS One* **6,** e23352. doi:10.1371/journal.pone.0023352.

Nocker, A., Cheung, C. Y. & Camper, A. K. (2006) Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. *J Microbiol Methods* **67,** 310-320. doi:10.1016/j.mimet.2006.04.015.

Ntrouka, V., Hoogenkamp, M., Zaura, E. & van der Weijden, F. (2011) The effect of chemotherapeutic agents on titanium-adherent biofilms. *Clin Oral Implants Res* **22,** 1227-1234. doi:10.1111/j.1600-0501.2010.02085.x.

Oliveira, S. C., Slot, D. E., Celeste, R. K., Abegg, C., Keijser, B. J. & Van der Weijden, F. A. (2015) Correlations between two different methods to score bleeding and the relationship with plaque in systemically healthy young adults. *J Clin Periodontol* **42,** 908-913. doi:10.1111/jcpe.12435.

Ouverney, C. C., Armitage, G. C. & Relman, D. A. (2003) Single-cell enumeration of an uncultivated TM7 subgroup in the human subgingival crevice. *Appl Environ Microbiol* **69,** 6294-6298.

Ozkavaf, A., Aras, H., Huri, C. B., Mottaghian-Dini, F., Tozum, T. F., Etikan, I., Yamalik, N. & Caglayan, F. (2000) Relationship between the quantity of gingival crevicular fluid and clinical periodontal status. *J Oral Sci* **42,** 231-238.

Page, R. C., Offenbacher, S., Schroeder, H. E., Seymour, G. J. & Kornman, K. S. (1997) Advances in the pathogenesis of periodontitis: summary of developments, clinical implications and future directions. *Periodontol 2000* **14,** 216-248.

Palmer R J, D. R., Lamont RJ, Nyvad B, & Teles RP (2011) Human Oral Bacterial Biofilms Composition, Dynamics, and Pathogenesis. In: *Biofilm Infections*, (ed.) J. P. Bjarnsholt T, Moser C, and Høiby N, pp. 35–68. New York: Springer.

Paraskevas, S., Danser, M. M., Timmerman, M. F., Van der Velden, U. & van der Weijden, G. A. (2005) Optimal rinsing time for intra-oral distribution (spread) of mouthwashes. *J Clin Periodontol* **32,** 665-669. doi:10.1111/j.1600-051X.2005.00731.x.

Paraskevas, S., Rosema, N. A., Versteeg, P., Timmerman, M. F., van der Velden, U. & van der Weijden, G. A. (2007) The additional effect of a dentifrice on the instant efficacy of toothbrushing: a crossover study. *J Periodontol* **78,** 1011-1016. doi:10.1902/jop.2007.060339.

Paster, B. J., Boches, S. K., Galvin, J. L., Ericson, R. E., Lau, C. N., Levanos, V. A., Sahasrabudhe, A. & Dewhirst, F. E. (2001) Bacterial diversity in human subgingival plaque. *J Bacteriol* **183,** 3770-3783. doi:10.1128/JB.183.12.3770-3783.2001.

Pavicic, M. J., van Winkelhoff, A. J. & de Graaff, J. (1992) *In vitro* susceptibilities of *Actinobacillus actinomycetemcomitans* to a number of antimicrobial combinations. *Antimicrob Agents Chemother* **36,** 2634-2638.

Pavicic, M. J., van Winkelhoff, A. J., Douque, N. H., Steures, R. W. & de Graaff, J. (1994) Microbiological and clinical effects of metronidazole and amoxicillin in *Actinobacillus actinomycetemcomitans*-associated periodontitis. A 2-year evaluation. *J Clin Periodontol* **21,** 107-112.

Periasamy, S. & Kolenbrander, P. E. (2010) Central role of the early colonizer *Veillonella* sp. in establishing multispecies biofilm communities with initial, middle, and late colonizers of enamel. *J Bacteriol* **192,** 2965-2972. doi:10.1128/JB.01631-09.

Peyyala, R., Kirakodu, S. S., Ebersole, J. L. & Novak, K. F. (2011) Novel model for multispecies biofilms that uses rigid gas-permeable lenses. *Appl Environ Microbiol* **77,** 3413-3421. doi:10.1128/aem.00039-11.

Peyyala, R., Kirakodu, S. S., Novak, K. F. & Ebersole, J. L. (2013) Oral epithelial cell responses to multispecies microbial biofilms. *J Dent Res* **92,** 235-240. doi:10.1177/0022034512472508.

Pollanen, M. T., Overman, D. O. & Salonen, J. I. (1997) Bacterial metabolites sodium butyrate and propionate inhibit epithelial cell growth in vitro. *J Periodontal Res* **32,** 326-334.

Pretty, I. A., Edgar, W. M., Smith, P. W. & Higham, S. M. (2005) Quantification of dental plaque in the research environment. *J Dent* **33,** 193-207. doi:10.1016/j.jdent.2004.10.017.

Preza, D., Olsen, I., Aas, J. A., Willumsen, T., Grinde, B. & Paster, B. J. (2008) Bacterial profiles of root caries in elderly patients. *J Clin Microbiol* **46,** 2015-2021. doi:JCM.02411-07 [pii]10.1128/JCM.02411-07.

Preza, D., Olsen, I., Willumsen, T., Boches, S. K., Cotton, S. L., Grinde, B. & Paster, B. J. (2009a) Microarray analysis of the microflora of root caries in elderly. *Eur J Clin Microbiol Infect Dis* **28,** 509-517. doi:10.1007/s10096-008-0662-8.

Preza, D., Olsen, I., Willumsen, T., Grinde, B. & Paster, B. J. (2009b) Diversity and site-specificity of the oral microflora in the elderly. *Eur J Clin Microbiol Infect Dis* **28,** 1033-1040. doi:10.1007/s10096-009-0743-3.

Qiqiang, L., Huanxin, M. & Xuejun, G. (2012) Longitudinal study of volatile fatty acids in the gingival crevicular fluid of patients with periodontitis before and after nonsurgical therapy. *J Periodontal Res* **47,** 740-749. doi:10.1111/j.1600-0765.2012.01489.x.

Quigley, G. A. & Hein, J. W. (1962) Comparative cleansing efficiency of manual and power brushing. *J Am Dent Assoc* **65,** 26-29.

Quirynen, M., Dekeyser, C. & van Steenberghe, D. (1991) The influence of gingival inflammation, tooth type, and timing on the rate of plaque formation. *J Periodontol* **62,** 219-222. doi:10.1902/jop.1991.62.3.219.

Quirynen, M. & van Steenberghe, D. (1989) Is early plaque growth rate constant with time? *J Clin Periodontol* **16,** 278-283.

Quirynen, M., Vogels, R., Peeters, W., van Steenberghe, D., Naert, I. & Haffajee, A. (2006) Dynamics of initial subgingival colonization of 'pristine' peri-implant pockets. *Clin Oral Implants Res* **17,** 25-37. doi:10.1111/j.1600-0501.2005.01194.x.

Raggio, D. P., Braga, M. M., Rodrigues, J. A., Freitas, P. M., Imparato, J. C. & Mendes, F. M. (2010) Reliability and discriminatory power of methods for dental plaque quantification. *J Appl Oral Sci* **18,** 186-193.

Raja, M., Hannan, A. & Ali, K. (2010) Association of oral candidal carriage with dental caries in children. *Caries Res* **44,** 272-276. doi:10.1159/000314675.

Rasiah, I. A., Wong, L., Anderson, S. A. & Sissons, C. H. (2005) Variation in bacterial DGGE patterns from human saliva: over time, between individuals and in corresponding dental plaque microcosms. *Arch Oral Biol* **50,** 779-787. doi:10.1016/j.archoralbio.2005.02.001.

Reeder, J. & Knight, R. (2010) Rapidly denoising pyrosequencing amplicon reads by exploiting rank-abundance distributions. *Nat Methods*.7(**9**):668-9. doi: 10.1038/nmeth0910-668b.

Renvert, S. & Polyzois, I. (2015) Risk indicators for peri-implant mucositis: a systematic literature review. *J Clin Periodontol* **42 Suppl 16,** S172-186. doi:10.1111/jcpe.12346.

Renvert, S. & Quirynen, M. (2015) Risk indicators for peri-implantitis. A narrative review. *Clin Oral Implants Res* **26 Suppl 11,** 15-44. doi:10.1111/clr.12636.

Riep, B., Edesi-Neuss, L., Claessen, F., Skarabis, H., Ehmke, B., Flemmig, T. F., Bernimoulin, J. P., Gobel, U. B. & Moter, A. (2009) Are putative periodontal pathogens reliable diagnostic markers? *J Clin Microbiol* **47,** 1705-1711. doi:10.1128/JCM.01387-08.

Robitaille, N., Reed, D. N., Walters, J. D. & Kumar, P. S. (2016) Periodontal and Peri-implant Diseases: Identical or Fraternal Infections? *Mol Oral Microbiol*. **31**, 285-301. doi:10.1111/omi.12124

Rosema, N., Slot, van Palenstein Helderman, W., Wiggelinkhuizen, L. & Van der Weijden, G. (2014) The efficacy of powered toothbrushes following a brushing exercise: a systematic review. **14**, 29-41. *Int J Dent Hyg*. doi:10.1111/idh.12115

Rosenberg, E. S., Torosian, J. P., Hammond, B. F. & Cutler, S. A. (1993) Routine anaerobic bacterial culture and systemic antibiotic usage in the treatment of adult periodontitis: a 6-year longitudinal study. *Int J Periodontics Restorative Dent* **13,** 213-243.

Rosier, B. T., De Jager, M., Zaura, E. & Krom, B. P. (2014) Historical and contemporary hypotheses on the development of oral diseases: are we there yet? *Front Cell Infect Microbiol* **4,** 92. doi:10.3389/fcimb.2014.00092.

Rowshani, B., Timmerman, M. F. & Van der Velden, U. (2004) Plaque development in relation to the periodontal condition and bacterial load of the saliva. *J Clin Periodontol* **31,** 214-218. doi:10.1111/j.0303-6979.2004.00468.x.

Rudney, J. D., Chen, R., Lenton, P., Li, J., Li, Y., Jones, R. S., Reilly, C., Fok, A. S. & Aparicio, C. (2012) A reproducible oral microcosm biofilm model for testing dental materials. *J Appl Microbiol* **113,** 1540-1553. doi:10.1111/j.1365-2672.2012.05439.x.

Safety, S. C. O. C. (2010) Opinion on Sodium Perborate and Perboric Acid. Luxembourg.

Sanz, M., Lau, L., Herrera, D., Morillo, J. M. & Silva, A. (2004) Methods of detection of *Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis* and *Tannerella forsythensis* in periodontal microbiology, with special emphasis on advanced molecular techniques: a review. *J Clin Periodontol* **31,** 1034-1047. doi:CPE609 [pii]10.1111/j.1600-051X.2004.00609.x.

Schatzle, M., Loe, H., Burgin, W., Anerud, A., Boysen, H. & Lang, N. P. (2003) Clinical course of chronic periodontitis. I. Role of gingivitis. *J Clin Periodontol* **30,** 887-901.

Schlafer, S., Riep, B., Griffen, A. L., Petrich, A., Hubner, J., Berning, M., Friedmann, A., Gobel, U. B. & Moter, A. (2010) *Filifactor alocis* - involvement in periodontal biofilms. *BMC Microbiol* **10,** 66.

Schwarz, F., Mihatovic, I., Golubovic, V., Eick, S., Iglhaut, T. & Becker, J. (2014) Experimental peri-implant mucositis at different implant surfaces. *J Clin Periodontol* **41,** 513-520. doi:10.1111/jcpe.12240.

Schwarz, F., Sculean, A., Wieland, M., Horn, N., Nuesry, E., Bube, C. & Becker, J. (2007) Effects of hydrophilicity and microtopography of titanium implant surfaces on initial supragingival plaque biofilm formation. A pilot study. *Mund Kiefer Gesichtschir* **11,** 333-338. doi:10.1007/s10006-007-0079-z.

Sender, R., Fuchs, S. & Milo, R. (2016) Are We Really Vastly Outnumbered? Revisiting the Ratio of Bacterial to Host Cells in Humans. *Cell* **164,** 337-340. doi:10.1016/j.cell.2016.01.013.

Serrano, J., Escribano, M., Roldan, S., Martin, C. & Herrera, D. (2014) Efficacy of adjunctive chemical plaque control in managing gingivitis. A systematic review and meta-analysis. *J Clin Periodontol*. doi:10.1111/jcpe.12331.

Shaddox, L. M., Alfant, B., Tobler, J. & Walker, C. (2010) Perpetuation of subgingival biofilms in an *in vitro* model. *Mol Oral Microbiol* **25,** 81-87. doi:10.1111/j.2041-1014.2009.00549.x.

Shaddox, L. M., Huang, H., Lin, T., Hou, W., Harrison, P. L., Aukhil, I., Walker, C. B., Klepac-Ceraj, V. & Paster, B. J. (2012) Microbiological characterization in children with aggressive periodontitis. *J Dent Res* **91,** 927-933. doi:10.1177/0022034512456039.

Shaddox, L. M. & Walker, C. (2009) Microbial testing in periodontics: value, limitations and future directions. *Periodontol 2000* **50,** 25-38.

Shapiro, S., Giertsen, E. & Guggenheim, B. (2002) An in vitro oral biofilm model for comparing the efficacy of antimicrobial mouthrinses. *Caries Res* **36,** 93-100. doi:57866.

Shchipkova, A. Y., Nagaraja, H. N. & Kumar, P. S. (2010) Subgingival microbial profiles of smokers with periodontitis. *J Dent Res* **89,** 1247-1253. doi:10.1177/0022034510377203.

Shi, B., Chang, M., Martin, J., Mitreva, M., Lux, R., Klokkevold, P., Sodergren, E., Weinstock, G. M., Haake, S. K. & Li, H. (2015) Dynamic changes in the subgingival microbiome and their potential for diagnosis and prognosis of periodontitis. *MBio* **6,** e01926-01914. doi:10.1128/mBio.01926-14.

Signoretto, C., Burlacchini, G., Faccioni, F., Zanderigo, M., Bozzola, N. & Canepari, P. (2009) Support for the role of *Candida* spp. in extensive caries lesions of children. *New Microbiol* **32,** 101-107.

Simon-Soro, A., Belda-Ferre, P., Cabrera-Rubio, R., Alcaraz, L. D. & Mira, A. (2013a) A tissue-dependent hypothesis of dental caries. *Caries Res* **47,** 591-600. doi:10.1159/000351663.

Simon-Soro, A. & Mira, A. (2015) Solving the etiology of dental caries. *Trends Microbiol* **23,** 76-82. doi:10.1016/j.tim.2014.10.010.

Simon-Soro, A., Tomas, I., Cabrera-Rubio, R., Catalan, M. D., Nyvad, B. & Mira, A. (2013b) Microbial geography of the oral cavity. *J Dent Res* **92,** 616-621. doi:10.1177/0022034513488119.

Simonsson, T., Ronstrom, A., Rundegren, J. & Birkhed, D. (1987) Rate of plaque formation--some clinical and biochemical characteristics of "heavy" and "light" plaque formers. *Scand J Dent Res* **95,** 97-103.

Siqueira, J. F., Jr. & Rocas, I. N. (2009) Diversity of endodontic microbiota revisited. *J Dent Res* **88,** 969-981. doi:10.1177/0022034509346549.

Sissons, C. H. (1997) Artificial dental plaque biofilm model systems. *Adv Dent Res* **11,** 110-126.

Slot, D. E., Wiggelinkhuizen, L., Rosema, N. A. & Van der Weijden, G. A. (2012) The efficacy of manual toothbrushes following a brushing exercise: a systematic review. *Int J Dent Hyg* **10,** 187-197. doi:10.1111/j.1601-5037.2012.00557.x.

Slots, J. (2011) Herpesvirus periodontitis: infection beyond biofilm. *J Calif Dent Assoc* **39,** 393-399.

Slots, J. & Slots, H. (2011) Bacterial and viral pathogens in saliva: disease relationship and infectious risk. *Periodontol 2000* **55,** 48-69. doi:10.1111/j.1600-0757.2010.00361.x.

Slots, J. & Ting, M. (2002) Systemic antibiotics in the treatment of periodontal disease. *Periodontol 2000* **28,** 106-176.

Soares, G. M., Teles, F., Starr, J. R., Feres, M., Patel, M., Martin, L. & Teles, R. (2015) Effects of azithromycin, metronidazole, amoxicillin, and metronidazole plus amoxicillin on an in vitro polymicrobial subgingival biofilm model. *Antimicrob Agents Chemother* **59,** 2791-2798. doi:10.1128/aac.04974-14.

Socransky, S. S., Haffajee, A. D., Cugini, M. A., Smith, C. & Kent, R. L., Jr. (1998) Microbial complexes in subgingival plaque. *J Clin Periodontol* **25,** 134-144.

Soderling, E. M. (2009) Xylitol, mutans streptococci, and dental plaque. *Adv Dent Res* **21,** 74-78. doi:10.1177/0895937409335642.

Sousa, V., Mardas, N., Spratt, D., Boniface, D., Dard, M. & Donos, N. (2016) Experimental models for contamination of titanium surfaces and disinfection protocols. *Clin Oral Implants Res*. doi:10.1111/clr.12735.

Tanner, A., Maiden, M. F., Macuch, P. J., Murray, L. L. & Kent, R. L., Jr. (1998) Microbiota of health, gingivitis, and initial periodontitis. *J Clin Periodontol* **25,** 85-98.

Tanner, A. C., Paster, B. J., Lu, S. C., Kanasi, E., Kent, R., Jr., Van Dyke, T. & Sonis, S. T. (2006) Subgingival and tongue microbiota during early periodontitis. *J Dent Res* **85,** 318-323. doi:85/4/318 [pii].

Tastepe, C. S., Liu, Y., Visscher, C. M. & Wismeijer, D. (2013) Cleaning and modification of intraorally contaminated titanium discs with calcium phosphate powder abrasive treatment. *Clin Oral Implants Res* **24,** 1238-1246. doi:10.1111/j.1600-0501.2012.02536.x.

Teles, R. P., Bogren, A., Patel, M., Wennstrom, J. L., Socransky, S. S. & Haffajee, A. D. (2007) A three-year prospective study of adult subjects with gingivitis II: microbiological parameters. *J Clin Periodontol* **34,** 7-17. doi:10.1111/j.1600-051X.2006.01015.x.

Teles, R. P., Haffajee, A. D. & Socransky, S. S. (2006) Microbiological goals of periodontal therapy. *Periodontol 2000* **42,** 180-218.

ten Cate, J. M. & Zaura, E. (2012) The numerous microbial species in oral biofilms: how could antibacterial therapy be effective? *Adv Dent Res* **24,** 108-111. doi:10.1177/0022034512450028.

ter Steeg, P. F. & van der Hoeven, J. S. (1990) Growth stimulation of Treponema denticola by periodontal microorganisms. *Antonie Van Leeuwenhoek* **57,** 63-70.

ter Steeg, P. F., Van der Hoeven, J. S., de Jong, M. H., van Munster, P. J. & Jansen, M. J. (1987) Enrichment of subgingival microflora on human serum leading to accumulation of Bacteroides species, Peptostreptococci and Fusobacteria. *Antonie Van Leeuwenhoek* **53,** 261-272.

Teughels, W., Durukan, A., Ozcelik, O., Pauwels, M., Quirynen, M. & Haytac, M. C. (2013) Clinical and microbiological effects of Lactobacillus reuteri probiotics in the treatment of chronic periodontitis: a randomized placebo-controlled study. *J Clin Periodontol* **40,** 1025-1035. doi:10.1111/jcpe.12155.

Teughels, W., Kinder Haake, S., Sliepen, I., Pauwels, M., Van Eldere, J., Cassiman, J. J. & Quirynen, M. (2007) Bacteria interfere with A. actinomycetemcomitans colonization. *J Dent Res* **86,** 611-617.

Teughels, W., Loozen, G. & Quirynen, M. (2011) Do probiotics offer opportunities to manipulate the periodontal oral microbiota? *J Clin Periodontol* **38 Suppl 11,** 159-177. doi:10.1111/j.1600-051X.2010.01665.x.

Tew, J. G., Marshall, D. R., Burmeister, J. A. & Ranney, R. R. (1985) Relationship between gingival crevicular fluid and serum antibody titers in young adults with generalized and localized periodontitis. *Infect Immun* **49,** 487-493.

Theilade, E. (1996) The experimental gingivitis studies: the microbiological perspective. *J Dent Res* **75,** 1434-1438.

Theilade, E., Wright, W. H., Jensen, S. B. & Loe, H. (1966) Experimental gingivitis in man. II. A longitudinal clinical and bacteriological investigation. *J Periodontal Res* **1,** 1-13.

Thomas, R. Z., van der Mei, H. C., van der Veen, M. H., de Soet, J. J. & Huysmans, M. C. (2008) Bacterial composition and red fluorescence of plaque in relation to primary and secondary caries next to composite: an in situ study. *Oral Microbiol Immunol* **23,** 7-13. doi:10.1111/j.1399-302X.2007.00381.x.

Thompson, H., Rybalka, A., Moazzez, R., Dewhirst, F. E. & Wade, W. G. (2015) In vitro culture of previously uncultured oral bacterial phylotypes. 81**,** 8307-8314. doi:10.1128/aem.02156-15.

Thurnheer, T. & Belibasakis, G. N. (2015) Integration of non-oral bacteria into in vitro oral biofilms. *Virulence***, 6**, 258-640. doi:10.4161/21505594.2014.967608.

Thurnheer, T. & Belibasakis, G. N. (2016) Incorporation of staphylococci into titanium-grown biofilms: an in vitro "submucosal" biofilm model for peri-implantitis. *Clin Oral Implants Res* **27,** 890-895. doi:10.1111/clr.12715.

Thurnheer, T., Bostanci, N. & Belibasakis, G. N. (2016) Microbial dynamics during conversion from supragingival to subgingival biofilms in an in vitro model. *Mol Oral Microbiol* **31,** 125-135. doi:10.1111/omi.12108.

Timmerman, M. F., Van der Weijden, G. A., Hart, A. A., Abbas, F., Winkel, E. G. & Van der Velden, U. (2002) How do data from deepest pocket per quadrant relate to full-mouth scores? Progression of untreated periodontal disease in young Indonesians. *J Clin Periodontol* **29,** 219-223.

Tsuda, H., Ochiai, K., Suzuki, N. & Otsuka, K. (2010) Butyrate, a bacterial metabolite, induces apoptosis and autophagic cell death in gingival epithelial cells. *J Periodontal Res* **45,** 626-634. doi:10.1111/j.1600-0765.2010.01277.x.

Turesky, S., Gilmore, N. D. & Glickman, I. (1970) Reduced plaque formation by the chloromethyl analogue of victamine C. *J Periodontol* **41,** 41-43. doi:10.1902/jop.1970.41.41.41.

Turesky, S. S. (1970) What is the role of dental calculus in the etiology and progression of periodontal disease? *J Periodontol* **41,** 285-286.

Turnbaugh, P. J., Ley, R. E., Hamady, M., Fraser-Liggett, C. M., Knight, R. & Gordon, J. I. (2007) The human microbiome project. *Nature* **449,** 804-810. doi:10.1038/nature06244.

Urzua, B., Hermosilla, G., Gamonal, J., Morales-Bozo, I., Canals, M., Barahona, S., Coccola, C. & Cifuentes, V. (2008) Yeast diversity in the oral microbiota of subjects with periodontitis: *Candida albicans* and *Candida dubliniensis* colonize the periodontal pockets. *Med Mycol* **46,** 783-793. doi:10.1080/13693780802060899.

Uzel, N. G., Teles, F. R., Teles, R. P., Song, X. Q., Torresyap, G., Socransky, S. S. & Haffajee, A. D. (2011) Microbial shifts during dental biofilm re-development in the absence of oral hygiene in periodontal health and disease. *J Clin Periodontol* **38,** 612-620. doi:10.1111/j.1600-051X.2011.01730.x.

van den Bosch, W. F. (2000) Method for Preparing a Preparation for Bleaching Teeth or forTreating Skin Complaints and Mucous Membrane Disorders. In: *The United States Patent and Trade mark Office (USPTO)*, (ed.) D. W. A. V. V. Aruba, AN.

van der Reijden, W. A., Bosch-Tijhof, C. J., Strooker, H. & van Winkelhoff, A. J. (2006) prtH in Tannerella forsythensis is not associated with periodontitis. *J Periodontol* **77,** 586-590. doi:10.1902/jop.2006.050271.

van der Veen, M. H., Thomas, R. Z., Huysmans, M. C. & de Soet, J. J. (2006) Red autofluorescence of dental plaque bacteria. *Caries Res* **40,** 542-545. doi:10.1159/000095655.

van der Veen, M. H., Volgenant, C. M., Keijser, B., Ten Cate, J. B. & Crielaard, W. (2016) Dynamics of red fluorescent dental plaque during experimental gingivitis-A cohort study. *J Dent* **48,** 71-76. doi:10.1016/j.jdent.2016.02.010.

van der Velden, U., Abbas, F. & Hart, A. A. (1985) Experimental gingivitis in relation to susceptibility to periodontal disease. (I.) Clinical observations. *J Clin Periodontol* **12,** 61-68.

van der Waal, S. V., Scheres, N., de Soet, J. J., Wesselink, P. R. & Crielaard, W. (2015) Cytotoxicity, interaction with dentine and efficacy on multispecies biofilms of a modified salt solution intended for endodontic disinfection in a new in vitro biofilm model. *Int Endod J* **48,** 153-161. doi:10.1111/iej.12294.

van der Weijden, F. & Slot, D. E. (2011) Oral hygiene in the prevention of periodontal diseases: the evidence. *Periodontol 2000* **55,** 104-123.

van der Weijden, F. A. & Slot, D. E. (2015) Efficacy of homecare regimens for mechanical plaque removal in managing gingivitis a meta review. *J Clin Periodontol* **42** Suppl 16, 77-91. doi:10.1111/jcpe.12359.

van der Weijden, F. A., Van der Sluijs, E., Ciancio, S. G. & Slot, D. E. (2015) Can Chemical Mouthwash Agents Achieve Plaque/Gingivitis Control? *Dent Clin North Am* **59,** 799-829. doi:10.1016/j.cden.2015.06.002.

van der Weijden, G. A., Timmerman, M. F., Nijboer, A., Reijerse, E. & Van der Velden, U. (1994a) Comparison of different approaches to assess bleeding on probing as indicators of gingivitis. *J Clin Periodontol* **21,** 589-594.

van der Weijden, G. A., Timmerman, M. F., Saxton, C. A., Russell, J. I., Huntington, E. & Van der Velden, U. (1994b) Intra-/inter-examiner reproducibility study of gingival bleeding. *J Periodontal Res* **29,** 236-241.

van Essche, M., Loozen, G., Godts, C., Boon, N., Pauwels, M., Quirynen, M. & Teughels, W. (2012) Bacterial Antagonism Against Periodontopathogens. *J Periodontol*. doi:10.1902/jop.2012.120261.

van Leeuwen, M. P., Rosema, N. A., Versteeg, P. A., Slot, D. E., Van Winkelhoff, A. J. & Van der Weijden, G. A. (2015) Long-term efficacy of a 0.07% cetylpyridinium chloride mouth rinse in relation to plaque and gingivitis: a 6-month randomized, vehicle-controlled clinical trial. *Int J Dent Hyg* **13,** 93-103. doi:10.1111/idh.12082.

van Leeuwenhoek, A. (1683) to Francois Aston. In: *Early Letters*, pp. 11. Delft in Holland, 12 September 1683. English extract printed in *Philosophical Transactions* 14, 568.

van Strydonck, D. A., Slot, D. E., Van der Velden, U. & Van der Weijden, F. (2012) Effect of a chlorhexidine mouthrinse on plaque, gingival inflammation and staining in gingivitis patients: a systematic review. *J Clin Periodontol* **39,** 1042-1055. doi:10.1111/j.1600-051X.2012.01883.x.

van Winkelhoff, A. J., Herrera, D., Oteo, A. & Sanz, M. (2005) Antimicrobial profiles of periodontal pathogens isolated from periodontitis patients in The Netherlands and Spain. *J Clin Periodontol* **32,** 893-898.

van Winkelhoff, A. J. & Winkel, E. G. (2005) Microbiological diagnostics in periodontics: biological significance and clinical validity. *Periodontol 2000* **39,** 40-52.

van Winkelhoff, A. J. & Winkel, E. G. (2009) Antibiotics in periodontics: right or wrong? *J Periodontol* **80,** 1555-1558.

Vartoukian, S. R., Adamowska, A., Lawlor, M., Moazzez, R., Dewhirst, F. E. & Wade, W. G. (2016) In Vitro Cultivation of 'Unculturable' Oral Bacteria, Facilitated by Community Culture and Media Supplementation with Siderophores. *Appl Environ Microbiol* **11,** e0146926. doi:10.1128/aem.02156-1510.1371/journal.pone.0146926.

Vianna, M. E., Conrads, G., Gomes, B. P. & Horz, H. P. (2009) T-RFLP-based mcrA gene analysis of methanogenic archaea in association with oral infections and evidence of a novel Methanobrevibacter phylotype. *Oral Microbiol Immunol* **24,** 417-422. doi:10.1111/j.1399-302X.2009.00539.x.

Vianna, M. E., Holtgraewe, S., Seyfarth, I., Conrads, G. & Horz, H. P. (2008) Quantitative analysis of three hydrogenotrophic microbial groups, methanogenic archaea, sulfate-reducing bacteria, and acetogenic bacteria, within plaque biofilms associated with human periodontal disease. *J Bacteriol* **190,** 3779-3785.

Vickerman, M. M., Brossard, K. A., Funk, D. B., Jesionowski, A. M. & Gill, S. R. (2007) Phylogenetic analysis of bacterial and archaeal species in symptomatic and asymptomatic endodontic infections. *J Med Microbiol* **56,** 110-118.

Volgenant, C. M., van der Veen, M. H., de Soet, J. J. & ten Cate, J. M. (2013) Effect of metalloporphyrins on red autofluorescence from oral bacteria. *Eur J Oral Sci* **121,** 156-161. doi:10.1111/eos.12045.

Wade, W. G. (2011) Has the use of molecular methods for the characterization of the human oral microbiome changed our understanding of the role of bacteria in the pathogenesis of periodontal disease? *J Clin Periodontol* **38 Suppl 11,** 7-16. doi:10.1111/j.1600-051X.2010.01679.x.

Wade, W. G. (2013) The oral microbiome in health and disease. *Pharmacol Res* **69,** 137-143. doi:10.1016/j.phrs.2012.11.006.

Walker, C. & Karpinia, K. (2002) Rationale for use of antibiotics in periodontics. *J Periodontol* **73,** 1188-1196.

Walker, C. & Sedlacek, M. J. (2007) An in vitro biofilm model of subgingival plaque. *Oral Microbiol Immunol* **22,** 152-161. doi:10.1111/j.1399-302X.2007.00336.x.

Warnakulasuriya, S., Dietrich, T., Bornstein, M. M., Casals Peidro, E., Preshaw, P. M., Walter, C., Wennstrom, J. L. & Bergstrom, J. (2010) Oral health risks of tobacco use and effects of cessation. *Int Dent J* **60,** 7-30.

Wennstrom, J. & Lindhe, J. (1979) Effect of hydrogen peroxide on developing plaque and gingivitis in man. *J Clin Periodontol* **6,** 115-130.

Wennstrom, J. L. (1988) Mouthrinses in "experimental gingivitis" studies. *J Clin Periodontol* **15,** 511-516.

Wikler, M. A. (2009) Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically: Approved Standard. Clinical and Laboratory Standards Institute. Clinical & Institute, L. S. .

Willner, D., Furlan, M., Schmieder, R., Grasis, J. A., Pride, D. T., Relman, D. A., Angly, F. E., McDole, T., Mariella, R. P., Jr., Rohwer, F. & Haynes, M. (2011) Metagenomic detection of phage-encoded platelet-binding factors in the human oral cavity. *Proc Natl Acad Sci U S A* **108** Suppl 1, 4547-4553. doi:10.1073/pnas.1000089107.

Winkel, E. G., Van Winkelhoff, A. J., Timmerman, M. F., Van der Velden, U. & Van der Weijden, G. A. (2001) Amoxicillin plus metronidazole in the treatment of adult periodontitis patients. A double-blind placebo-controlled study. *J Clin Periodontol* **28,** 296-305.

Woese, C. R., Kandler, O. & Wheelis, M. L. (1990) Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. *Proc Natl Acad Sci U S A* **87,** 4576-4579.

Xie, G., Chain, P. S., Lo, C. C., Liu, K. L., Gans, J., Merritt, J. & Qi, F. (2010) Community and gene composition of a human dental plaque microbiota obtained by metagenomic sequencing. *Mol Oral Microbiol* **25,** 391-405. doi:10.1111/j.2041-1014.2010.00587.x.

Ximenez-Fyvie, L. A., Haffajee, A. D. & Socransky, S. S. (2000) Comparison of the microbiota of supra- and subgingival plaque in health and periodontitis. *J Clin Periodontol* **27,** 648-657.

Yamabe, K., Maeda, H., Kokeguchi, S., Tanimoto, I., Sonoi, N., Asakawa, S. & Takashiba, S. (2008) Distribution of Archaea in Japanese patients with periodontitis and humoral immune response to the components. *FEMS Microbiol Lett* **287,** 69-75.

Yang, F., Zeng, X., Ning, K., Liu, K. L., Lo, C. C., Wang, W., Chen, J., Wang, D., Huang, R., Chang, X., Chain, P. S., Xie, G., Ling, J. & Xu, J. (2012) Saliva microbiomes distinguish caries-active from healthy human populations. *ISME J* **6,** 1-10. doi:10.1038/ismej.2011.71.

Yanine, N., Araya, I., Brignardello-Petersen, R., Carrasco-Labra, A., Gonzalez, A., Preciado, A., Villanueva, J., Sanz, M. & Martin, C. (2013) Effects of probiotics in periodontal diseases: a systematic review. *Clin Oral Investig* **17,** 1627-1634. doi:10.1007/s00784-013-0990-7.

Zambon, J. J. (1997) Principles of evaluation of the diagnostic value of subgingival bacteria. *Ann Periodontol* **2,** 138-148.

Zaura-Arite, E., van Marle, J. & ten Cate, J. M. (2001) Conofocal microscopy study of undisturbed and chlorhexidine-treated dental biofilm. *J Dent Res* **80,** 1436-1440.

Zaura, E. (2012) Next-generation sequencing approaches to understanding the oral microbiome. *Adv Dent Res* **24,** 81-85. doi:10.1177/0022034512449466.

Zaura, E., Keijser, B. J., Huse, S. M. & Crielaard, W. (2009) Defining the healthy "core microbiome" of oral microbial communities. *BMC Microbiol* **9,** 259. doi:10.1186/1471-2180-9-259.

Zaura, E. & Ten Cate, J. M. (2015) Towards understanding oral health. *Caries Res* **49** Suppl 1**,** 55-61. doi:10.1159/000377733.

Zhou, Y., Gao, H., Mihindukulasuriya, K. A., La Rosa, P. S., Wylie, K. M., Vishnivetskaya, T., Podar, M., Warner, B., Tarr, P. I., Nelson, D. E., Fortenberry, J. D., Holland, M. J., Burr, S. E., Shannon, W. D., Sodergren, E. & Weinstock, G. M. (2013) Biogeography of the ecosystems of the healthy human body. *Genome Biol* **14,** doi:10.1186/gb-2013-14-1-r1.

Zhuang, L. F., Watt, R. M., Steiner, S., Lang-Hua, B. H., Wang, R., Ramseier, C. A. & Lang, N. P. (2014) Subgingival microbiota of Sri Lankan tea labourers naive to oral hygiene measures. *J Clin Periodontol* **41,** 433-441. doi:10.1111/jcpe.12230.

Zijnge, V., van Leeuwen, M. B., Degener, J. E., Abbas, F., Thurnheer, T., Gmur, R. & Harmsen, H. J. (2010) Oral biofilm architecture on natural teeth. *PLoS One* **5,** e9321. doi:10.1371/journal.pone.0009321.

Zitzmann, N. U. & Berglundh, T. (2008) Definition and prevalence of peri-implant diseases. *J Clin Periodontol* **35,** 286-291. doi:10.1111/j.1600-051X.2008.01274.x.

Zollinger, L., Schnyder, S., Nietzsche, S., Sculean, A. & Eick, S. (2015) In-vitro activity of taurolidine on single species and a multispecies population associated with periodontitis. *Anaerobe* **32,** 18-23. doi:10.1016/j.anaerobe.2014.11.008.

Glossary of common terms used in this thesis

Term	Definition
16S ribosomal RNA (16S rRNA)	An RNA molecule that is encoding small ribosomal subunit in prokaryotes. 16S has a conserved region (common to all prokaryotes) and hypervariable regions that are unique to particular species
Biofilm	A sessile community of microbes characterized by cells that are attached to a surface or to each other and embedded in a matrix of extracellular polymeric substances (Hall-Stoodley et al., 2004)
Dysbiosis	The state of imbalance in the relative abundance or influence of species within a microbial community that is associated with a disease (Hajishengallis and Lamont, 2012, Lamont and Hajishengallis, 2014)
Ecosystem	A community of living organisms in conjunction with the nonliving components of their environment interacting as a system (Zaura and Ten Cate, 2015)
Genus	kind of living micro /organisms within the taxonomic rank (plural: genera)
Homeostasis:	A property of a system in which variables are regulated so that internal conditions remain stable and relatively constant (Zaura and Ten Cate, 2015)
Incipient dysbiosis	In non-susceptible individuals dysbiosis does not progress beyond gingivitis (Meyle and Chapple, 2015)
Microbiome	The totally of microbes, their genetic, and the environment in which they interact (Cho and Blaser, 2012)
Microbiota	All living microbial organisms constituting the microbiome (Cho and Blaser, 2012)

Term	Definition
Next-generation sequencing (NGS)	A term describing modern high throughput sequencing technologies
Operational taxonomic unit (OTU)	The grouping of bacterial 16S rRNA gene sequences by their similarity. Sequences are typically grouped at a value between 97% and 99%.
Pathobionts	Commensal microorganisms with the potential to induce pathology under conditions of disrupted homeostasis (Simon-Soro and Mira, 2015, Lamont and Hajishengallis, 2014).
Phylotype	A type of bacterium defined by its placement in a phylogenetic tree based on its sequence of 16S rRNA gene
Resilience	The ability to withstand disturbance, is a central concept in ecology (Cho and Blaser, 2012)
Species	Distinct groups of bacteria that have been isolated, cultured and named
Symbionts	Commensal microorganism (Simon-Soro and Mira, 2015)
Symbiosis	Two or more species living closely together in a long-term relationship
Taxa	Grouped organisms (singular: taxon)
Taxonomic rank	Taxonomic hierarchy
Taxonomy	The biological systematic classification of living organisms

Financial justification of the research

The thesis was financed with a commission from ACTA Dental Research BV. ACTA Research BV received financial support from NGen Oral Pharma N.V. (Curacao) for the role of the Departments of Periodontology and Preventive Dentistry of ACTA in this project. NGen Oral Pharma N.V. provided study products. The author reports that this company had no influence on the design, content, results and publication of any chapter included in this thesis.

Acknowledgements

Acknowledgements

I extend my sincere gratitude to the members of the reading committee for their time and effort to read the manuscript and to participate in the defense ceremony.

After all these years, an important period in my life has come to an end. This thesis would never have been possible without the guidance, supervision and support of important friends and supervisors which I would like to thank.

Dear **Wim Crielaard**, my promotor, thank you for inspiring and motivating me to learn and allow me to develop my research project in the field which I like the most in science; microbial ecology. Your open and clear mind have been of great support. You were always ready to volunteer when I needed a donor for my experiments on chapter three. Many thanks for the opportunities that you have given me.

My sincere gratitude to **Fridus van der Weijden**. When you agreed to be my promotor I knew that it was going to be a great opportunity to learn from you. Thank you so much for all your guidance, valuable comments, and sharp corrections. I am very grateful to you for all your time and expertise.

Dear **Wouter Beertsen**, no words can express my deepest gratitude for your essential guidance and each and every contribution of time, support and ideas. I am certain that I wouldn't have made it this far without your encouragement. I thoroughly admire you as a Professor and for your outstanding kind personality.

Foremost, I would like to thank Prof. dr. **Egija Zaura**, my copromotor. Thanks for being so patient with me during the last years, while I was learning about a whole new world to me; oral microbial ecology. I admire you as a person, woman, mother, researcher, and now Professor! You had a great influence in my professional and personal life. When feeling confused, I'd always remember your wise words and advice, they comfort me. You taught me so much about "symbiosis" within biofilms, plants and people. Muchisimas gracias!!

My deep appreciation for **Rob Exterkate**, through all these years there have been so many times that you helped me at the lab with your extended knowledge and expertise with biofilms. Thanks for all your critical and constructive advice. I enjoyed working together with you.

Mark Buijs, your meticulous work and expertise in the data processing, although often time consuming, were essential to my research, but your kindness and also your attitude while helping me at the lab will be unforgettable. Thank you!

Wendy de Wit, you helped me with countless matters at the lab. But as my dear friend you were there with me through all my hardships. I treasured all the precious moments we shared. Thank you for your friendship!

Dear **Joyce van der Horst**, your cooperation with the samples collection was very important for the biofilm studies, but above all I want to express my gratitude for your friendship, your positive attitude, and for being my paranimf.

To my roommates **Jessica Koopman**, **Bob Rosier, Aurea Simón – Soro**, **Josiane Almeida**, and **Bruno Matos**; your presence at our room on the 12th floor never failed to cheer me up!. Your friendship is one of the best things about doing my PhD at ACTA.

Special thanks to my dear friends and collegues working (or who have worked) at the department of preventive dentistry: **Prof. dr. Bob Ten Cate, Prof. dr. Cor van Loveren, Monique van der Veen, Hans de Soet, Alexa Laheij, Michel Hoogekamp, Bernd Brand, Bastiaan Krom, Ilona Persoon, Catherine Volgenant, Charifa Zemouri, Suzette van der Waal, Nina Scheres, Marleen Janus, Dong-Mei Deng, Carolien Bosch, Elly Deutekom, Ali May, and Sultan Imangaliyev**, and to all other colleagues from the 12th and 13th floor.

Dear **Wil van der Reijden**, thanks for teaching me about how to work in the lab. You taught me the basic tools to be able to go through all these years. Your enthusiasm about bacteria had great impact on me: resulting in this thesis.

I would like to also express my gratitude to **Nienke Hennequin-Hoenderdos** , **Dagmar Slot** en **Eveline van der Sluijs**; for their expertise, guidance, and motivation during the clinical trial (chapter 4a). Special thanks to **Esther Martin and Guylaine van Anraat, Sam Supranoto, Rosalien Keukenmeester** for the data collection, your time, and effort you put into it.

I am grateful to **Prof. Dr. Bruno Loos** for cooperating with the accessibility to the periodontitis patients from the department of periodontology. **Martijn Rosema**, your help on a critical moment finishing this thesis was of great value. Thanks!. **Marisa Voll** and **Bibi van Etten** thank you for helping me to collect my samples at the Perio-department.

Pilar Amalia, you made the cover and layout of this thesis, thank you for your great effort!

My dear **Jorn**, thanks for your encouragement and for being there, in your way, bringing countless happy moments.

To my greatest treasure, my daughter **Alejandra**. Gracias mi amor por todo el apoyo, compression y cariño que me brindaste durante todos estos años. Tu eres la fuente de mi energia, mi verdad. Mi bonita estoy muy orgullosa de ti !!

To my mother, for her unconditional support and greatest love, **Mami**, te quiero tanto, gracias por estar siempre conmigo, aunque la distancia nos separe.

Last but not least, I want to express my gratitude to my dear family and friends, who have always cheered me up and believed in me. Specially to **Elly Lubbers, Fanny Elena Arguedas, Nico van Niekerk, en Mirjam Mush**: because you are always there when I need you and I want to be there for you too. You are my family in The Netherlands.

Doing what you like is freedom,

Liking what you do is happiness;

Therefore I am happy.

About the author

Mercedes Fernandez y Mostajo graduated as a dentist (DDS) in 2002 at the University of "Nuestra Señora de La Paz", in La Paz, Bolivia. Her interest in periodontology brought her to the University of Buenos Aires to attend a specialized post-graduate course in Periodontology (2004). In 2005 Fernandez was awarded with a Nuffic fellowship program to attend an advanced international course in oral health development, including new concepts in education and basic oral care at the WHO Collaborating Centre for oral health care which is located at "Radboud university medical center" in Nijmegen, The Netherlands. After that, she followed the 3-year post-graduate master in periodontology at the Periodontology Department from the same university in a combined program with the Periodontology Department from the Academic Centre for Dentistry Amsterdam (ACTA). In 2010 she obtained the degree of MSc in Periodontology accredited by the European Federation of Periodontology (EFP). While working in general practice since 2011 Fernandez has been working as a part-time researcher at the Department of Preventive dentistry and obtained her PhD in 2017 in the field of oral microbial ecology. Currently she is working in different dental practices in the Netherlands as a Periodontist undertaking all aspects of periodontal therapy including non-surgical, surgical, muco-gingival and implant therapy.

List of publications

- Fernandez y Mostajo, M., Exterkate, R. A. M., Buijs, M. J., Beertsen, W.,van der Weijden, F., Zaura E., Crielaard W. (2017)

  A reproducible microcosm biofilm model of subgingival microbial communities. J Periodontal Res. (submitted)

- Fernandez y Mostajo M., Exterkate R. A.M., Buijs, M. J., Crielaard, W. & Zaura, E. (2016)

  Effect of mouthwashes on the composition and metabolic activity of oral biofilms grown in vitro. Clin Oral Investig. doi:10.1007/s00784-016-1876-2.

- Volgenant, C. M., Fernandez, y Mostajo M., Rosema N. A., van der Weijden F. , ten Cate J. M., van der Veen M. H. (2016)

  Comparison of red autofluorescing plaque and disclosed plaque-a cross-sectional study. Clin Oral Investig. doi:10.1007/s00784-016-1761-z.

- Zaura E., Fernandez y Mostajo M., Koopman M. J., Crielaard W. (2014)

  The oral microbiome. In: The Human Microbiota and Microbiome,

  (ed.) J. R. Marchesi, pp. 20 - 31. Cardiff CAB International.

- Fernandez y Mostajo, M., van der Reijden W. A., Buijs M. J., Beertsen W., van der Weijden F., Crielaard W. & Zaura E. (2014)

  Effect of an oxygenating agent on oral bacteria in vitro and on dental plaque composition in healthy young adults. Front Cell Infect Microbiol 4, 95. doi:10.3389/fcimb.2014.00095.

- Fernandez y Mostajo M., Zaura E., Crielaard W. & Beertsen W. (2011)

  Does routine analysis of subgingival microbiota in periodontitis contribute to patient benefit? Eur J Oral Sci 119, 259-264. doi:10.1111/j.1600-0722.2011.00828.x.

Participations at congresses

aug 2015: **A multispecies subgingival biofilm model from periodontitis and peri-implantitis patients** (presentation)
International Association for Dental Research ANZ
Australia & New Zealand group 2015
Dunedin, New Zealand

Poster publications:

june 2015: **A novel reproducible model of subgingival biofilms**
M. Fernandez y Mostajo, R.A.M. Exterkate, M.J. Buijs, W. Beertsen, E. Zaura, W.Crielaard
EuroPerio 8 *(Abstract 1779)*
London, UK

september 2013: **Effects of different plaque and saliva donors on oral microcosms *in- vitro***
M. Fernandez y Mostajo, R.A.M. Exterkate, E. Zaura, M.J. Buijs, W. Crielaard Eurobiofilms
Gent, Belgium

februari 2010: **Antimicrobial effect of an active oxygen releasing compound on periodontitis and caries associated microorganisms *(in-vitro)***
M. Fernandez y Mostajo, W. van der Reijden, W. Beertsen
IOT Dental Research Meeting
Lunteren, The Netherlands

april 2005: **Prevalence of caries in relation with the education level and nutrition in three rural communities in La Paz, Bolivia**
M. Rosales, *M. Fernandez y Mostajo*, J. Wilde
International Association of Dental Research (Abstract 3172)
Baltimore, USA

june 2003: **The oral health related with the nutrition levels and education in a suburban community of La Paz, Bolivia**
I. Wilde, *M. Fernandez y Mostajo*, J. Jacobsen, J. Wilde.
International Association of Dental Research (Abstract 1560)
Goteborg, Sweden