Infection and disinfection of transmucosal implant surfaces *in vitro*

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Abstract

Peri-implantitis is an inflammatory process, which is initiated by a biofilm-induced pathway causing a reversible inflammatory reaction in the soft tissues surrounding the implant; however, it may progress into irreversible damage in the surrounding alveolar bone and result in loss of attachment. Modelling any kind of oral biofilm is challenging as up to 700 species have been identified on various surfaces within the mouth. Thus, also data on bacterial colonization and biofilm formation on dental implant surfaces is limited and mostly based on identification of species. However, knowledge about this process, in particular in its early stages, is essential for the development of strategies to prevent and control microbial adherence and biofilm formation.

The aims of this study were (i) to develop and validate a flow chamber model to assess peri-implantitis related multispecies biofilm formation; (ii) to test antimicrobial susceptibility of this biofilm; (iii) to evaluate the reliability of commonly used methods and novel isothermal microcalorimetry (IMC) analyses for vitality testing of adherent microorganisms *in vitro*.

An *in vitro* flow chamber model was developed to study single- and multispecies adhesion to protein-coated surface in reduced nutrient conditions that mimic the clinical situation of peri-implant disease.

Adherent single-species model with *S. sanguinis* was used for antimicrobial susceptibility testing (Original study 1 in Chapter 4) to answer two questions: firstly, how efficient is disinfectant treatment and secondly, to identify discrepancies between the results revealed by different vitality detection methods (commonly applied: staining by Live/dead Baclight kit, conventional culturing; and a novel approach by IMC). Chlorhexidine has been considered for many years the standard in oral infection control, however, the three other disinfectants (povidone-iodine, octenidine dihydrochloride, polyhexanide) showed comparable efficacy against the adherent microorganism. The interpretation of the results of staining and traditional culturing is difficult as major discrepancies were present. The disagreement between the results of the methods can be attributed to different aspects: (i) staining is based on the destruction or integrity of the cell wall; (ii) conventional culturing requires bacteria to be re-suspended prior to cultivation and subsequent quantification of colony-forming units. However, several factors – such as physical damage to the cells by re-suspension or lack of growth due to suboptimal culture conditions – may cause false

negative results; (iii) the population of cells that are viable but not cultivable (VBNC), but are still present by staining and microscopy.

The IMC is a very sensitive method, which detects heat-flow from all metabolic processes in any living microorganism. The principle of the method is that all living systems produce heat and heat release can be measured calorimetrically without any interference with the processes. In other words, IMC measures heat production or consumption, which is proportional to the rate at which any given chemical, physical or biological process is taking place (i.e., metabolic heat from bacterial growth). Our results on adherent disinfectant-treated *S. sanguinis* indicate that IMC is a useful tool to avoid the problem with VBNC cells and provides a novel approach in oral microbiology that detects reliably all the cells that are able to resuscitate after disinfectant treatment and thereby may lead to the expression of virulence factors in the colonized sites.

In order to study antimicrobial efficacy on a more complex system, a 72-hour biofilm model including *S. sanguinis*, *F. nucelatum* and *P. gingivalis* was created (Original study 2 in Chapter 5). After 72 h, the three species were always detected with similar proportions throughout experiments by microscopic methods. Even, when such stability in the proportions and structure was found, the metabolic activity of biofilms in IMC revealed high heterogeneity. Thus, this heterogeneity suggests that bacteria appear to have intra-species variance affecting their behaviour in biofilms, which is dependent on different factors that are hitherto not well understood.

IMC analysis of the behaviour of the three-species biofilm provided a novel opportunity to observe how an intact biofilm acts as an entity when exposed to antibiotics (Original study 3 in Chapter 6). The clinical efficacy of adjuvant antibiotic therapy in the peri-implantitis treatment is unclear; in order to gain better understanding the most frequently used antibiotics: amoxicillin, metronidazole and their combination were tested. Additionally, in order to obtain comparable data to the previously published information (Chapter 4), the vitality of adherent single-species of *S. sanguinis* and *P. gingivalis* after antibiotic treatment was assessed by vitality staining, conventional culturing and IMC. The results of staining and culturing correlated poorly. This can be due to reasons mentioned earlier, such as limitations of the methods and VBNC cells, but can also be due to the bacteriostatic effects of antibiotics that cannot be reliably detected by staining. Although amoxicillin and metronidazole are considered bactericidal, IMC results suggest that when applied alone, the antibiotics seem to work primarily through bacteriostatic

effects; when combined, the efficacy is increased by their synergistic bactericidal action. Furthermore, this indicates that it might not be possible to strictly divide the effects of antibiotics into bacteriostatic and bactericidal, as the results here imply that the effect seems to be dependent on their concentration and combination plus the nature of targeted cells.

In conclusion, this study emphasises the need to critically evaluate the results of live/dead staining and conventional culturing as many aspects can affect the outcome of these methods leading to miscalculations on the proportion of cells that are able to resuscitate and express virulence factors on treated sites.

Thus, as a novel alternative in oral biofilm research IMC gives new insights helping to monitor the efficacy and dynamics of biofilms and their antimicrobial susceptibility *in vitro*.

1 Introduction

Peri-implantitis

Peri-implantitis is an inflammatory process, which affects the soft and hard tissues around an implant and results in loss of attachment (Albrektsson & Isidor, 1993, Mombelli & Décaillet, 2011). Its prevalence appears to be rapidly and constantly increasing due to the use of dental implants, thus efficient prevention and control strategies are of the highest importance (Persson*, et al.*, 2006).

The etiology and pathogenesis of peri-implantitis is comparable to those of periodontal diseases with a biofilm-induced pathway causing initially reversible inflammatory reaction (mucositis) in the surrounding soft tissues, which may progress into usually irreversible damage in the surrounding alveolar bone. Mucositis appears in 80% of the patients and in 50% of the implants, while peri-implantitis is observed in 28-56% of the patients, and in 12-43% of the implants (Zitzmann & Berglundh, 2008). However, also mechanical stress caused by occusal or lateral overloading may initiate a formation of microfractures that trigger a cascade of reactions in the close peri-implant proximity (Mombelli & Lang, 1998, Quirynen*, et al.*, 2003). Implant failures that occur in the early phase after installation are related to an absence of osseointegration due to host-associated reactions such as wound infection or initial overload. So-called late failures are observed after the first year in function and affect an initially successful osseo-integrated implant. Biomechanical reasons with overload are responsible for a sudden mobility and subsequent implant loss, while peri-implantitis entails an inflammatory process affecting the peri-implant bone in the marginal region leading to the loss of osseo-integration and to clinical failure.

Biofilms associated with peri-implantitis

Bacteria frequently live in heterogenic well-established communities, commonly called biofilms, which are attached to surfaces. Biofilm can be defined as a sessile community of cells irreversibly attached to substratum embedded in an extracellular polysaccharide matrix that they have produced (Costerton*, et al.*, 2005, Mombelli & Décaillet, 2011). Biofilm formation includes distinct stages. First, an acquired pellicle is formed followed by reversible adhesion involving weak long-range physiochemical interactions between bacterial cell surface and pellicle, which can lead to stronger adhesin-receptor mediated

attachment. These early colonizers grow and modify the environment to make it suitable for more fastidious bacteria that are often obligatory anaerobes. Thus, the biofilm grows and co-adhesion of secondary colonizers to already attached cells takes place (Kolenbrander, *et al.*, 2000). Attached organisms synthesize exopolysaccharides (i.e., glucans) that form a matrix, which acts as a scaffold for the biofilm, is biologically active and able to retain water, nutrients and enzymes (Marsh, 2006). The advantages for bacteria bound to biofilm over their planktonic counterparts include increased metabolic efficiency, substrate accessibility, enhanced resistance to environmental stress and inhibitors/antimicrobials, and an increased ability to cause infection and disease (Percival, *et al.*, 2012).

Once established, biofilms are difficult to remove or eradicate completely. In this environment, the microbial cells exhibit a different phenotype than their planktonic counterparts, particularly relating to decreased growth rate and altered gene transcription (Marsh, *et al.*, 2011). The gene expression patterns have been studied mostly on *Streptococcus* species that predominate in supragingival plaque, but similar principles can also be applied to subgingival organisms (Li & Burne, 2001, Svensäter, *et al.*, 2001, Welin, *et al.*, 2004). During the first 2 h of streptococcal attachment 33 proteins were found expressed differently - 25 proteins were up- and 8 down-regulated (Welin, *et al.*, 2004). Moreover, novel proteins of yet unknown functions were detected by biofilms that were not present in planktonic cells (Li & Burne, 2001). Also, genes involved in growth and biosynthesis of cofactors are down-regulated while transport and binding proteins are up-regulated (Lo, *et al.*, 2009). However, during plaque formation, bacteria not only bind to host proteins, but also co-aggregate with other organisms. Exposing *Streptococcus gordonii* to saliva induces genes encoding adhesins that promote binding to salivary glycoproteins and co-aggregation with *Actinomyces* species (Dû & Kolenbrander, 2000). Differential expression of proteins by *Porphyromonas gingivalis* that leads to community development was observed when *S. gordonii* and *Fusobacterium nucelatum* were present (Kuboniwa, *et al.*, 2009). Additionally, small diffusible molecules are expressed that facilitate quorum sensing throughout the biofilm and enable bacteria to act as a complex multicellular entity (Kolenbrander, *et al.*, 2002, Suntharalingam & Cvitkovitch, 2005).

A mature biofilm contains bacteria that live in their own heterogeneous (micro)environmental niches. The metabolic activities of the cells together with diffusion processes result in concentration gradients of oxygen and nutrients, signalling compounds

and bacterial waste within biofilms. As bacteria respond to these gradients and to changing local chemical conditions, biofilms can be exhibit considerable structural, chemical and biological heterogeneity. Thus, cells found in biofilms can be not only physiologically different from planktonic cells, but also differ from each other as the biofilm development proceeds (Stewart & Franklin, 2008, Marsh*, et al.*, 2011).

The heterogeneity, down-regulation of growth related genes and the matrix of exopolysaccharides that covers the cells is generally thought to explain the increased tolerance to antimicrobial agents. For example, 10 – 50 times higher concentrations of chlorhexidine are needed to eliminate *Streptococcus sanguinis* in biofilms compared to their planktonic counterparts (Larsen & Fiehn, 1996), however in case of *Streptococcus sobrinus* it can be up to 300 times higher (Shani*, et al.*, 2000). Biofilms of several species have also shown to be more tolerant to antibiotics (e.g. amoxicillin, minocycline and metronidazole), although the susceptibility differs between the species (Larsen, 2002, Socransky & Haffajee, 2002, Takahashi*, et al.*, 2007). Additionally, confocal microscopy studies demonstrate that only outer layers of biofilms are affected by antimicrobials, suggesting either quenching of the agent at the biofilm surface or lack of penetration into the deeper layers (Zaura-Arite*, et al.*, 2001).

The knowledge of mechanisms of bacterial adhesion might provide a tool to control or influence biofilm formation; however, in strategies like blocking adhesin-receptors thereby eliminating attachment or co-adhesion the bacterial cells have shown the ability to express multiple types of adhesins that are invoked when major receptor is blocked (Hasty*, et al.*, 1992, Zhang*, et al.*, 2005). Additionally, although adhesion is necessary for colonization, the final proportions of a species within a mixed culture biofilm are dependent on the ability of the organisms to grow, outcompete neighbouring cells and adapt to local conditions within the biofilm (Marsh, 2006).

Oral microbiota that adhere and accumulate on implant surfaces as established biofilms and cause peri-implantitis is mixed, variable in its contents and in most cases dominated by diverse Gram-negative anaerobic bacteria (Mombelli & Décaillet, 2011). In the early stages of biofilm formation, adhesion is the primary interaction between cells and a predominance of facultative anaerobic streptococci are commonly found while Gram-negative strict anaerobic rods are present only in minor relative proportions (Mombelli*, et al.*, 1988, Pontoriero*, et al.*, 1994, 2002). Upon defects in the soft-tissue closure, implant surface comes in contact with proteins present in saliva (and possibly serum) that produce

an interphase between the surface of the implant and the initial microbial colonizers. Oral streptococci have been constantly shown to be the major primary colonizers constituting up to 80% of plaque bacteria within 4 to 8 h (Diaz*, et al.*, 2006, Dige*, et al.*, 2009). Viridans streptococci express numerous protein and lipoprotein adhesins that provide them with a broad capacity for binding human or bacterial receptors. For example, the genome of *S. sanguinis* encodes over 90 polypeptides that are predicted to be anchored on the surface of cells, potentially involved in adhesion and creating a series of prior conditions for the adhesion of secondary colonizers (Xu*, et al.*, 2007). However, subsequent growth and further co-adhesion of bacteria from the surrounding environment increase the local cell density and lead to the development of matured biofilm where Gram-negative anaerobes gain a more important role (Mombelli & Mericske-Stern, 1990). *F. nucleatum* coaggregates with virtually all other bacteria. It appears to act as bridging organism by binding other early colonizers such as streptococci, and later colonizers including well-described periodontal pathogens, such as *P. gingivalis*, *Prevotella intermedia*, *Treponema denticola* and *Tannerella forsythia* (Hultin*, et al.*, 2002).

Surface characteristics that influence biofilm formation

Titanium is hitherto the most used dental implant material. Its advantages are biocompability, biomechanical resistance, chemical inertia, low density and absence of toxicity (Grosgogeat*, et al.*, 1999, Siddiqi*, et al.*, 2011). The first generation of successfully used titanium implants were machined with smooth surface, while the second generation underwent chemical and topographical modifications as surface roughness, surface free energy, hydrophilicity, and surface chemistry are important factors for the biological process of osseointegration (Albrektsson & Wennerberg, 2004). For titanium, most of the surface properties are associated with the chemically stable and corrosion-resistant surface oxide (TiO_2) that spontaneously develops on implant surface when it is exposed to air (Rådegran*, et al.*, 1991). Surface oxide can vary in thickness and in microstructure; therefore, it plays a role in the surface topography whether the roughness of the surface is determined by the bulk material or by the thickness of formed oxide (Sul*, et al.*, 2002). Thus, many of the currently used oral titanium implants are not well characterized with respect to their surface properties, and whether the improved bone response is due to the surface roughness or the surface composition has remained unclear (Palmquist*, et al.*, 2010).

While these surface characteristics, although not well-understood, are important for the osseointegration of the implant, they also affect the microbial adherence on the implant. Bacterial adhesion to a bare material of known surface free energy can be well-estimated on the basis of the interfacial thermodynamics (Pratt-Terpstra*, et al.*, 1989). However, upon insertion to the oral cavity, materials are covered with a proteinaceous layer termed pellicle. This layer is composed of absorbed proteins, amongst them several enzymes, glucoproteins and other macromolecules and mediates interactions between the solid material, oral fluids and microorganisms (Lendenmann*, et al.*, 2000, Hannig*, et al.*, 2005, Hannig & Joiner, 2006). Thus, adhesion to protein-coated surfaces is a complex process. The initial pellicle formation is mediated by ionic and hydrophobic interactions, and van der Waals forces followed by unfolding and conformational changes of proteins that allow continuous adsorption of biomolecules (Hannig & Joiner, 2006, Hannig & Hannig, 2009). As mentioned above, the initial bacterial adhesion passes through a phase of weak and reversible binding before an irreversible attachment is established. Reversible initial binding occurs preferentially in the surface irregularities where bacteria are protected against mechanical shear forces (Quirynen & Bollen, 1995, Carlén*, et al.*, 2001, Marsh, 2006). Although some heterogeneity of the pellicles is expected, it was observed that pellicles on different substrata have a certain uniformity; the differences are not pronounced and seem to be attributed to the selective mechanisms of protein adsorption during the initial stages (Hannig & Hannig, 2009).

Pellicle formed *in vivo* reduces bacterial adhesion considerably irrespective of the underlying substratum and has a masking effect on the specific surface characteristics of the material. It has been noted that surface properties seem to influence mostly the early bacterial adherence and not the plaque maturation (Konradsson*, et al.*, 2006), as the composition of plaque on different surfaces featuring comparable roughness do not differ considerably (Quirynen*, et al.*, 1990). Interestingly, materials with low critical surface tension, so-called theta-surfaces, are unable to retain thick biofilms, at critical thickness the layers of plaque peel away (Baier, 2006).

In conclusion, physico-chemical surface properties of materials are only in part counterbalanced by pellicle formation. Despite the homogeneity and the masking effects of the pellicle, the conformational changes of the absorbed proteins on different materials are the most relevant factors for the bacterial adhesin receptors or for the effective sites of enzymes (Norde, 1995, Quinn*, et al.*, 2006, Goobes*, et al.*, 2008).

Peri-implantitis-related in vitro biofilms

Biofilm formation on teeth, restorative and implant materials is a complex process in the oral cavity that involves the microflora, salivary proteins and surface characteristics. Thus, numerous biofilm models have been developed to study different aspects of the formation, structure, gene expression and antimicrobial susceptibility of oral biofilms preferably in physiological conditions similar or close to those in the oral cavity (Busscher & van der Mei, 2006, McBain, 2009). *In vitro* model systems mimic the plaque formation with species-specific biofilms including up to 10 different species as demonstrated in the so-called Zurich biofilm model (Ammann*, et al.*, 2012). Microtiter plates are among the most-frequently-used biofilm model systems to study the structure (Thurnheer*, et al.*, 2004, Ammann*, et al.*, 2012) or antimicrobial susceptibility (Shapiro*, et al.*, 2002, Brändle*, et al.*, 2008). As small volumes of reagents are needed, there is an opportunity to run a large scale screening of various antimicrobials by varying multiple experiment parameters. The *in vitro* modelling system offers straightforward and user-friendly handling that can explain its widespread use. However, also several weaknesses are evident: it is a closed system, no flow goes into or out of the reactor during the experiment, thus causing the environment to change – the nutrients become depleted, signalling molecules accumulate, toxic waste builds up, etc (McBain, 2009). On the contrary, in the flow chamber systems, the bacterial suspension circulates allowing biofilm formation on the substrate while nutrients are renewed and waste products removed (Larsen & Fiehn, 1996, Weiger*, et al.*, 1999, Decker*, et al.*, 2003, 2003, Busscher & van der Mei, 2006, Corbin*, et al.*, 2011). Although oral cavity or implanted biomaterial surfaces are considered to be relatively stagnant environments where transport through liquid flow of suspended organisms plays a smaller role than sedimentation and diffusion, the fluid flow is found to be beneficial also to the adhesion of microorganisms (Busscher & van der Mei, 2006). It has been shown that increased fluid flow towards the surface results in faster adhesion of microorganisms due to higher mass transport, despite the presence of higher shear forces that stimulate their detachment. However, it is important to bear in mind that should the flow exceed a critical limit, wall shear rates become high enough to prevent adhesion or even lead to detachment (Liu & Tay, 2002, Busscher & van der Mei, 2006).

The flow chamber system that was applied throughout this series of studies has been successfully used in various other studies that have investigated initial bacterial adhesion

to different materials (Weiger*, et al.*, 1999, Hauser-Gerspach*, et al.*, 2007, 2008) or antimicrobial susceptibility of adherent cells (Decker*, et al.*, 2003, 2003, 2008, Vig Slenters*, et al.*, 2008).

Characterization of in vitro and in situ biofilms

The common practice is to analyze the microflora present in a biofilm by using tests based on DNA (microarray, PCR). While these enable to draw conclusions about the presence of specific pathogens, no further information about structure or viability of the biofilm can be obtained (Porras*, et al.*, 2002, Colombo*, et al.*, 2009).

Microscopic analyses have proven to be invaluable tools in describing the biofilms and providing indiscriminate views of the structure. Especially scanning electron microscopy (SEM) that allows high resolution and magnification is often used to illustrate biofilm formation. However, the images of biofilms might be compromised by the fact that bacteria embedded in the exopolysaccharide matrix (EPS) cannot be easily visualized (Al-Ahmad*, et al.*, 2009). Furthermore, as the bacterial cells adapt to growth in surface-associated communities, they often change their characteristic shape and size known from the planktonic growth, thus the identification of single species can be challenging (Stewart & Franklin, 2008).

Fluorescence *in situ* hybridization (FISH) combined with confocal laser scanning microscopy (CLSM) allows the visualization of spatial organization and quantification throughout the different layers of bacteria in biofilms. The technique enables 16S RNA based labelling of the bacteria, despite the EPS and has been frequently used in many studies over the last decade (Wecke*, et al.*, 2000, Thurnheer*, et al.*, 2004, Al-Ahmad*, et al.*, 2009, Dige*, et al.*, 2009, Schaudinn*, et al.*, 2009). It is worth noting that when the vitality status of the cells needs to be assessed neither of these microscopic approaches is able to give information.

Various approaches can be applied to assess vitality within biofilms after antimicrobial treatment. The widely used commercial kit Live/dead Baclight contains a pair of nucleic acid stains: a green fluorescent stain, Syto 9, which is membrane permeant and expected to stain all cells, and a red fluorescent stain, propidium iodide, which is membrane impermeant and should only stain cells with compromised membranes. This staining approach combined with confocal microscopy has been used to visualize the spatial patterns of antimicrobial action in biofilms (van der Mei*, et al.*, 2006, Aziz*, et al.*, 2010, Shen*, et al.*, 2011, Ordinola-Zapata*, et al.*, 2013). However, this kit is prone to potential

artefacts, such as the non-specific binding of propidium iodide, which result in flow cytometry experiments that the cells are scored both live and dead simultaneously (Stocks, 2004, Berney*, et al.*, 2007). Thus, the suitability of the staining should be critically evaluated for each system to ensure accurate estimation of bacterial vitality levels.

In contrast, conventional culture methods have proven to be efficient in testing the susceptibility of bacterial cells to antibiotics either by E-tests or by adding the antibiotic directly into the suitable growth agar (Skucaite*, et al.*, 2010, Mouratidou*, et al.*, 2011, Walter*, et al.*, 2011). As described earlier, cells in biofilms are often found with altered behaviour compared to their planktonic counterparts and cultivation of that kind of cells may pose a difficulty. In suboptimal growth conditions, the size of a cell population that is labelled viable but non-cultivable (VBNC) and defined by low levels of metabolic activity is complicated to estimate (Gilbert*, et al.*, 2002, Oliver, 2010). Moreover, these cells are able to switch again to being cultivable when the growth conditions improve, thus they influence the outcome of conventional tests and predispose patients to a possible (re)infection (Oliver, 2010).

Another method that is gaining more recognition in microbiology is isothermal microcalorimetry (IMC). Although also based on a conventional culturing principle, the method allows the monitoring of bacterial metabolic activity and bacterial growth in various types of samples, from soil to food microbiology (Braissant*, et al.*, 2010). Furthermore, as a quantitative analytical technique IMC provides real time information about the lag time and growth rate of bacteria or defines the relation between the quantities of substances that take part in a reaction or form a compound in a specific process (Buchholz*, et al.*, 2010, Braissant*, et al.*, 2013). The principle of the method is that all living systems produce heat and heat evolution can always be measured calorimetrically without any interference with the processes. In other words, IMC measures heat production or consumption, which is proportional to the rate at which any given chemical, physical or biological process is taking place (i.e., metabolic heat from bacterial growth). However, it must be noted that the detected heat-flow signal is non-specific and can be only handled as net signal related to the sum of all processes (Wadsö, 2002). Therefore, only carefully planned experiments are able to benefit fully from the sensitivity, accuracy, simplicity, and the amount of data the method provides. Unlike conventional culturing, IMC can detect a relatively small number of microorganisms – when considered that a single bacteria is reported to produce approximately 2 pW when

active, therefore only 10^5 bacterial cells are required to generate a detectable signal. Additionally, the range in which heat-flow can be measured reaches up to 2 W, that is orders of magnitude higher than microbes usually produce when cultivated in 1 – 3 ml of media (0.2 – 500 μW). As the baseline drift of the machine is typically around 0.2μW/24 h, the accuracy of the heat-flow measures in intermediate range (20 – 100 μW) can be expected to be close to 1% (Braissant*, et al.*, 2010).

The main challenge of the method is the translation of the data into useful microbiological data. When these data are not supported by any other measurement such as optical density (Alklint*, et al.*, 2005), cell counts (Kong*, et al.*, 2009), or other metabolic assays, conclusions are not easily drawn. However, microbial growth in batch culture goes through four phases: the lag phase, exponential growth phase, stationary growth phase and death phase, and these are reflected also in a typical heat curve (integrated heat-flow). Thus a growth curve modelling can be applied using Gompertz's growth curve equation that defines relationship between heat, maximum growth and lag time (Braissant*, et al.*, 2013). Many other models exists and have been thoroughly reviewed (Zwietering*, et al.*, 1990), however the use of these on IMC data is restricted and has been described in detail (Braissant*, et al.*, 2013).

A knowledgeable analysis of the data allows IMC to be used in different areas of microbiology. Liquid cultures are often preferred for rapid detection of bacterial growth in clinical settings. For example early detection of methicillin resistance in clinical isolates of *Staphylococcus aureus* (Baldoni*, et al.*, 2009) or experimental meningitis in cerebrospinal fluid (Trampuz*, et al.*, 2007) have been conducted using IMC. Since the method measures heat passively, culture growth on a solid or viscous medium as well as in a matrix can also be evaluated (Mihhalevski*, et al.*, 2011, Kabanova*, et al.*, 2012). Also, antimicrobial susceptibility has been tested by IMC as the inhibition of bacterial growth can be measured. Different studies have demonstrated antibiotic susceptibility with strains of *Staphylococcus aureus* (Baldoni, et al., 2009), *Enterococcus faecalis* (Furustrand Tafin*, et al.*, 2011) and antifungal susceptibility of *Aspergillus* species (Furustrand Tafin*, et al.*, 2012). Additionally, Howell & colleagues show that IMC is able to detect drug-resistant *Mycobacterium* strains more rapidly than the standardized conventional culturing and the method also allows the examination of new antimicrobial agents for their minimal inhibitory concentrations and their effects on bacterial cells (Howell*, et al.*, 2012).

Treatment of biofilms, infection control

A reasonable approach for the prevention of peri-implantitis would be to inhibit bacterial colonization already in the initial phase of biofilm formation. However, the modifications in surface characteristics and other factors of the implant material not only affect the microbial colonization, but play a role also in the osseointegration. Studies including an animal model observed that re-osseointegration failed to occur in the area previously exposed to bacterial contamination, but consistently occurred at sites where a new sterile implant component was placed in the bone defect following surgical debridement (Persson*, et al.*, 2001). It was further observed that substantial re-osseointegration occurred with implants having rough sandblasted and acid-etched surfaces (SLA), while bone growth on a previously exposed smooth surface (turned) was minimal (Persson*, et al.*, 2001).

Due to its infection-induced nature, the treatment of peri-implantitis is generally based on the control of implant-associated biofilms (Mombelli & Décaillet, 2011). Here it is important to note that bacteria in biofilms are always found with increased tolerance towards antimicrobial agents. They are up to 100 - 1000 times less susceptible to antibiotics than their planktonic counterparts as the antibiotics have restricted penetration through the biofilm (Gilbert*, et al.*, 2002). For example, to eliminate *S. sanguinis* in biofilms compared to their planktonic counterparts 10 – 50 times higher concentrations of chlorhexidine are needed (Larsen & Fiehn, 1996) and in case of *S. sobrinus* it can be up to 300 times (Shani*, et al.*, 2000). Moreover, microorganisms within biofilms respond to local environmental conditions through various ways, such as altering their gene-expression patterns or undertaking physiological activities to adapt to a particular condition (i.e., stress through antimicrobials, changes in pH). (Gilbert*, et al.*, 2002, Stewart & Franklin, 2008, Høiby*, et al.*, 2010) Thus, most commonly mechanical debridement combined with systemic antibiotic treatment is applied in the clinical treatment of peri-implantitis.

In general the treatment is laborious, time-consuming and the patient's compliance in terms of daily oral hygiene routines plays an essential role. Mechanical debridement, laser or ultrasonic devices, combined with antiseptics and/or antibiotics can be used against adherent microorganisms (Karring*, et al.*, 2005, Schwarz*, et al.*, 2005, Persson*, et al.*, 2006, Renvert*, et al.*, 2006, Salvi*, et al.*, 2007, Heitz-Mayfield*, et al.*, 2012). Regenerative surgical approaches can be considered within selected indications (Romeo*, et al.*, 2005,

Schwarz*, et al.*, 2006, 2007, 2008, Mombelli*, et al.*, 2012).

Therefore the physical and chemical characteristics of implant surfaces should be analysed in detail in terms of primary microbial adherence (Quirynen*, et al.*, 1999, Pier-Francesco*, et al.*, 2006). Moreover, in order to evaluate the efficiency of strategies for the elimination of biofilms, there is a need for validated *in vitro* models that allow effective investigation of different detection approaches on primary adherence and biofilm formation as well as post-treatment examination of the bacteria. Thus, as the supragingival-subgingival implant-associated biofilm formation is a complex process, modelling it remains a challenging quest even more so as this important investigation area is of major clinical importance.

2 Study aims

Data on the colonization of dental implant surfaces by oral microorganisms is limited and covers mostly the identification of microbial species. However, knowledge on the colonization, in particular on the early stages, is essential for the development of strategies to control microbial adherence and biofilm formation on implant surfaces.

The aims of this study were:

1) To utilize three different methods – live/dead staining, conventional culture method and IMC – to quantify vital adherent *S. sanguinis* cells after direct exposure to disinfectants on protein covered titanium test specimens. Furthermore, to determine whether the results were in agreement or provided a more complete picture together than any method used alone.
2) To develop and characterize a peri-implantitis-related three-species biofilm by well-established commonly-used microscopic methods and to complement this information by using IMC to determine various measures of the metabolic activity.
3) To determine the metabolic activity of our *in vitro* three-species biofilm associated with peri-implantitis in the presence of amoxicillin, metronidazole or their combination by IMC.

3 Hypotheses

1) The anaerobic flow-chamber model provides an efficient tool allowing the analysis of microbial adherence *in vitro*. Adherent microorganisms and their vitality status can be examined by fluorescence microscopy and IMC analysis.
2) Efficacy of disinfection protocols is difficult to estimate as vitality staining and methods based on culturing might pose discrepancies in bacterial survival rates.
3) Adhesion of pathogenic bacteria found in peri-implantitis, *P. gingivalis* and *F. nucleatum*, is enhanced in the presence of primary colonizer, *S. sanguinis*.
4) An increased resistance in comparison to planktonic microorganisms is detected by single and multispecies biofilms on implant surface *in vitro*.
5) IMC allows investigation of intact biofilms at low metabolic levels and assessment of the antimicrobial effect of antibiotics.

4 Quantification of vital adherent *Streptococcus sanguinis* cells on protein-coated titanium after disinfectant treatment

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Quantification of vital adherent *Streptococcus sanguinis* cells on protein-coated titanium after disinfectant treatment

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Abstract The quantification of vital adherent bacteria is challenging, especially when efficacy of antimicrobial agents is to be evaluated. In this study three different methods were compared in order to quantify vital adherent *Streptococcus sanguinis* cells after exposure to disinfectants. An anaerobic flow chamber model accomplished initial adhesion of *S. sanguinis* on protein-coated titanium. Effects of chlorhexidine, Betadine®, Octenidol®, and ProntOral® were assessed by quantifying vital cells using Live/Dead BacLight™, conventional culturing and isothermal microcalorimetry (IMC). Results were analysed by Kruskal–Wallis one-way analysis of variance. Live/dead staining revealed highest vital cell counts ($P < 0.05$) and demonstrated dose-dependent effect for all disinfectants. Microcalorimetry showed time-delayed heat flow peaks that were proportioned to the remaining number of viable cells. Over 48 h there was no difference in total heat between treated and untreated samples ($P > 0.05$), indicating equivalent numbers of bacteria were created and disinfectants delayed growth but did not eliminate it. In conclusion, contrary to culturing, live/dead staining enables detection of cells that may be viable but non-cultivable. Microcalorimetry allows unique evaluation of relative disinfectant effects by quantifying differences in time delay of regrowth of remaining vital cells.

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1 Introduction

Reliable quantification of vital adherent microorganisms on surfaces of various biomaterials is challenging. Fluorescence microscopy and conventional culturing methods have commonly been applied for the quantification of vital bacteria after direct exposure to disinfectants [1–9].

Determination of vital and dead bacterial cells by fluorescence microscopy is based on the intactness of the cell membrane. The fluorescence agent Syto 9 labels all the cells, whereas propidium iodide attaches only on cells with damaged membranes. Intact and damaged cells appear green and red when exposed to wavelengths of 450–490 and 546 nm, respectively [10]. This practical technique has been widely used in assessment of the vitality status of planktonic and adherent microorganisms [1–10]. Due to the three-dimensional nature of well-established biofilms such Syto 9/propidium iodide-based fluorescence microscopy has commonly been combined with confocal laser scanning microscopy. This enables observations also in the deeper layers of the biofilm [4, 6, 11, 12]. Determination of the vitality of adherent microorganisms by conventional culturing requires their resuspension prior to cultivation and subsequent quantification of colony-forming units. Several factors—such as physical damage to the cells by resuspension or lack of growth due to suboptimal culture conditions—may cause the culture method to yield partially false negative results. Consequently, using both fluorescent microscopy and

conventional culture methods commonly give results which are not always in accord [13]. This may also be due to viable but non-cultivable (VBNC) microorganisms [14]. Despite their typically low levels of metabolic activity, bacteria may switch again to being cultivable when the culture conditions improve, a process called resuscitation [15].

Isothermal microcalorimetry (IMC) has become a promising tool for monitoring heat flow generated by various (micro)biological metabolic activities in vitro. Modern microcalorimeters are very sensitive and can measure heat production rates of less than a microwatt—equivalent to the combined metabolic output of a few hundred thousand active bacteria [16]. Thus, metabolism and growth of relatively limited numbers of bacteria can be monitored continuously and accurately at any chosen temperature [17–19]. In microbiology, microcalorimetry has been used to determine replication rates of bacterial cells [20, 21], effects of biocides on microbial activity [22, 23], and bacterial coaggregation [24] as well as to identificy bacterial species by the patterns of their heat flow rate curves [25–28]. Hauser-Gerspach et al. [29] showed that IMC allows estimating the rate of bacterial adhesion onto surfaces. In that study IMC could also differentiate effects of buffer (saline vs. saliva) and material surface area, using different baseline numbers of readily-adherent *Streptococcus sanguinis*.

Based on its ability to measure growth and also detect bactericidal (and bacteriostatic) effects, IMC could be suitable for the determination of the number of vital adherent bacteria on various surfaces. Integration of the heat flow curves (μW = vs. μJ/s vs. time) obtained by IMC provides the cumulative amount of heat (J vs. time) which is proportional to the cumulative amount of biomass formed. This allows determination of time the bacteria need to reach the exponential growth phase using the growth model of Gompertz [30, 31]. This lag time depends on the bacterial concentration of the sample; therefore defining it as a dependent variable to detect differences between standardized concentrations, provides a mathematical relationship between the time and concentration applicable for any treated sample. Additionally, as IMC allows following the microbial activity of the bacteria over a longer period of time, and thus it can be used to detect whether resuscitation of the VBNC occurs under a given set of conditions.

The aims of this study were to (a) utilize three different methods—live/dead staining, conventional culture method and IMC—to quantify vital adherent *S. sanguinis* cells after direct exposure to disinfectants on protein covered titanium test specimens and (b) determine whether the results were in agreement—or perhaps together provided a more complete picture than any single method.

2 Materials and methods

2.1 Microorganism

Streptococcus sanguinis (DSM 20068; German Collection of Microorganisms and Tissue Culture Cells, Braunschweig, Germany) was used throughout the study. A 10 μl inoculum of *S. sanguinis* in skim milk solution (from −20°C) was suspended in 5 ml Schaedler broth (BBL™, Becton–Dickinson, Basel, Switzerland) and incubated at 37°C for 8 h. From the 8-h-old culture a new subculture (1:50) was made, which was grown at 37°C for 16 h. Thereafter the culture was ultrasonicated for 30 s (30 W; Vibracell, Sonics & Materials, Newtown, CT); centrifuged at 8500 rpm for 5 min, washed with 0.9% NaCl and the cells were harvested again by centrifugation. Bacteria were resuspended in simulated body fluid (SBF) [32] to a density of 10^8 cells/ml.

2.2 Saliva and serum

The saliva was collected (using paraffin chewing stimulation to augment production) from three healthy volunteers according to a recently reported procedure [33]. Briefly, the collected saliva was ultrasonicated for 30 s (30 W; Vibracell, Sonics & Materials, Newtown, CT), filtered through a 70 μm filter (Cell Strainer, Becton–Dickinson, Basel, Switzerland) and centrifuged at 22,000×*g* for 40 min at 4°C. The supernatant was filtered through two connected filters (0.45 and 0.22 μm; Millex-HV and Millex-GV, respectively; Millipore, Switzerland) and frozen at −20°C in aliquots of 10 ml corresponding the need for each individual experiment.

Serum used was a pool from five persons (Blutspendezentrum SRK, Basel, Switzerland). The pH of the serum/saliva mixture was adjusted to 7.2 by adding potassium and sodium phosphate buffers (0.067 mol/l).

2.3 Substrate for adhesion

Polished titanium disks (mean roughness of 120 nm), 5 mm diameter and 1 mm thick (commercial pure titanium grade 2, ASTM F-67, Straumann AG, Waldenburg, Switzerland) sterilized by steam autoclaving and gamma irradiation were used as substrates for *S. sanguinis* adherence. The sterile disks were exposed for 15 min to freshly mixed serum/saliva (1:10) solution prior to each experiment in order produce protein pellicle formation [33].

2.4 Anaerobic flow chamber model

The flow chamber model has recently been described in detail [33]. Briefly, the system is comprised of a flow

chamber with the test specimen, a polytetrafluoroethylene dispenser containing the bacterial suspension and a peristaltic pump with an integrated speed controller (flow rate at 0.8 ml/min), all the parts are connected by polyvinylchloride tubes. The dispenser and flow chamber are placed on a shaker (260 rev/min) to maintain the homogeneity of the suspension. Circulating bacteria were allowed to adhere on the protein-coated disks under anaerobic conditions (MACS MG, Don Whitley Scientific Ltd; atmosphere of 80% N_2, 10% H_2 and 10% CO_2) at 37°C for 2 h.

2.5 Treatment of adherent *S. sanguinis* cells with disinfectants

Four different disinfectants were used in this study: chlorhexidine (chlorhexidin-D Mundspülung, 0.125% chlorhexidine digluconate; Universitätsspital Basel, Basel, Switzerland), Betadine® (0.75% povidone-iodine; Mundipharma, Medical Company, Basel, Switzerland), Octenidol® (0.1% octenidine; Schülke & Mayr GmbH, Norderstedt, Germany) and ProntOral® (0.1–0.25% polyhexamethylene biguadine; B. Braun Medical AG, Sempach, Switzerland). Each of the four was tested at four different concentrations, which are shown in Table 1. The protein-coated disks with adherent *S. sanguinis* cells were removed from the anaerobic flow chamber after 2 h, dipped gently in sterile saline to remove any non-adherent cells and placed in disinfectant solutions for 30 s. The disk incubated in sterile saline served as a control. After the exposure to the solutions, the disks were gently dipped in sterile saline. The sides and the bottom of the disks were decontaminated with 70% ethanol for 30 s. Thereafter the specimens were prepared either for live/dead staining and fluorescence microscopy, for culturing by the conventional method or for IMC.

2.6 Detection of the vitality status of the adherent bacteria

2.6.1 Live/dead staining and fluorescence microscopy

The adherent cells were stained by dual fluorescent dyes (Live/Dead BacLight™ bacterial Viability Kit; MoBiTec, Luzern, Switzerland), and analyzed microscopically (x40; Provis AX70, Olympus AG, Volketswil, Switzerland). It has been hypothesized that the use of two fluorescent dyes allows differentiation between vital and dead cells. Syto9 stains all cells but propidium iodide only stains those with damaged membranes. Microscopic examination using FITC and Cy3 light filters respectively reveals first all cells and then those which are stained by both dyes and are thus "dead". From each disk five randomly selected microscopic fields each of 0.024 mm^2 were photographed and the bacteria on the area counted by Analysis program (Olympus AG, Volketswil, Switzerland). All experiments were done three times.

2.6.2 Conventional culture methods

The specimens for conventional culture methods were placed in 1 ml 0.9% NaCl, vortexed for 1 min and treated with ultrasound for 15 s (22.5 W; Vibracell, Sonics & Materials, Newtown, CT) to remove adherent cells from the disks. Serial dilutions were made in sterile saline and aliquots of 0.1 ml were plated on Columbia blood agar plates (BBL™, Becton–Dickinson, Basel, Switzerland; supplemented with 50 ml/l of human blood, 0.5 mg/l of menadione, and 5 mg/l of hemin) in duplicates and incubated anaerobically at 37°C for 48 h. Based on colony morphology and cellular characteristics the purity of the cultures was controlled, colonies were counted and quantified (CFU/ml). All experiments were done three times.

2.6.3 Microcalorimetric analysis

The IMC instrument (TAM 48, TA Instruments, New Castle DE USA) was first set at 37°C. The titanium disks were then placed in microcalorimetric ampoules filled with 3 ml of Schaedler broth. Each sample was ultrasonicated for 5 s (7.5 W; Vibracell). The ampoules were closed under aerobic conditions and each was placed in the equilibration position in one of the 48 chambers of the IMC instrument for 15 min to allow initial thermal equilibration. After this equilibration phase, the samples were lowered into measuring position and measurement started after 45 min (i.e., the time required to obtain full thermal

Table 1 Disinfectants and their concentrations used in this study

Product name	Antimicrobial agent	Stock concentration (%)	Used concentrations in this study			
			1:1 (%)	1:2 (%)	1:4 (%)	1:8 (%)
Chlorhexidine	Chlorhexidine digluconate	0.125	0.125	0.063	0.031	0.016
Betadine®	Povidone-iodide	0.750	0.750	0.375	0.188	0.094
Octenidol®	Octenidine HCl	0.100	0.100	0.050	0.025	0.013
ProntOral®	Polyhexamethylene biguanide	0.100–0.250	0.100–0.250	0.05–0.125	0.025–0.063	0.013–0.031

equilibration). During all the measurement time, the instrument maintained the set temperature of 37°C with an accurracy of 0.02°C and a temperature drift less than 2μ°C and heat flow (μW = μJ/s) versus elapsed time was recorded for each ampoule. Calibration of the microcalorimetric experiments was done by determining the lag times of different concentrations of *S. sanguinis* suspension (10^2, 5×10^2, 10^3, 5×10^3, 10^4, 5×10^4, 10^5 CFU/ml) in 3 ml Schaedler broth (BBL™, Becton–Dickinson, Basel, Switzerland) in duplicates. The samples were ultrasonicated for 5 s (7.5 W; Vibracell), closed and placed into the IMC at 37°C for analysis. Each experiment was done three times.

For each sample the lag time was calculated by fitting the Gompertz's equation for describing growth curves to the integrated heat-flow curve (i.e. J vs. time) [30, 31]. Curve fitting was performed using the R software and the "grofit" package. So that heat flow (growth) data for the disinfectant-treated samples could be analyzed, the relationship between the CFU counts and lag time from the calibration assays was determined. The linear regression model was obtained by Stata 10 resulting in equation: $\ln(\text{CFU}) = 17.972 - 1.072 * \text{lag time(h)}$, with $R^2 = 0.9896$. The equation allowed determination of the initial concentration of bacteria in each sample.

Additionally, the maximum growth rate of the bacteria and the accumulated heat over 48 h were determined using the Gompertz equation as well, in order to see if there were differences in these parameters between disinfectant-treated and untreated control samples.

2.7 Statistical analysis

For all three methods the results were evaluated as vitality percentages of the samples. Therefore, the vital cell counts in each of the treated samples were divided by the vital cell counts for untreated control samples and multiplied by 100. All the results were analyzed by Stata 10 software for the Kruskal–Wallis analysis of variance.

3 Results

3.1 Anaerobic flow chamber

Bacterial suspension inside the anaerobic flow chamber system maintained the pH and CFU counts at the same level throughout the 2 h, indicating that the conditions for the bacteria did not change during the experiment. At the beginning and at the end of the experiment the CFU counts per ml were $6.38 \times 10^8 \pm 2.18 \times 10^7$ and $6.40 \times 10^8 \pm 1.84 \times 10^7$, respectively. The corresponding pH values ranged from 7.27 ± 0.05 and 7.20 ± 0.06, respectively.

3.2 Live/dead staining and fluorescence microscopy

Vitality percentages of the adherent cells after the different disinfectant treatments are presented in Fig. 1. The vitality percentage was calculated in relation to the control that was present in each disinfectant group. A dose-dependent effect was observed for chlorhexidine, Betadine® and ProntOral®. Octenidol® treatment resulted in a small statistically not significant inconsistency between the 1:8 and 1:4 dilutions, which could be explained by the high efficacy of the disinfectant, resulting overall in a very small percentage of vital cells at both dilutions. Disinfectant treatment resulted in significant differences among the different concentrations of the same disinfectant in all four cases ($P < 0.05$).

Almost no effect on the vitality of the adherent *S. sanguinis* cells was detected by the lowest concentration of chlorhexidine and Betadine®. In comparison, Octenidol® and ProntOral® reduced the vitality of the cells far more efficiently at the three lowest concentrations than did chlorhexidine or Betadine®. No significant differences were observed among the undiluted original solutions.

3.3 Conventional culture method

The results of the colony forming unit (cfu-based) experiments are shown in Fig. 2. (In untreated control samples an average of 2.3×10^4 CFU/ml was detected.) A dose-dependent effect was observed only with chlorhexidine by this method. Significant differences between the different concentrations of the same disinfectant were present in the chlorhexidine group ($P < 0.05$); the other three disinfectants gave no significant differences for different concentrations. The vitality of the cells was reduced to less than 1% by the treatment with Betadine®, Octenidol® and ProntOral® at all concentrations except 1:8 Octenidol®.

A remarkably lower percentage of vital cells were detected by the CFU count experiments than by fluorescent

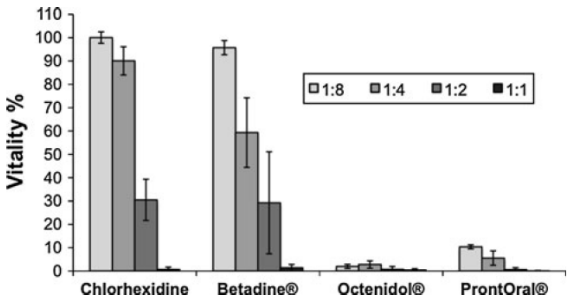

Fig. 1 Vitality percentage of disinfectant-treated samples compared to the untreated control measured by live/dead staining method. As a result mean values with standard deviations are given ($n = 3$)

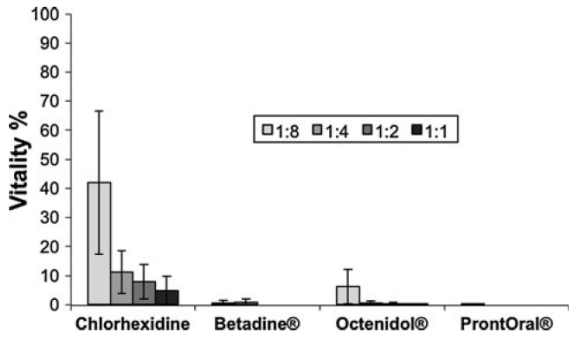

Fig. 2 Vitality percentage of disinfectant-treated samples compared to the untreated control measured by conventional culturing method. Mean values with standard deviations are given ($n = 3$)

staining. The biggest difference between the two methods was detected for the treatment with Betadine®. The differences in results for the Octenidol® and ProntOral® groups were smaller between the two methods, but still significant at lower concentrations.

3.4 Isothermal microcalorimetry (IMC)

The vitality percentages determined by IMC are shown in Fig. 3. (In the untreated control 2.8×10^4 CFU/ml were detectable). No dose-dependent effect was observed by this method for reasons explained later. Significant differences among the different concentrations of the same disinfectant were present only in the chlorhexidine group ($P < 0.05$), the other three disinfectants had no significant differences among the results for different concentrations.

The vitality percentages of the cells were lower when determined by IMC compared to either of the other two methods. The highest vitality rate was observed after the treatment with 1:8 dilution of chlorhexidine (73.32% ± 17.24). More than 1% of vital cells were detected just by four other solutions: 2.74% ± 1.16 by 1:4 of chlorhexidine, 14.49% ± 2.87 by 1:8 of Octenidol®, 10.21% ± 2.49 by 1:8 of ProntOral® and 4.28% ± 3.28 by 1:2 of ProntOral®.

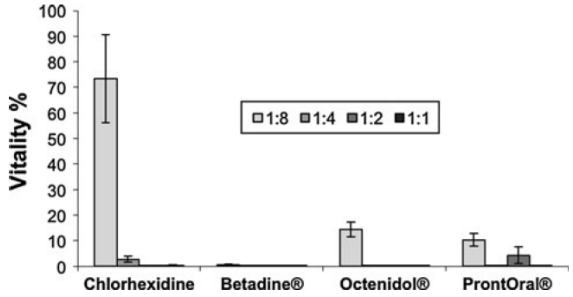

Fig. 3 Vitality percentage of disinfectant-treated samples compared to the untreated control measured by isothermal microcalorimetry. Mean values with standard deviations are given ($n = 3$)

The mean maximum growth rate in control samples was $1.14 \pm 0.21\ h^{-1}$ and for the treated samples grouped all together $1.14 \pm 0.78\ h^{-1}$. The average accumulated heat produced by control samples in 48 h was 6.54 ± 0.13 J and this of the treated samples grouped all together 5.74 ± 0.25 J. No significant difference was detected between the untreated control sample and any of the treated samples for either of these parameters ($P > 0.05$), which indicates that equivalent numbers of bacteria were created and disinfectants delayed growth but were not able to eliminate it.

4 Discussion

In the present study three methods were used to analyse the vitality rates of adherent bacteria after direct exposure to four disinfectants in order to compare the methods and define their optimal usage. The techniques applied were: live/dead staining and two methods based on the cultivability of the cells—the conventional culture method to count survived bacteria as colonies on a solid media, and IMC—using the cumulative heat production versus time curves of the samples to quantify the amount of the cells in the liquid media. To achieve comparable data, the results revealed by each method were converted into vitality percentages ± SD.

It is well-known that bacterial response to chemical and environmental stress results in activating low-metabolism survival mechanisms. A common related response to such stress on bacterial cells is an inability to develop into colonies on routine culture media, even though the cells may remain viable for long periods of time. As cells in the viable but non-cultivable (VBNC) state are no longer culturable, alternate nonculture methods must be used to demonstrate their vitality. A commonly used method is staining (e.g. the Live/Dead BacLight™ assay) designed to demonstrate the percentage of cell exhibiting cytoplasmic membrane integrity (and presumed viability), through direct microscopic examination [4, 10]. Despite their typically low levels of metabolic activity, these bacteria are again culturable upon resuscitation. The VBNC cells are one of the major hindrances each treatment protocol might encounter. Under suitable conditions the VBNC cells are able to overcome the stress and re-grow to the same density the cell population had before the applied treatment. Therefore, monitoring the behaviour of the disinfectant-treated bacteria in their favourable environment leads to a better insight of what is happening to the VBNC cells.

All disinfectants showed dose-dependent effects throughout the study with a minor and not significant exception in the Octenidol® group in results of the live/dead staining. This can be explained by the high efficacy of the disinfectant reducing the percentage of vital cells to less than 5%. The vitality percentages measured by live/dead staining were considerably higher than those measured by either the conventional culture methods or IMC, especially for chlorhexidine and Betadine®. On one hand, a possible high population of VBNC cells (with intact membranes) may lead to higher viability percentages for live/dead staining than the methods based on culturing [15]. On the other hand, lower viability percentages for the culture methods could be due to killing of more cells through the necessary handling procedures. However, bacteriostatic effects of disinfectants remain beyond the sensitivity of the live/dead method. In case of Betadine®, which is based on an iodide complex, the interference with the propidium iodide in the staining step should be considered as a confounding factor that might reduce the percentage of dead cells correctly stained. Thus, for the first time ever the present results indicate that live/dead staining may have severe limitations for in vitro susceptibility testing due to possible interactions with iodine-based antimicrobial agents.

The CFU/ml determined in untreated control samples showed no significant difference between the conventional culture method and microcalorimetry, thereby making the discrepancy in the results between the methods even more obvious. An immense difference in results was observed in the samples treated with chlorhexidine, where a dose-dependent effect was detected by conventional culture and by live/dead staining, but IMC only detected a significant amount of bacteria in the sample treated with lowest concentration of the disinfectant.

The IMC experiments were run at 37°C for 48 h in a suitable media for *S. sanguinis* to grow. Such a setup enables one to monitor also the long-term bactericidal effects of the disinfectants because any unkilled cells need considerable time to become metabolically active again and replicate sufficiently to result in a detectable heat flow. Over 48 h there was no difference in total heat between treated and untreated samples ($P > 0.05$), indicating equivalent numbers of bacteria were created and disinfectants delayed growth but did not eliminate it. The sample treated with a 1:8 dilution of chlorhexidine was the only one which showed a high rate of vitality by microcalorimetry at the beginning of the experiment.

In this study model, 48 h was enough to observe by IMC the resuscitation and growth of the disinfectant-treated bacteria to the same level as the untreated control, despite the significant differences in the starting disinfectant concentrations.

5 Conclusions

Live/dead staining is intended to detect bacterial cell death based on destruction of the cell membrane. The higher percent viable cells—right after treatment—found by this method compared to the culture methods indicates that the higher percentage of viable cells determined by live/dead staining also includes VBNC cells.

The conventional culture method used was not able to detect VBNC cells and thereby underestimates the count of bacteria able to re-colonize the surface. Although IMC is based on culturing, too, it allows unique evaluation of relative disinfectant effects by quantifying differences in time delay of regrowth of remaining vital cells—i.e. cells that would be classified as VBNC cells by conventional culture. As a completely "non-invasive" monitoring method with high sensitivity, reproducibility and simplicity, IMC is a more appealing method of choice than conventional culture method to observe the efficacy—and dynamics—of disinfectants in vitro.

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5 Isothermal microcalorimetry provides new insights into biofilm variability and dynamics

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RESEARCH LETTER

Isothermal microcalorimetry provides new insights into biofilm variability and dynamics

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oral biofilm; metabolic activity; heat flow; scanning electron microscopy; fluorescence *in situ* hybridization; isothermal microcalorimetry.

Abstract

The purpose of this study was to investigate a three-species *in vitro* biofilm with peri-implantitis-related bacteria for its variability and metabolic activity. *Streptococcus sanguinis*, *Fusobacterium nucleatum*, and *Porphyromonas gingivalis* were suspended in simulated body fluid containing 0.2% glucose to form biofilms on polished, protein-coated implant-grade titanium disks over 72 h using a flow chamber system. Thereafter, biofilm-coated disks were characterized by scanning electron microscopy and fluorescence *in situ* hybridization/confocal laser scanning microscopy. To assess metabolic activity within the biofilms, their heat flow was recorded for 480 h at 37 °C by IMC. The microscopic methods revealed that the total number of bacteria in the biofilms varied slightly among specimens ($2.59 \times 10^4 \pm 0.67 \times 10^4$ cells mm^{-2}), whereas all three species were found constantly with unchanged proportions (*S. sanguinis* 41.3 ± 4.8%, *F. nucleatum* 17.7 ± 2.1%, and *P. gingivalis* 41.0 ± 4.9%). IMC revealed minor differences in time-to-peak heat flow (20.6 ± 4.5 h), a trend consistent with the small variation in bacterial species proportions as shown by microscopy. Peak heat flow (35.8 ± 42.6 μW), mean heat flow (13.1 ± 22.0 μW), and total heat over 480 h (23.5 ± 37.2 J) showed very high variation. These IMC results may be attributed to differences in the initial cell counts and relative proportions of the three species, their distribution and embedment in exopolysaccharide matrix on the test specimens. The present results provide new insights into variability and dynamics of biofilms on titanium disks, aspects that should be explored in future studies of dental surfaces.

Introduction

Biofilms can be described as communities of microbiota with associated extracellular polymeric matrix on a substrate. Microorganisms within such biofilms respond to local environmental conditions in various ways, such as altering their gene-expression patterns or undertaking physiological activities to adapt to a particular condition/location within the biofilm. This reveals heterogeneities within the bacterial population (Stewart & Franklin, 2008). Furthermore, as the microbial cells adapt their growth within surface-associated communities, they often change their characteristic shape and size from those that they exhibit during planktonic growth, thus making their microscopic identification challenging (Costerton, 1999; Webster *et al.*, 2004). Natural variants within biofilms increase tolerance of antimicrobial agents (Drenkard & Asubel, 2002) and help to adapt to environmental conditions (Klein *et al.*, 2010).

Well-developed biofilms on dental implant surfaces cause peri-implantitis, an infection-induced inflammation that is one of the main causes of dental implant failure (Paquette *et al.*, 2006). Due to the complex nature of the supragingival/subgingival implant-associated biofilm formation, *in vitro* modeling is challenging. However, it may offer an efficient approach for studying biomaterials and biofilms, including their responses to therapeutic interventions. Recent reports on early colonization and biofilm

formation on implant surfaces indicate the urgent need for further developments in dental materials science and infection control (Quirynen *et al.*, 2006; Fürst *et al.*, 2007; Heuer *et al.*, 2007; Salvi *et al.*, 2008; Pye *et al.*, 2009; Mombelli & Décaillet, 2011).

Microscopic analyses have proven to be invaluable tools in describing biofilms in terms of their structure and association with a surface. Scanning electron microscopy (SEM) allows a high-resolution and magnification. However, SEM cannot be used to visualize bacteria embedded in the exopolysaccharide matrix (EPS) (Marrie *et al.*, 1982). As a complement to SEM, fluorescence *in situ* hybridization (FISH) combined with confocal laser scanning microscopy (CLSM) allows the observations of the spatial organization and quantification of bacterial biofilms using 16S rRNA gene-labeled probes even within EPS matrix (Amann, 1995; Paster *et al.*, 1998; Schwartz *et al.*, 2003; Thurnheer *et al.*, 2004; Al-Ahmad *et al.*, 2009). In various studies over the last decade, these methods have facilitated direct observations to characterize the bacterial distribution within oral biofilms (Wecke *et al.*, 2000; Thurnheer *et al.*, 2004; Dige *et al.*, 2009; Schaudinn *et al.*, 2009). Neither of these microscopic approaches, however, is sufficient to give real-time information about the dynamics of the metabolic activity and biomass formation within biofilms; rather, they only provide sequential periodic 'snapshots,' over time, of the structure and composition of the biofilm.

Isothermal microcalorimetry (IMC) is a highly sensitive analytical tool that provides, in real time, the progress of a chemical and physical process. All such processes produce or consume heat. The heat flow ($W = J\ s^{-1}$) is proportional to the reaction rate, and total heat over time (J) is a measurement of the extent of the process. IMC captures heat flow in the microwatt (μW) range and enables detection of the metabolic heat evolved from ca. 10 000 mammalian cells or ca. 100 000 bacteria (Braissant *et al.*, 2010). Thus, IMC has the potential to provide real-time quantitative data on metabolic activity, aggregation, and biomass formation in biofilms *in situ*. The sensitivity of IMC has been exploited in evaluating metabolism and growth of living cells in culture in medical and environmental microbiology (Howell *et al.*, 2012). While IMC has been applied to study the co-aggregation of different strains of biofilm-forming bacteria (Postollec *et al.*, 2003), studies that focus on the use of this technique for investigating *in vitro* multispecies biofilms are scarce.

The purpose of this study was to characterize a peri-implantitis-related biofilm by well-established commonly used microscopic methods and to complement this information using IMC to determine various measures of the metabolic activity. A three-species biofilm was allowed to form on surfaces of protein-coated titanium disks in a newly developed anaerobic flow chamber system. The selected bacterial species were an early colonizer, *Streptococcus sanguinis*; a pathogenic bridging organism, *Fusobacterium nucleatum*; and a common periodontal and peri-implant pathogen, *Porphyromonas gingivalis* (Quirynen *et al.*, 2006; Fürst *et al.*, 2007; Heuer *et al.*, 2007).

Materials and methods

Bacterial suspensions

Streptococcus sanguinis (DSM 20068), *F. nucleatum* (ATCC 10953), and *P. gingivalis* (DSM 20709) were used for the biofilm formation. A 10 μL inoculum of *S. sanguinis* in skim milk solution (stored at −20 °C) was suspended in 5 mL Schaedler broth (BBL™; Becton Dickinson, Basel, Switzerland) and incubated aerobically at 37 °C for 8 h. The bacterial suspension was used as an inoculum for a new subculture (1 : 50), which was incubated aerobically at 37 °C for 16 h. The culture was ultrasonicated for 30 s (22.5 W; Vibracell, Sonics & Materials, Newtown, CT), centrifuged at 5700 ***g*** for 5 min at room temperature, washed with physiological saline, and harvested by centrifugation. The *S. sanguinis* cells were resuspended in simulated body fluid (Cho *et al.*, 1995) to a density of $1.1 \times 10^8 \pm 6.2 \times 10^7$CFU mL^{-1}. *Fusobacterium nucleatum* and *P. gingivalis* were maintained in Microbank® blue vials (Chemie Brunschwig AG, Basel, Switzerland) at −70 °C. One pearl of each frozen culture was inoculated into 10 mL thioglucolate aliquots (Biomerieux SA, Geneva, Switzerland), enriched with 5 μg mL^{-1} hemin (Fluka, Buchs, Switzerland) and 0.5 μg mL^{-1} menadione (VWR International, Dietikon, Switzerland), and incubated anaerobically at 37 °C for 96 h. The cultures were harvested; *F. nucleatum* and *P. gingivalis* were suspended to a density of $3.2 \times 10^7 \pm 1.9 \times 10^6$ CFU mL^{-1} and $2.1 \times 10^9 \pm 9.3 \times 10^8$ CFUmL^{-1}, respectively.

Anaerobic flow chamber

Details and uses of the flow chamber system have been presented previously (Weiger *et al.*, 1999; Decker *et al.*, 2003a, b, 2008; Hauser-Gerspach *et al.*, 2007; Meier *et al.*, 2008; Vig Slenters *et al.*, 2008); thus only brief descriptions of its main parts are given here. The system consists of an anaerobic flow chamber (Minucells, Bad Abbach, Germany) with (1) a test specimen mounted with its test surface not facing the flow direction; (2) a Teflon® dispenser (Multimed GmbH, Kirchheim unter Teck, Germany) containing the bacterial suspension; and (3) a peristaltic pump (Spetec GmbH, Erding, Germany) with an integrated speed controller. In this study, the system was modified to mimic conditions related to peri-implantitis, namely an anaerobic

atmosphere and a slow-flowing, nutrient-poor environment containing three different strains of peri-implantitis-related bacteria. Specifically, the circulating bacteria were allowed to adhere to the protein-coated titanium specimens under anaerobic conditions (MACS MG; Don Whitley Scientific Ltd; atmosphere of 80% N_2, 10% H_2 and 10% CO_2) at 37 °C for 72 h.

Biofilm formation

Sterile polished disks of commercially pure titanium (Grade 2, ASTM F-67), 5 mm diameter and 1 mm thickness, with a mean surface roughness of 120 nm (Straumann AG, Basel, Switzerland), were sterilized by steam autoclaving and gamma irradiation and used as substrates. The disks were placed for 15 min in freshly mixed serum/saliva mixture (1 : 10) prior to each experiment in order to allow protein pellicle formation (Hauser-Gerspach *et al.*, 2007). Fasting stimulated saliva of three healthy volunteers was homogenized, filtered through a 70-μm filter (Cell Strainer; Becton Dickinson), and centrifuged at 22 000 ***g*** for 45 min at 4 °C. The supernatant was filter-sterilized (45 and 0.22 μm; Millex-HV and Millex-GV respectively; Millipore, Switzerland) and mixed with pooled serum (Blutspendezentrum, Basel, Switzerland).

The protein-coated substrates were placed in the anaerobic flow chamber, 0.2% glucose was added to the bacterial suspension, and the suspension was circulated at 0.8 mL min^{-1} for 72 h. To compensate for the decrease in pH of the bacterial suspension (7.26 ± 0.07 to 4.84 ± 0.21), it was renewed in 24-h intervals. After 72 h, the biofilm-coated titanium disks were evaluated using SEM, CLSM, and IMC.

SEM

The biofilms were fixed overnight in 2% glutaraldehyde solution (Sigma, Buchs, Switzerland), washed once with PBS, and dehydrated in stepwise increasing concentrations of ethanol – 30%, 50%, 70%, 90%, 2 × 100% for 10 min each. The samples (n = 3) were then critical-point-dried and coated with 10 nm of gold and examined (Fei Nova NanoSEM 230®, Eindhoven, the Netherlands).

FISH and CLSM

Oligonucleotide DNA probes, labeled at the 5′-end with Cy3 and Cy5 or with 6-carboxyfluorescein (FAM) and additionally labeled at the 3′-end (Microsynth AG, Balgach, Switzerland), are listed with their sequences and specificities in Table 1. Appropriate probe sequences for the specific detection of each bacterial strain in the biofilm have been described previously (Paster *et al.*, 1998; Thurnheer *et al.*, 2004; Guggenheim *et al.*, 2009).

The biofilms were fixed in 4% paraformaldehyde (Sigma) for 1 h at 4 °C and washed once with PBS. Thereafter, the biofilm-associated microorganisms were permeabilized by exposure to lysozyme (Sigma; 70 000 U mL^{-1}) for 2 min at room temperature and rinsed with physiological saline. FISH was carried out using a modification of a method previously described (Thurnheer *et al.*, 2004). The biofilms were pre-incubated for 15 min at 48 °C in final hybridization buffer (0.9 M NaCl, 20 mM L^{-1} Tris–HCl pH 7.5, 0.01% SDS) containing 30% formamide and then placed for 3 h at 48 °C in the same solution with the oligonucleotide probes added (5 μg mL^{-1} for STR405 and LNA-Pging, 15 μg mL^{-1} for FUS664). After hybridization, the biofilms were immersed for 15 min at 48 °C in washing buffer (102 mM L^{-1} NaCl, 20 mM L^{-1} Tris–HCl 7.5, 5 mM L^{-1} EDTA, 0.01% SDS). Thereafter, the disks were embedded upside-down in 10 μL Mowiol mounting solution and stored at room temperature in the dark at least 6 h. Biofilms were examined using a Leica SP5® microscope (Leica, Wetzlar, Germany) fitted with three lasers: He-Ne, argon and DPSS. Filters were set to 490–530 nm for FAM, 570–610 nm for Cy3, and 650–730 nm for Cy5. The fluorescence signal from Cy5 was assigned to blue color for better differentiation from Cy3. Confocal images were obtained using a 63× (numeric aperture 1.4) oil immersion objective. Each biofilm was scanned at three random positions at the center of the disk. Z-direction series were generated by vertical optical sectioning at every position with the thickness of the slices set to 0.3 μm. Proprietary Leica confocal software was used to acquire digital images of 1024 × 1024 pixels in size that were the average of 32 frames. The counts of the bacteria in the biofilm were made using image analysis software (Olympus AG,

Table 1. Characteristics of 16S rRNA gene-directed oligonucleotide probes used for FISH (underlined bases have locked ribose conformation)

Probe	Tag	Target	Sequence (5′ → 3′)	Reference
STR405	Cy5	*S. sanguinis*	TAG CCG TCC CTT TCT GGT	(Thurnheer *et al.*, 2004)
FUS664	FAM2	*F. nucleatum*	CTT GTA GTT CCG CYT ACC TC	(Paster *et al.*, 1998)
LNA-Pging	Cy3	*P. gingivalis*	GTT TTC ACC ATC MGT CAT C	(Guggenheim *et al.*, 2009)

Volketswil, Switzerland) and verified manually on random views to exclude possible errors due to not counting bacteria present in bundles. The experiment was repeated twice, resulting in six disks that were scanned at three random position in the central area.

IMC

Three milliliters of Columbia agar (BBL™; Becton Dickinson) supplemented with 5% human blood (Blutspendezentrum), 5 μg mL^{-1} hemin (Fluka, Buchs, Switzerland), and 0.5 μg mL^{-1} menadione (VWR International, Dietikon, Switzerland) were placed in sterile IMC ampoules and incubated anaerobically for 48 h. Specimens with the biofilms were placed in ampoules, enabling continuous contact between the biofilm and the agar. A sterile titanium disk with no biofilm on it served as the negative control. Each of the ampoules was immediately sealed under anaerobic conditions and inserted into one of the individual microcalorimeters in the 48-microcalorimeter instrument used (TAM 48®; TA Instruments, New Castle, DE). The heat flow (μJ s^{-1} = μW), due to the heat given up during bacterial activity in the biofilm ($n = 24$), was recorded continuously for 480 h. From the data, the following parameters were determined: time-to-peak heat flow, peak heat flow, mean heat flow (that is, heat flow averaged over the period, 72–420 h, as during this time it stayed on a stable plateau level after reaching the peak heat flow), and total heat produced up to 480 h (computed as the integral of the heat-flow-vs.-time curve between 0 and 480 h).

Statistical analysis

For each of the four IMC parameters determined, the median, mean, and standard deviation were computed. Test of significance was conducted using the Kruskal–Wallis analysis of variance method and a commercially available software package (STATA Statistical Software, release 16; StataCorp, College Station, TX). Significance was denoted at $P < 0.05$.

Results

SEM revealed that a biofilm was present over the entire surface of a protein-coated titanium disk (Fig. 1a), although minor variations were observed in the density and areas covered by the EPS matrix (Fig. 1b).

FISH/CLSM showed bundles of *F. nucleatum* cells that formed the framework of the biofilm in which both coccal species were observed to bind (Fig. 2). Hardly, any

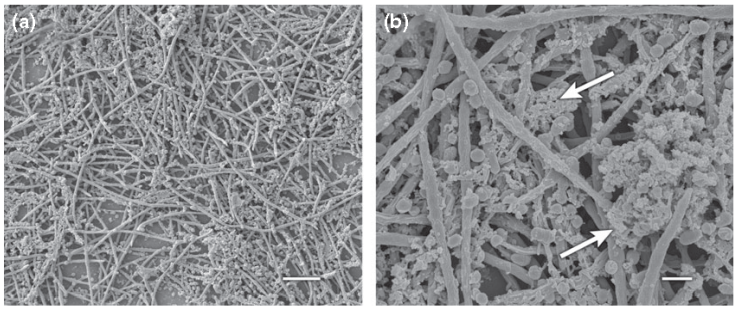

Fig. 1. SEM scans of 72-h-old *in vitro* three-species biofilm. Bars on the pictures indicate 5 μm on (a) and 1 μm on (b). A continuous biofilm formation is detected all over the surface (a); in detailed view of (a), the formation of EPS is shown on (b) with arrows.

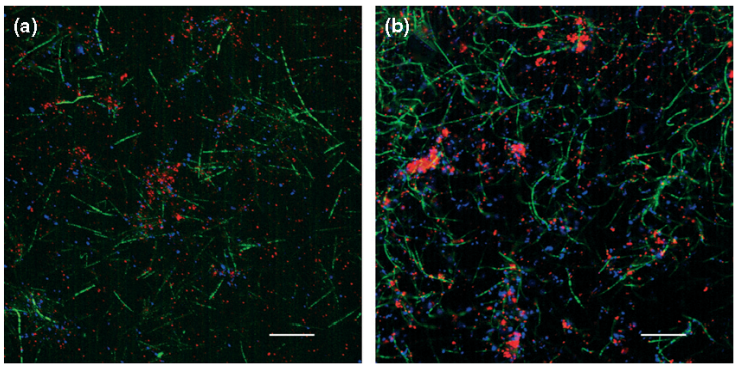

Fig. 2. Biofilms detected by FISH/CLSM *Streptococcus sanguinis* shown in blue (STR405-Cy5), *Fusobacterium nucleatum* in green (FUS664-FAM2) and *Porphyromonas gingivalis* in red (LNA-Pging-Cy3). The variability in the total counts of the bacteria is shown on panels (a) and (b). Bar indicates 50 μm.

co-adherence between the two coccal species was detected. While the relative proportions of the bacterial species stayed unchaged within the biofilms, the total bacterial count showed significant differences ($P < 0.05$); however, the biological meaning of the minor differences in total counts is doubtful (Table 2).

A typical IMC heat-flow-vs.-time plot is given in Fig. 3. In 17 of 24 samples, after the heat flow reached a peak, it gradually reduced and finally returned to baseline value. In each of the other seven samples, however, after the heat flow reached a peak, it did not reduce to baseline value but remained at a high plateau. While the variability in the time-to-reach peak, heat flow is low, each of the other three parameters determined showed large variability (Table 3).

Table 2. Bacterial proportions and total counts of *Streptococcus sanguinis*, *Fusobacterium nucleatum* and *Porphyromonas gingivalis* in the 72-h-old biofilm detected by FISH/CLSM

Species	Strain	Proportion (%)	Total cell count (cells mm^{-2})
S. sanguinis	DSM 20068	41.3 ± 4.8	$1.07 \times 10^4 \pm 2.97 \times 10^3$
F. nucleatum	ATCC 10953	17.7 ± 2.1	$4.90 \times 10^3 \pm 2.08 \times 10^3$
P. gingivalis	DSM 20709	41.0 ± 4.9	$1.06 \times 10^4 \pm 4.13 \times 10^3$

Mean ± SD are given ($n = 6$).

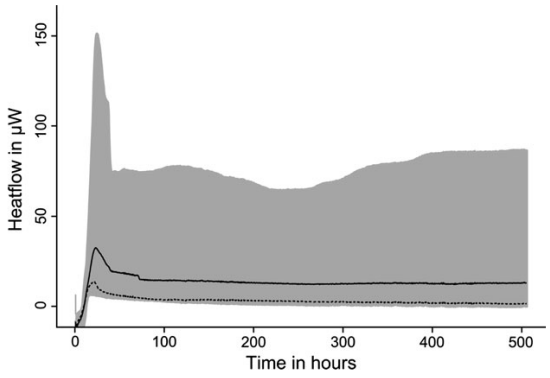

Fig. 3. Typical heat flow-vs.-time curve, as obtained using IMC. The range of the curve is shown as the gray area, the mean curve is the continuous curve and median curve is the dotted curve ($n = 24$).

Table 3. Summary of IMC results ($n = 24$)

	Time-to-peak heat flow (h)	Peak heat flow (µW)	Mean heat flow (µW)	Total heat produced (J)
Median	20.5	14.9	2.7	5.5
Mean	20.6	35.8	13.1	23.5
Standard deviation	4.5	42.6	22.0	37.2

Discussion

Microscopic analyses have proven to be invaluable tools in describing biofilms in terms of their structure and association with a surface; however, no real-time information about the dynamics of the metabolic activity and biomass formation can be obtained. The present study is the first of its kind characterizing a multispecies *in vitro* biofilm using both well-established microscopic methods (SEM and FISH/CLSM) and a very sensitive calorimetric method (IMC).

Imaging by SEM was used to gain the first overview of the formed biofilm. As intended, the scans revealed biofilms that covered the entire surface with bacteria partially embedded in EPS, indicating that 0.2% glucose (Tenuta *et al.*, 2006; Filoche *et al.*, 2007) was sufficient to induce EPS formation. In addition, SEM showed that *F. nucleatum* served as the central 'bridging organism' or framework in the biofilm architecture, demonstrating inter- and intraspecies cellular binding (Lancy *et al.*, 1983; Kaplan *et al.*, 2009; Merritt *et al.*, 2009). SEM was not able to distinguish between the two coccal species present in the biofilm partly because of their similar shape and size, and partly because of embedding in EPS.

FISH/CLSM allowed the discrimination between *S. sanguinis* and *P. gingivalis* and determination of the relative proportions of all three species. A partially heterogeneous architecture of the biofilm, which may be due competitive binding, was observed. However, the distribution of the relative proportions of the three species in all experiments stayed unchanged.

The heat flow at a given time (as determined using IMC) was a measure of metabolic activities of all bacteria present, and it thus declines correspondingly if bacterial activity diminishes. Similarly, heat over time (i.e. the integral of the heat flow) is a proxy for the growth curve and approaches a maximum when metabolic activity decreases (Braissant *et al.*, 2010). This metabolic decline and asymptotic biomass accumulation pattern is due to changes in the IMC ampoule internal environment that occur during bacterial metabolism; that is, exhaustion of available nutrients or electron acceptors or build up of metabolic waste products. The pattern of rise and decline of the metabolic activity of the biofilm was seen in the first 50 h (Fig. 3) exhibiting similarities in the behavior of the biofilm to common liquid or solid culture studies (Braissant *et al.*, 2010). Thus, cumulative heat correlates with cumulative bacterial biomass only during this early part when the biofilm still grows and until heat flow peak is reached. Once the heat flow has stabilized at a constant level, the accumulation of heat is most probably not related to a net increase in bacterial numbers and production of fresh biomass, but, rather, to metabolic activities

related to maintainance of the mature biofilm and survival of the present cells. Alternatively, it can be hypothesized that during this steady state of the heat flow, growth rate is equal to bacterial death rate, resulting in a stable metabolically active bacterial population. This latter hypothesis is in line with the first one if equally low growth and death rates are considered.

In the present study, between 72–480 h ca. 70% of the samples ($n = 17$) showed a low steady state heat flow comprised between 0.8 and 1.8 μW, whereas in the remaining 30% ($n = 7$), the values were found much higher (reaching from 8.6 to 86.0 μW). Assuming a heat flow of 2 pW per active bacterial cell (James, 1987), we calculated the number of active bacteria in the biofilm. This suggests that in the present samples showing the lowest steady state heat flow, ca. 4×10^5 to 9×10^5 bacteria remained active on the surface of the titanium disk (5 mm^2), whereas this number is up to 4.3×10^7 in the samples having the highest heat flow. This result emphasizes major variability within biofilms that appear similar in microscopic analyses. On the other hand, the time required to reach the maximum heat flow showed only moderate specimen-to-specimen variability. This can be understood as a consequence of the static conditions of measurement – constant temperature, sealed and unstirred ampoules, a fixed initial chemical environment, plus, as shown by FISH/CLSM, unchanged relative initial proportions of the three bacteria (Table 2).

In contrast to the time required to reach the maximum heat flow peak, each of the three parameters computed from the IMC data varied widely (Table 3), showing that biofilm maturation rapidly diverges between originally similar samples. These results indicate heterogenecity of the aggregate metabolic activity of all bacteria present and reflect the differences in remaining active cells after 480 h. These findings regarding the heat flow and the total heat must, by definition, reflect the total number of bacteria present at the time or the time interval over which the parameters are calculated. At this point, it should be remembered that, in contrast to microscopic analyses that provide generalized data based on number of scans taken, IMC allows the measurement of the whole surface of the test specimen harboring the biofilm. Therefore, the variability of the IMC results may be explained by differences in the initial cell counts and bacterial distributions within the biofilm on the titanium disks that cannot be detected by microscopy where the whole surface area cannot be studied in detail.

In conclusion, (1) three-species biofilm formed on protein-coated titanium was documented by SEM and FISH/CLSM; specifically, the species present, their proportions, and their approximate surface distribution were determined; (2) IMC detected a surprisingly high variability within biofilms as the measurement includes the whole surface area harboring the biofilm rather than generalized data based on number of areas scanned; (3) these new insights may be beneficial, and, thus, should be considered in future research into biofilms on dental surfaces.

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6 Microcalorimetric determination of the effects of amoxicillin, metronidazole and their combination on an *in vitro* biofilm

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Microcalorimetric determination of the effects of amoxicillin, metronidazole and their combination on an *in vitro* biofilm

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Abstract

Background: The mechanism of action of adjuvant antibiotic therapy in the treatment of peri-implantitis is not well understood. The aim of the study was to investigate antibiotic susceptibility of an *in vitro* biofilm by isothermal microcalorimetry (IMC).

Methods: Titanium disks containing a 72 h three-species biofilm (*Streptococcus sanguinis* ATCC20068, *Fusobacterium nucleatum* ATCC10953, and *Porphyromonas gingivalis* DSM20709) were placed in series of IMC ampoules with nutrient agar supplemented with increasing concentrations of amoxicillin, metronidazole or their combination and incubated anaerobically for 10 d. Lag time and maximum growth rate were determined from continuous heat-flow recordings of metabolic activity. To validate the IMC biofilm results, adherent *S. sanguinis* and *P. gingivalis* were incubated anaerobically in media supplemented with antibiotics at 37°C for 24 h, and their viability was determined by live/dead staining, conventional culturing, and IMC.

Results: In all biofilm samples incubated with antibiotics a prolonged lag phase was observed compared to controls ($p<0.05$). Maximum growth rate was significantly lower for samples either treated with amoxicillin or metronidazole in comparison to controls ($p<0.05$). Combining the antibiotics did not improve this effect. Concentrations exceeding 10xMIC completely inhibited the growth of adherent *S. sanguinis* and *P. gingivalis*, whereas lower concentrations resulted in only a delay in the lag phase. A poor correlation was observed between live/dead staining and conventional culturing.

Conculsion: IMC gives new evidence about antibiotic effects on oral biofilms and is more informative than conventional culture and live/dead assays. The combination of antibiotics was found more efficient than metronidazole alone. However, only minor differences in the growth inhibition compared to amoxicillin alone were detected.

Keywords: calorimetry, amoxicillin, metronidazole, biofilms, microbial sensitivity tests

Introduction

The major goal of periodontal therapy is to reduce or eliminate pathogenic species and to maintain colonization by host-compatible species in the oral cavity. [1, 2] The pathogenic microbiota forms biofilms on implant surfaces and when left unattended leads to inflammatory reactions and progresses into peri-implantitis. [3] Different therapeutic options have been advocated for the treatment and prevention of periodontal/peri-implant diseases. In particular, the combination of metronidazole and amoxicillin is frequently used, which is known to have a synergistic or additive antimicrobial effect and to improve clinical results against periodontal infections. [4-8] In surgical peri-implantitis treatment, the use of amoxicillin and clavulanic acid has been recommended due to the observed presence of *Staphylococcus aureus* species, while in patients with a history of periodontal disease, the combination of amoxicillin and metronidazole is indicated. [9-11] However, it is important to note that bacteria in biofilms have been shown to be 100 - 1000 times less susceptible to antibiotics than their planktonic counterparts as the antibiotics have restricted penetration through the biofilm. [12] Additionally, microorganisms within biofilms respond to local environmental conditions in several ways, such as altering their gene-expression patterns or undertaking physiological activities to adapt to a particular condition (i.e., stress through antimicrobials). [12-14] Thus most commonly mechanical debridement combined with systemic antibiotic treatment is applied in the clinical treatment of peri-implantitis. [9, 15]

Various approaches have been used to quantify the effects of antibiotic treatment on oral microbiota. The common practice is to analyze the microflora via conventional culture methods that have proven to be efficient in testing the susceptibility of bacterial cells to antibiotics. [8, 16, 17] Nevertheless, viable but non-cultivable (VBNC) cells, a population of cells that is defined by low levels of metabolic activity and for their ability to switch

again to being cultivable when the growth conditions improve, may influence the outcome of conventional tests and predispose patients to (re)infection. [14]

Fluorescence microscopy combined with live/dead staining has also been used to detect the effects of antibiotics on adherent microorganisms. [18-20] While the conventional culture method includes only cultivable bacteria, which are able to proliferate, live/dead staining combined with fluorescence microscopy enables the quantification of viable cells based on the intactness of their membranes including also the VBNC population of cells. Combined with confocal laser scanning microscopy, it is possible to assess the viability throughout biofilms. [14]

Isothermal microcalorimetry (IMC) is a highly sensitive culture-based technique that allows for the measurement of heat generated by microbial activities. Bacteria replicate in a suitable culture medium, resulting in an exponential increase in the heat production rate that can be recorded in real-time by IMC (i.e., heat-flow curve). When using IMC, broth cultures are often preferred for rapid detection of bacterial growth in clinical settings [21-23], but since the method measures heat passively, culture growth on solid or viscous medium as well as in matrix can also be evaluated. [24, 25] Also the aspect of heterogeneity of biofilms can be assessed by this method. [26] As antimicrobials inhibit bacterial growth, IMC has been used to determine antimicrobial susceptibility, as demonstrated with strains of *S. aureus*, *Enterococcus faecalis* and fungi. [21, 27-30]

As the mechanism of action of adjuvant antibiotic therapy in the treatment of peri-implantitis is not well understood, the aim of this study was to determine the metabolic activity of an *in vitro* three-species biofilm [26] associated with periodontitis and peri-implantitis in the presence of amoxicillin, metronidazole or their combination by IMC.

Materials and Methods

Susceptibility testing of planktonic bacteria

The susceptibilities of planktonically grown Streptococcus sanguinis DSM20068, Fusobacterium nucleatum ATCC10953, and Porphyromonas gingivalis DSM20709 were determined for amoxicillin, metronidazole and the combination of these drugs in a 3:2 ratio 4 by the macrodilution method according to the Clinical and Laboratory Standards Institute (CLSI) protocol. 31 Based on these data, the antibiotic concentrations were calculated for susceptibility testing of the biofilm and adherent single-species experiments. Previously, adherent bacteria and microorganisms in established biofilms have been reported to demonstrate an increased resistance to antibiotics in comparison to their planktonic counterparts. 13, 32 Thus, to detect the antibiotic effects by the applied methodology multiplications (range 0.25 - 100x) of minimal inhibitory concentration (MIC) values were applied.

Susceptibility testing of adherent S. sanguinis and P. gingivalis

A 10 µL inoculum of *S. sanguinis* in skim milk solution (stored at -20 °C) was suspended in 10 mL Schaedler broth‖ and incubated aerobically at 37 °C for 16 h. *P. gingivalis* was maintained in vials¶ at -70 °C. One pearl of frozen culture was inoculated into 10 mL thioglucollate‖ enriched with 0.5 mg/L of menadione, and 5 mg/L of hemin, and incubated anaerobically# for 96 h. *S. sanguinis* and *P. gingivalis* were harvested in stationary growth phase, washed with physiological saline, and resuspended in simulated body fluid (SBF) (7.996 g NaCl, 0.35 g $NaHCO_3$, 0.224 g KCl, 0.228g K_2HPO_4 x $3H_2O$, 0.305g $MgCl_2$ x $6H_2O$, 0.278 g $CaCl_2$, 0.071 g Na_2SO_4, 6.057 g $(CH_2OH)_3CNH_2$ dissolved in 1 L of ultra-pure water, pH adjusted to 7.25 with 1 mol/L HCl). [33] Ionic concentrations of the SBF are similar to those of the human extracellular fluid and may thus mimic some *in vivo* conditions and improve the reproducibility in an *in vitro* peri-

implantitis model. Titanium disks[**] were coated with 2 mL pooled serum-saliva mixture (1:10) prior to each experiment for 15 min at room temperature as described previously in detail. [34] Circulating bacteria (approximately $5x10^8$ for *S. sanguinis* and $1x10^9$ for *P. gingivalis*) were allowed to adhere to the protein-coated disks at 37°C for 2 h in an anaerobic[#] flow chamber system described elsewhere in detail. [34] Subsequently, the disks were incubated anaerobically[#] for 24 h in antibiotic solutions at concentrations of 1x, 10x, 50x and 100x MIC of amoxicillin, metronidazole and their combination. Disks incubated in 0.9% NaCl served as untreated controls. Live/dead staining and conventional culturing were used to assess the vitality of cells after 24 h treatment.

The metabolic activity of the cells in the presence of antibiotics up to 7 d was measured by IMC. The titanium disks[**] with adherent bacteria were placed in ampoules filled with 3 mL of enriched thioglycollate[‖] (0.5 mg/L of menadione, and 5 mg/L of hemin) supplemented with antibiotic solutions as mentioned above. Adherent bacteria on titanium disks incubated on in pure thioglycollate[‖] without any antibiotics supplemented were used as positive controls in each experiment. Sterile titanium disks without inoculated bacteria served as negative controls. Additionally, ampoules filled with thioglycollate[‖] were used as blank samples for baseline detection. The ampoules were closed in strictly anerobic conditions[#] and placed in the IMC device[‡‡] at 37°C and the heat-flow was recorded up to 10 d. The IMC experiments were calibrated by determining the lag times of a set of five increasing starting concentrations (CFU/mL on blood agar plates[††]) of *S. sanguinis* and *P. gingivalis* in duplicate to evaluate the survival rates of treated samples (n=3). A linear regression model describing the relationship between the CFU counts and lag time for *S. sanguinis* and *P. gingivalis* was obtained with statistical software[§§] and resulted in the following equations: ln(CFU) = 17.972 – 1.072 * lag time(h) with $R^2 = 0.9896$ and ln(CFU) = 18.376 – 0.133* lag time(h) with $R^2 = 0.9646$,

respectively. Additionally, the maximum growth rate of the bacteria was determined using the same equation to detect differences between antibiotic-treated and untreated control samples.

The adherent cells stained by dual fluorescent dyes‖‖ were analyzed microscopically¶¶ using FITC and Cy3 light filters at 5 randomly selected microscopic fields, each 0.024 mm^2 and the number of live and dead bacteria on the area counted¶¶. Vitality percentage of each sample was calculated by the equation (live cell count/total cell count)*100%.

For conventional culture methods, the specimens were placed in 1 mL 0.9% NaCl, vortexed for 1 min and treated with ultrasound for 15 s## to remove adherent cells from the disks without affecting their viability. [34] Serial dilutions were made in sterile saline, and aliquots of 0.1 mL were plated on agar plates†† (supplemented with 50 mL/L of human blood, 0.5 mg/L of menadione, and 5 mg/L of hemin) in duplicate and were incubated anaerobically# at 37ºC for 48 h (*S. sanguinis*) or 10 days (*P. gingivalis*). Based on colony morphology and cellular characteristics, the purity of the cultures was controlled, and colonies were counted and quantified (CFU/mL). Additionally, to be able to compare the staining results to conventional culturing, the vitality was expressed as a percentage of the corresponding control, i.e., (CFU/mL in treated sample/CFU/mL in untreated control sample)*100%.

Susceptibility testing of an in vitro biofilm

Biofilm formation has been described in detail previously by Astasov-Frauenhoffer and colleagues. [26] Briefly, *S. sanguinis, F. nucleatum* (storage and growth conditions the same as for *P. gingivalis*), and *P. gingivalis* were harvested in stationary growth phase, washed with physiological saline, and resuspended in SBF at densities of $1.1 \times 10^8 \pm 6.2 \times 10^7$, $3.2 \times 10^7 \pm 1.9 \times 10^6$ and $2.1 \times 10^9 \pm 9.3 \times 10^8$ CFU/mL, respectively. The bacterial suspension was allowed to form a biofilm on protein-coated disks** [34] in an anaerobic#

flow chamber for 72 h. The effects of the antibiotics on the metabolic activity of the biofilm were continuously recorded by IMC. Titanium disks covered by the biofilm were placed in microcalorimetric ampoules filled with 3 mL of agar[††] supplemented with 50 mL/L of human blood, 0.5 mg/L of menadione, and 5 mg/L of hemin and with antibiotic solutions corresponding to 0.25x, 0.5x, 1x, 10x, 50x, and 100x MIC. Of the three species, the MIC values are the highest for *S. sanguinis,* thus these were applied for the biofilm. Controls used were prepared with blood agar[††] otherwise the same type as in single-species experiments. The ampoules were closed in anerobic conditions and placed in the IMC instrument[‡‡] at 37°C to record heat-flow (µW = µJ/s) over 10 d. The data were analyzed by fitting the heat over time curve (i.e., resulting from the integration of the heat-flow curve) with Gompertz's equation with the "grofit" package in statistical software[***] (Fig.1). As outcome parameters, the maximum growth rate (1/h) and lag time (h) were estimated. [35]

Statistical analysis

The IMC data of test samples and controls were analyzed with the Mann–Whitney U test. A $p<0.05$ was considered statistically significant. The results of live/dead staining and CFU methods were made comparable by calculating the viable cell counts in each of the treated samples, dividing by the viable cell counts for untreated control samples and multiplying by 100% (vitality %). These results were analyzed with statistical software[§§] using the Kruskal-Wallis analysis of variance. The correlation coefficient between live/dead staining and conventional culturing was determined using Pearson´s product-moment correlation coefficient.

Results

MICs/MBCs determined for *S. sanguinis, F. nucleatum,* and *P. gingivalis* according to

CLSI protocol and using IMC are presented in Table 1 and 2, respectively. IMC showed that the growth of both adherent single strains in liquid media was delayed but not eliminated by solutions at the concentration of 1x MIC and metronidazole at 10x MIC. All solutions in higher concentrations were able to delay growth or eliminate it over the whole measurement period. For *S. sanguinis* 1x MIC amoxicillin, 10x MIC metronidazole and 1x MIC of the combination resulted in significant decrease in the growth rate and increase in the lag phase ($p<0.05$). The lag time for *P. gingivalis* was also significantly increased in all samples where growth was detected ($p<0.05$). However, the growth rate was only minimally affected by the treatment resulting in statistically not significant difference ($p>0.05$). The continuous recordings of IMC (Table 2) corresponded to the MIC values determined after 24 h exposure using the CLSI protocol (Table 1).

Conventional culturing revealed low viability percentages of *S. sanguinis* after amoxicillin treatment, with the exception of 1x MIC, which exhibited no reduction in viability compared to the control (Fig. 2A). Significant differences were observed in groups treated with amoxicillin and the combination of the two antibiotics ($p<0.05$) (Fig. 2A, C). The highest efficacy was detected for metronidazole, for which concentrations reduced the viability < 1% (Fig. 2B). The treatment of adherent *P. gingivalis* cells with amoxicillin or the combination of both antibiotics returned similar results (Fig. 2D, E). Again, metronidazole yielded a slightly higher efficacy (Fig. 2E). In comparison, live/dead staining detected an increased antibiotic efficacy only with 50x and 100x MICs of metronidazole (Fig. 2B, Fig 3). Although a statistically significant difference was measured between other groups (Fig. 2A, C-F), the marginal reduction in viability is questionable from a biological perspective. The Pearson correlation coefficient (0.31) revealed poor correlation between the two methods.

Monitoring the growth of the three-species biofilm at the interface between titanium disk

and solid medium IMC revealed that the MIC values for *S. sanguinis* (the highest values of the three species) used to test the susceptibility of the three-species biofilm in this study inhibited growth over 10 d. Therefore, concentrations corresponding to 0.25x and 0.5x MICs of antibiotics were also applied to get a better representation of the dose response. All biofilm samples, except for the 0.25x MIC of amoxicillin and metronidazole, exhibited prolonged lag phases in comparison to the control ($p<0.05$). The maximum growth rate was significantly lower for samples treated with amoxicillin and metronidazole than for control samples ($p<0.05$), and using the combination of the antibiotics did not affect this parameter. Compared to the IMC results of the single-species experiments where the liquid culture allows some planktonic growth, here the low growth rates indicate that only biofilm growth was observed. The results are summarized in Table 3.

Discussion

The mechanism of action of adjuvant antibiotic therapy in the treatment of peri-implantitis is not well understood. In this study, the antibiotic efficacy against an *in vitro* three-species biofilm model on titanium disks was investigated by isothermal microcalorimetry (IMC). Amoxicillin, metronidazole and their combination were used to evaluate the antibiotic susceptibility of the biofilm. The MICs for all antibiotic solutions used were obtained for each of the three species present in the biofilm: *S. sanguinis, F. nucleatum* and *P. gingivalis*. In comparison to the maximum dosages of amoxicillin (3-4 μg/mL) and metronidazole (8-10 μg/mL) attainable in human plasma, the detected MIC values were lower for both strains, with only one exception: the MIC for metronidazole against *S. sanguinis,* was markedly higher (>128 μg/mL). However, when used in combination with amoxicillin, a synergistic mode of action for these agents was detected, as the MIC value for metronidazole was reduced to 0.4 μg/mL.

IMC detects heat-flow, which is a measure of the metabolic activities of all bacteria present at a given time; thus reductions in heat-flow were observed in the presence of antimicrobial agents. The main advantage of IMC over other approaches is the quantitative measurements of the effects of the antimicrobials on parameters such as the growth rate and lag phase of the bacteria. Results showed that amoxicillin alone was always found more efficient in prolonging the bacterial lag phase in biofilms than metronidazole alone and demonstrated comparable results to the combination of antibiotics used. Similar results were detected for the single-species tested in this study. IMC provides knowledge on the nature of the dose response. A prolonged lag phase indicates bactericidal activity of the antimicrobial agent; a portion of the inoculum is killed, the growth rate is not affected or is only minimally affected. In contrast, a bacteriostatic agent interferes with cellular processes, and the increase in the lag phase duration is directly linked to a decrease in growth rate. [27, 30] Amoxicillin and metronidazole are both reported to be bactericidal. In experiments on treated adherent *S. sanguinis*, all three IMC parameters were affected. The inoculum was partially killed, resulting in a prolonged the lag time and in a reduced the growth rate, while no such effect was observed in the control samples. In experiments on adherent *P. gingivalis* and three-species biofilms, the same tendency was observed, although the growth rate remained mostly unchanged. Interestingly, when used alone, both antibiotics decreased the maximum growth rate of the biofilm but had a smaller effect on prolonging the lag phase, while biofilm samples treated with the combination of the two exhibited growth rates close to the control but more efficiently delayed growth. Thus, when applied alone, the antibiotics seem to work primarily through bacteriostatic effects; when combined, the efficacy is increased by their synergistic bactericidal action. Moreover, this finding demonstrates that the effects cannot be strictly divided into bacteriostatic and bactericidal,

as the results here indicate that the effect seems to be dependent on their concentration and combination and the nature of targeted cells. [27, 30] Additionally, this finding suggests that IMC should be considered for testing the effect of antimicrobial agents in general as the method proposes the possibility of efficiently studying material *in situ* for its susceptibility or resistance to any antibiotic solution without disturbing the complex structure of biofilms.

To relate the results reported in this study to previous investigations, live/dead staining and conventional culturing were used to test antibiotic susceptibility of adherent *S. sanguinis* and *P. gingivalis* cells to demonstrate a possible correlation between the two methods in viability rates in treated samples. [14, 34] Due to its poor capacity for attaching to surfaces when used as a single-species inoculum, adherent *F. nucleatum* ATCC10953 was not evaluated in this study. Both methods are commonly used in experiments on antibiotic susceptibility testing *in vitro.* [8, 16-20] However, poor correlation between the two methods was detected by the antibiotic susceptibility testing of adherent cells. Conventional culturing detects only bacteria that initiate cell division, and exposure to environmental stress factors (i.e., antimicrobials) makes them more sensitive to other culture conditions (i.e., temperature, the presence of oxygen and the duration of incubation). Additionally, live/dead staining is based only on the permeability of the cell membrane to specific chemicals; if the mechanisms of action of the antibiotics do not directly damage the cell wall, the data obtained by the staining method remain questionable. Moreover, a population of cells that are VBNC leads to possible under- or overestimation of cell counts, which may carry the potential for (re)infection. [14, 34, 36] Thus, using culturing or live/dead staining to present definitive answers on the antibiotic susceptibility of adherent cells should be used with great caution.

In conclusion, the combination of both antibiotics was more efficient than metronidazole

alone. However, only minor differences in growth inhibition compared to amoxicillin alone were detected. IMC appears to be more sensitive than live/dead staining and conventional culturing, providing insights into the effect of the antimicrobials through the data obtained on their influence on the growth rates and lag phases of biofilms and adherent microorganisms.

Footnotes

‖ BBL™, Becton Dickinson, Basel, Switzerland

¶ Microbank® blue Chemie Brunschwig AG, Basel, Switzerland

MACS MG, Don Whitley Scientific Ltd; atmosphere of 80% N_2, 10% H_2 and 10% CO_2; at 37ºC

** mean roughness of 120 nm, 5 mm diameter, 1 mm thickness, commercial pure titanium grade 2, ASTM F-67; Straumann AG, Basel, Switzerland

†† Columbia agar, BBL™, Becton Dickinson, Basel, Switzerland

‡‡ TAM 48, TA Instruments, New Castle, DE, USA

§§ Stata Statistical Software, release 10; StataCorp, College Station, TX, USA

‖‖ Live/Dead BacLight™ bacterial Viability Kit; MoBiTec, Luzern, Switzerland

¶¶ Analysis program, Provis AX70, Olympus AG, Volketswil, Switzerland

22.5 W; Vibracell, Sonics & Materials, Newtown, CT

*** R software, Package version 2.15.2, R Development Core Team, Vienna, Austria.

††† The maximum growth rate (1/h), lag time (h) and concentration (CFU/mL) of surviving bacteria (t = 0) in the samples where growth was detected throughout the measurement period (n = 6).

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Figure legends

Fig. 1. Example of data recorded by IMC and parameters calculated during the growth of three-species biofilm on solid medium over the first 48 h. A) Raw data showing the heat-flow. The maximum metabolic activity of the biofilm is reached when the curve reaches its maximum. B) Integrated data showing the heat produced over time (black line) and fitted Gompertz model (red line). This curve is used to determine the maximum growth rate (i.e., the maximum slope of the curve) and the duration of the lag phase (the intercept between a tangent to the maximum slope point and the y axis at 0) by fitting the Gompertz growth model to heat data.

Fig. 2. Results of live/dead staining (white bars) and conventional culturing (grey bars) of adherent *S. sanguinis* and *P. gingivalis* presented by mean values and standard deviations (n=9).

Fig. 3. Vitality of adherent *S. sanguinis* detected by live/dead staining after 24h of treatment with various concentrations of metronidazole (B)-(E). Cells appearing green are alive, red indicates dead cells. (A) untreated control sample, (B) 1x MIC, (C) 10x MIC, (D) 50x MIC, (E) 100x MIC.

Tables

Table 1. Minimal inhibitory and bactericidal concentrations (μg/mL) determined according to CLSI protocol M26-A. [31]

	Amoxicillin		Metronidazole		Amoxicillin/metronidazole	
Bacterial strain	*MIC*	*MBC*	*MIC*	*MBC*	*MIC*	*MBC*
S. sanguinis	0.5	2 - 4	> 128	> 128	0.6/0.4	1.2/0.8
F. nucleatum	0.125	0.25 - 2	< 0.06	0.5	0.075/0.05	0.3/0.2
P. gingivalis	< 0.06	0.25	0.125	0.125	0.038/0.025	0.15/0.1

Table 2. Parameters calculated from IMC data of the treated adherent cells.[†††]

	S. sanguinis DSM 20068			*P. gingivalis* DSM 20709		
Parameters	*maximum growth rate (1/h)*	*lag time (h)*	*concentration (CFU/mL)*	*maximum growth rate (1/h)*	*lag time (h)*	*concentration (CFU/mL)*
Control	0.463 ± 0.256	5.9 ± 0.4	$1.29x10^{5} \pm 5.85x10^{4}$	0.173 ± 0.026	22.1 ± 3.9	$5.64x10^{6} \pm 2.96x10^{6}$
1x MIC amoxicillin	0.2	41.1	*ND*	0.178 ± 0.015	119.0 ± 8.2	$1.77x10^{1} \pm 1.59x10^{1}$
1x MIC metronidazole	0.196 ± 0.019	8.7 ± 2.6	$1.97x10^{4} \pm 2.67x10^{4}$	0.169 ± 0.002	38 ± 8.1	$8.43x10^{5} \pm 7.05x10^{5}$
10x MIC metronidazole	0.122 ± 0.013	30.5 ± 0.6	*ND*	0.128 ± 0.062	134.2 ± 6.9	$2.06x10^{0} \pm 1.31x10^{0}$
1x MIC amoxicillin/metronidazole	0.049 ±0.007	68.9 ± 14.5	*ND*	0.145 ± 0.017	64.0 ± 28.0	$1.09x10^{5} \pm 9.56x10^{4}$

Table 3. Maximum growth rate (1/h) and lag time (h) calculated for samples of the *in vitro* three-species biofilm treated with antibiotics that revealed metabolic activity over 10 d (n = 6).

	Amoxicillin		Metronidazole		Amoxicillin/metronidazole	
Parameters	*maximum growth rate (1/h)*	*lag time (h)*	*maximum growth rate (1/h)*	*lag time (h)*	*maximum growth rate (1/h)*	*lag time (h)*
Control	0.046 ± 0.035	17.0 ± 3.7	0.046 ± 0.035	17.0 ± 3.7	0.046 ± 0.035	17.0 ± 3.7
0.25x MIC	0.007 ± 0.002	17.5 ± 8.9	0.008 ± 0.006	18.0 ± 3.0	0.06 ± 0.02	23.0 ± 4.7
0.5x MIC	0.005 ± 0.002	35.0 ± 10.1	0.009 ± 0.006	29.2 ± 8.7	0.03 ± 0.003	31.2 ± 2.3
1x MIC	no growth		0.007 ± 0.005	34.7 ± 15.5	no growth	

Figure 1

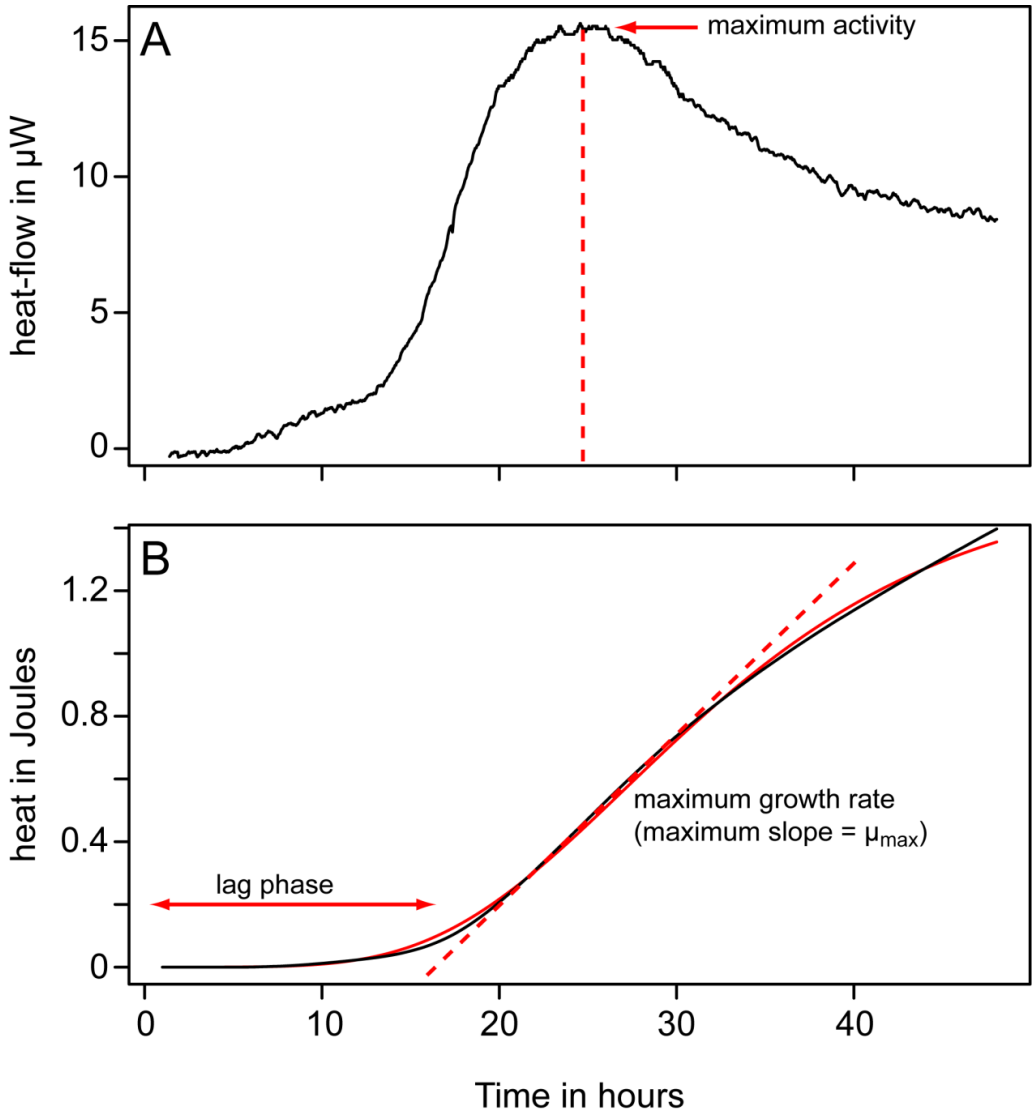

A
maximum activity
heat-flow in µW
15
10
5
0
B
heat in Joules
1.2
0.8
0.4
0
lag phase
maximum growth rate
(maximum slope = µmax)
0
10
20
30
40
Time in hours

Figure 2

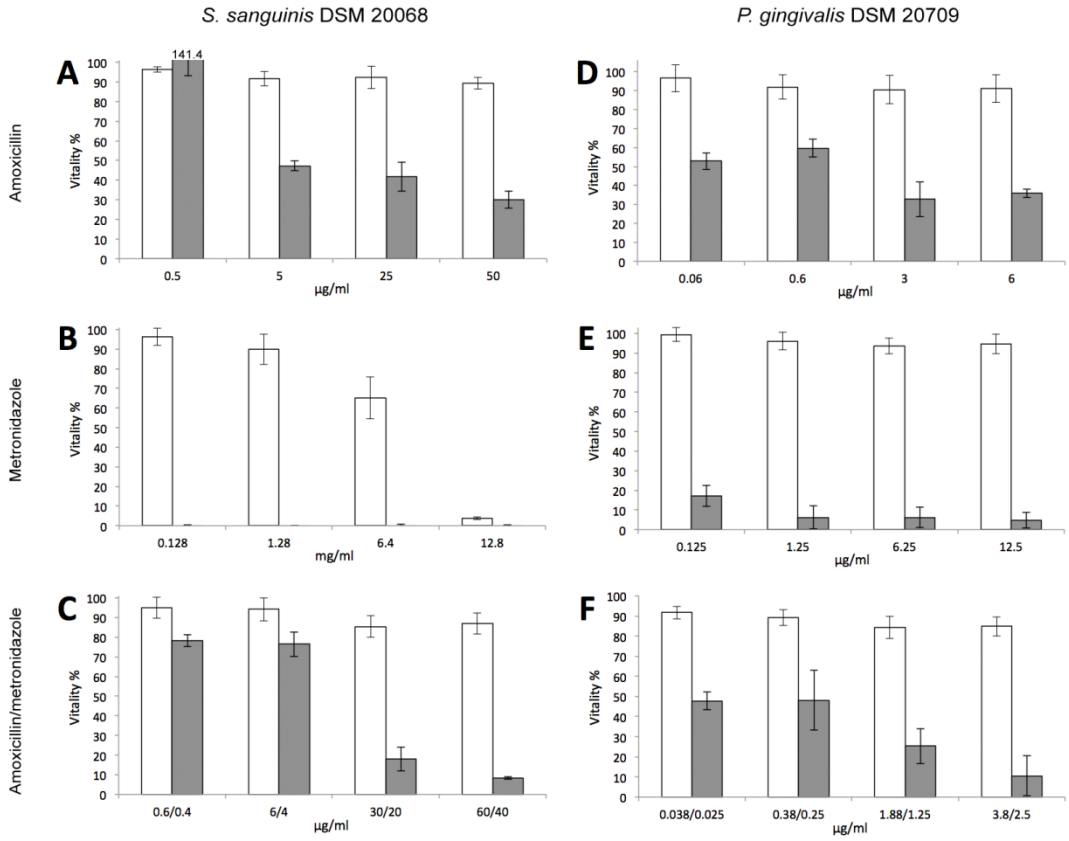
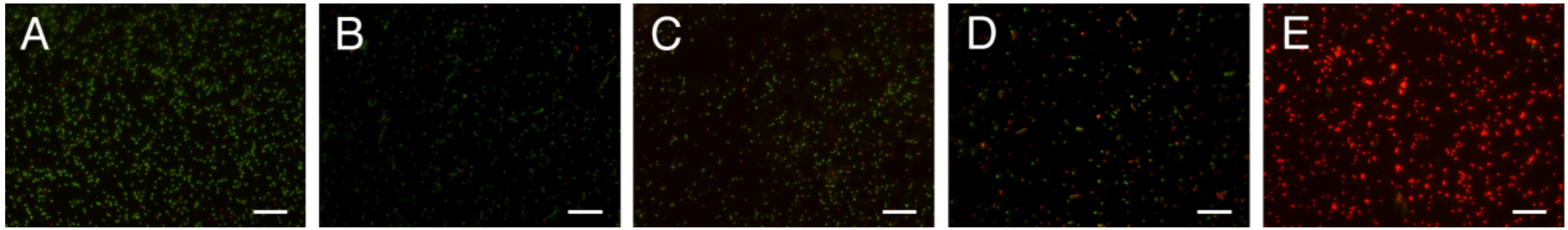

S. sanguinis DSM 20068
P. gingivalis DSM 20709
Amoxicillin
Metronidazole
Amoxicillin/metronidazole
A
B
C
D
E
F
Vitality %
141.4
0.5
5
25
50
µg/ml
0.06
0.6
3
6
0.128
1.28
6.4
12.8
mg/ml
0.125
1.25
6.25
12.5
0.6/0.4
6/4
30/20
60/40
0.038/0.025
0.38/0.25
1.88/1.25
3.8/2.5

Figure 3



7 Discussion

Modelling of any oral biofilm *in vitro* is challenging as up to 700 species have been identified in saliva and on various surfaces of the oral cavity. Most sites yield 20 – 30 different predominant species and number of species per individual range from 32 to 72 (Aas, *et al.*, 2005, Marsh, *et al.*, 2011). Thus, numerous models have been created to answer various specific questions concerning adherent oral microbiota. Different single- or multi-species models have been created to study changes in gene expression patterns in comparison to planktonic growth (Dû & Kolenbrander, 2000, Li & Burne, 2001, Svensäter, *et al.*, 2001, Welin, *et al.*, 2004, Kuboniwa, *et al.*, 2009, Lo, *et al.*, 2009), to study adherence to biomaterials (Hauser-Gerspach, *et al.*, 2007, 2008, Meier, *et al.*, 2008) and/or to test methods to clean the surfaces (Verkaik, *et al.*, 2010) as well as to test antimicrobial efficacy of treatments including antibiotics or disinfectants (Larsen, 2002, Shapiro, *et al.*, 2002, Busscher, *et al.*, 2008, Kim, *et al.*, 2008, Shen, *et al.*, 2011, Ordinola-Zapata, *et al.*, 2013). In this study, an anaerobic flow chamber model was used to study microbial adherence with the aim of developing a three-species biofilm including peri-implantitis pathogens. Furthermore, single species were allowed to adhere on a titanium surface to examine the efficiency of disinfectant and antibiotic treatment protocols. The vitality in antimicrobial treated cells was detected by commonly used Live/dead Baclight kit and by conventional culturing; additionally, a novel method in oral biofilm research, IMC, was used to assess vitality of adherent microorganisms and bacteria in biofilms based on their metabolic activity levels.

Anaerobic flow chamber model for bacterial adherence

The flow chamber used in this study has been applied in earlier work to evaluate initial bacterial adherence on different restorative materials (Weiger, *et al.*, 1999, Hauser-Gerspach, *et al.*, 2007, 2008, Meier, *et al.*, 2008) as well as to assess the efficacy of disinfectant treatment on adherent streptococci (Decker, *et al.*, 2003, 2003, Decker, *et al.*, 2008). Until now, no studies have reported the use of anaerobic pathogens in this model system; thus here, not only the adherence of facultative anaerobic species *S. sanguinis* was evaluated, but also that of strict anaerobic bacteria present in the microflora of peri-implantitis, namely *F. nucleatum* and *P. gingivalis*.

The adherence of bacteria was observed on protein-coated surface as upon insertion to

oral cavity, materials are covered with a proteinaceous layer termed pellicle. The layer includes different absorbed proteins, such as enzymes, glucoproteins and other macromolecules and mediates interactions between the surface of the material, oral fluids and microorganisms (Lendenmann*, et al.*, 2000, Hannig*, et al.*, 2005, Hannig & Joiner, 2006). Additionally, the bacteria were suspended in reduced nutrient conditions in simulated body fluid (Cho*, et al.*, 1995). These conditions were to mimic the physiological situation found in the oral cavity surrounding the implant, and were to promote adherence rather than to support the growth of the planktonic circulating bacteria. In all single species studies, the bacteria were allowed to adhere to the surface for 2 h as that is considered the time the cells need to attach to the surface via adhesin receptors/glycoproteins bindings (Welin*, et al.*, 2004, Hannig & Joiner, 2006, Hannig & Hannig, 2009).

Live/dead staining and conventional culturing in antimicrobial susceptibility testing of adherent single species

The anaerobic flow-chamber model proved to be an efficient tool allowing the study of specimens for microbial adherence *in vitro*. An adherent single-species model was used to answer two questions: firstly, how efficient are different treatment protocols of disinfectants and secondly, are there discrepancies between the results revealed by different vitality detection methods. Thus, disinfectant treatment on adherent *S. sanguinis* was applied (Chapter 4). Chlorhexidine has been for many years the standard in oral infection control, however, three other disinfectants (povidone-iodine, octenidine dihydrochloride, polyhexanide, respectively) showed comparable efficacy against the adherent microorganism as reported also in other studies (Rohrer*, et al.*, 2010, Tirali*, et al.*, 2012, de Lucena*, et al.*, 2013). Interestingly, the alternative agents showed high efficacy even when diluted down to 1/8 of the original solution while the same was not observed for chlorhexidine. Nevertheless, it must be noted, that the interpretation of the results is difficult (Shen*, et al.*, 2011). Major discrepancies were identified: the staining by live/dead kit detected high levels of vital cells in several groups of povidone-iodine and chlorhexidine treated samples; however, much lower levels of vitality were observed by conventional culturing. And although the results between the two methods correlate quite well (Pearson coefficient 0.6), the biological significance of those discrepancies is unclear. The disagreement between the methods is likely due to the VBNC population of cells that are no longer cultivable because of

their low levels of metabolic activity, but they are detectable by staining combined with microscopic methods (Oliver, 2010). The determination of the vitality of adherent microorganisms by conventional culturing requires their re-suspension prior to cultivation and subsequent quantification of colony-forming units. Several factors – such as physical damage to the cells by re-suspension or lack of growth due to suboptimal culture conditions – may cause the culture method to yield partially false negative results. Additionally, in the case of povidone-iodine, which is based on an iodide complex, the interference with the propidium iodide in the staining step cannot be excluded and therefore, it might be considered as a confounding factor that possibly reduces the percentage of dead cells correctly stained. Thus, the suitability of the staining protocol needs to be evaluated for each testing system to make sure no cells are mistakenly counted or double-labelled as other investigators have proposed for flow cytometry (Stocks, 2004, Berney*, et al.*, 2007). Moreover, no definite conclusion can be drawn by these methods as over- or underestimation of potential infectious cells can take place.

In order to verify the data on the problems of vitality detection and to compare these findings to previously published data (Chapter 4), (Shen*, et al.*, 2010), the vitality of adherent single species of *S. sanguinis* and *P. gingivalis* after antibiotic treatment was also assessed by vitality staining and conventional culturing (Chapter 6). Interestingly, although *P. gingivalis* is considered a late colonizer, it was able to adhere and form a monolayer on protein-coated surface in the absence of other bacteria, whereas *F. nucleatum* was not able to adhere in monoculture. Thus, the antibiotic susceptibility of adherent *F. nucleatum* as single species could not be studied.

Antibiotic concentrations up to 100x higher than the minimal inhibitory concentrations (MICs) for planktonic cells were applied, as adherent bacteria are known for their increased antimicrobial tolerance (Gilbert*, et al.*, 2002). However, it was kept in mind that such high concentrations used here could never be reached in human plasma; thus, the study design was to illustrate through the increased tolerance the difficulties of detecting the level of efficacy.

Possible reasons for discrepancies between staining and culturing were discussed earlier. In the case of antibiotic treatment, the correlation between the two methods was found poor using of both *S. sanguinis* and *P. gingivalis* species (Pearson

correlation coefficient 0.3). This can be attributed to the reasons mentioned above (VBNC cells, difficulties in cultivation), but also to the nature of the dose response. Unlike disinfectants that are mostly bactericidal, the antibiotics may act through bacteriostatic effects, thereby making the results of live/dead staining unreliable as the method is based solely on the destruction or integrity of the cell wall. In addition, the re-suspension of cells for conventional culturing can lead to false negative results, so discrepancies between the two methods are not surprising in this type of experiment. Therefore, conclusions based on only one of these methods when it comes to antimicrobial susceptibility testing should be handled with caution.

Thus, IMC was applied as a third method to provide real time information about the lag time and growth rate of bacteria and how the antimicrobial effect affects these parameters (Buchholz*, et al.*, 2010, Braissant*, et al.*, 2013).

IMC analysis of antimicrobial effect on adherent single species

IMC detects heat-flow from all metabolic processes that are present in any living cells. The principle of the method is that all living systems produce heat and heat evolution can always be measured calorimetrically without any interference with the processes. In other words, IMC measures heat production or consumption, which is proportional to the rate at which any given chemical, physical or biological process is taking place (i.e., metabolic heat from bacterial growth). However, it must be noted that the detected heat-flow signal is non-specific and can be only handled as net signal related to the sum of all processes (Wadsö, 2002). Thus, the main challenge of the method is the translation of the data into useful microbiological data (Braissant*, et al.*, 2013). Therefore, series of calibration experiments with known starting inoculums were completed to be able to relate measured microcalorimetric parameters to bacterial concentrations (CFU/ml) and to estimate inoculums of treated samples in following experiments. Here, no difference in comparison to controls in growth rates or amount of total heat produced over the first 48 h was observed in all disinfectant treated samples where growth was detected. Even in samples that had less than 300 CFU/ml in the beginning, up to 100x more bacteria were present in the control. However, the growth was detected in a lower number of samples by this method compared to the vitality staining and conventional culturing; this allows us to believe that, even though the treated samples are provided with the conditions for resuscitation, not all cells detected to be VBNC by staining were able to switch

themselves back to high metabolic activity. Thus, to avoid the issue with VBNC cells and the discrepancies between staining and conventional culturing, the IMC provides a novel and reliable approach that observes bacterial growth of all the cells that are able to resuscitate after disinfectant treatment and thereby may have a potential to the expression of the virulence factors in the colonized sites.

As IMC allows real time monitoring of the metabolic activity, unlike end-point determinations, the influence of amoxicillin, metronidazole and their combination on lag time and growth rates of adherent single species was observed. Additionally, previous studies have suggested that not only is the IMC useful for determining minimal inhibitory concentration (MIC), but also provides knowledge on the dose response, whether the effect of an antimicrobial is bacteriostatic or bacteriocidal (von Ah*, et al.*, 2008, Howell*, et al.*, 2012). The principle is that a bactericidal agent kills a proportion of the inoculum, thus an increase in lag phase is observed, but growth rate is not or only minimally affected. In case of a bacteriostatic agent, cellular processes are influenced and the decrease in growth rate can be linked to the prolonged lag phase.

Amoxicillin and metronidazole are both considered to be bactericidal. In experiments where adherent *S. sanguinis* was exposed to antibiotics, it was observed that the inoculum was partially killed, resulting in a prolonged lag time and in a reduced growth rate compared to the control samples. The same tendency was detected in experiments on adherent *P. gingivalis*, however the growth rate was only minimally affected. This indicates that the effects of antibiotics cannot be strictly divided into bacteriostatic and bactericidal, as the results here imply that the effect seems to be dependent on their concentration and combination plus the nature of targeted cells.

Thus, IMC is a useful tool to study also new drugs, whose antimicrobial effect is unclear. Furthermore, IMC allows monitoring of the effect in real time unlike vitality staining or conventional culturing that are based on end-point determinations.

Three-species biofilm model and its antibiotic susceptibility

In order to study antimicrobial efficacy on a more complex system, a 72-hour biofilm model was created (Chapter 5). The biofilm includes *S. sanguinis*, *F. nucelatum* and *P. gingivalis* that were also allowed to adhere on protein-coated surface in the same low nutrient system as the single species in the previous models. Modelling the

biofilm formation included series of experiments where all three species were allowed to adhere subsequent to each other over different time intervals (data not presented). However, no substantial differences were detected by microscopic analysis to the current model where all three species in mixed solution are allowed to adhere at the same time. Thus, no pattern of letting first early and then late colonizers adhere was applied and behaviour found in the oral cavity was mimicked, as bacteria there are also always present in a mixture.

Streptococci are known to decrease the pH of the environment to make it more suitable for them (Marsh, 2010). In order to promote the adherence of *F. nucelatum* and *P. gingivalis* rather than to prefer only *S. sanguinis* in the solution, the bacterial suspension was renewed in 24 h intervals. Surprisingly, as the bacteria were allowed to adhere on the surface over 72 h, the fluorescence *in situ* hybridisation of 16S rRNA of the bacteria showed that the species always adhered with similar proportions throughout experiments. This is in agreement with data published earlier as well as with new findings for batch-culture-based models (Thurnheer*, et al.*, 2004, Ammann*, et al.*, 2012). Despite the detailed reports on the structure of the biofilms, these studies provide no information on how the biofilms act on their metabolic activity patterns. Even when such stability in the proportions and structure was found in our experimental biofilm, they still revealed high heterogeneity by IMC. Only the time the biofilms needed to reach their heat-peak-flow varied slightly and that can be attributed to the fact that not such a high variance was found in the total count of bacteria or in the proportions of the species. However, all other parameters calculated from the data demonstrated high variance, supporting Stewart & Franklin (2008) that bacteria in biofilms not only act differently from their behaviour from planktonic cultures but also show intra-species differences within the same biofilm.

The main advantage of IMC in this study was that the structure of the biofilm was not disturbed. This offered the opportunity to observe how an intact biofilm acts as an entity when exposed to antibiotics (Chapter 6). Little is known about the clinical efficacy of adjuvant antibiotic therapy in peri-implantitis treatment on biofilms (van Winkelhoff, 2012), thus the most frequently used antibiotics: amoxicillin, metronidazole and their combination were tested by IMC.

Biofilms are well known for increased antibiotic tolerance, thus firstly the MIC for planktonic forms of the species present in the biofilm were determined and multiplications of those up to 100x were applied on the biofilm. Here, the antibiotic

efficacy was monitored only by IMC as the other commonly used vitality detection methods provide results that are difficult to interpret due to their limitations (Chapter 4), (Shen*, et al.*, 2010). Heat-flow profiles of biofilms exposed to antibiotics were followed up to 10 days to observe how the lag time and growth rate are affected. The growth rate that was found was lower than that of planktonically grown cultures, which leads us to believe that the incubation of the biofilm on agar created a system where the results are not interrupted by growth of free colonies that was observed in single species cultured in liquid media.

Interestingly, IMC revealed that the MIC values for *S. sanguinis* (the highest values of the three species) used to test the susceptibility of the three-species biofilm in this study inhibited growth over 10 d. That indicates that adherent *S. sanguinis* behaves differently in the presence of other bacteria and even its antibiotic susceptibility is affected. Results showed that amoxicillin alone was always found to be more efficient in prolonging the bacterial lag phase in biofilms than metronidazole alone and demonstrated comparable results to the combination of antibiotics used. In samples of treated three-species biofilms, prolonged lag phase was observed. However, the growth rate remained mostly unchanged. Interestingly, when used alone, both antibiotics decreased the maximum growth rate of the biofilm but had a smaller effect on prolonging the lag phase, while biofilm samples treated with the combination of the two exhibited growth rates close to the control but more efficiently delayed growth. Thus, when applied alone, antibiotics seem to work primarily through bacteriostatic effects; when combined, the efficacy is increased by their synergistic bactericidal action.

IMC proves to be an efficient way to study antibiotic susceptibility. Furthermore, when the model system excludes the growth of planktonic cultures it is still sensitive enough to detect the low levels of metabolic activity of treated samples.

8 Conclusions

In conclusion, this study emphasizes the need to critically evaluate the results of live/dead staining and conventional culturing as many aspects can affect the outcome of these methods leading to miscalculations on the proportion of cells that are able to resuscitate and lead to reinfection on treated sites. Furthermore, the downside of both of the methods is that they are based on end-point determinations of antimicrobial effects and little can be observed about the mode of action of the antimicrobials.

Thus, micro-calorimetric analysis provides a novel alternative approach in oral biofilm research that allows monitoring the efficacy and dynamics of biofilms and their antimicrobial susceptibility *in vitro*. The sensitivity of the method detects also intact biofilms at very low metabolic activity.

Furthermore, through the modifications to the anaerobic flow chamber modelling system, a multi-species biofilm model was created and validated in this study. The structure of the biofilm and presence of all three species was successfully determined by microscopic analysis. IMC allowed observation of the heterogeneity of the biofilms even when microscopically no structural differences were detected.

This multispecies biofilm model proposes an alternative to batch-culture-based models to estimate the initial bacterial colonization and biofilm formation of different implant materials and to test antimicrobial efficacy of new agents.

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Curriculum vitae

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EDUCATION

2008 - 2013	*University of Basel, Basel, Switzerland* PhD in Biomedical Engineering Title of thesis: "Infection and disinfection of transmucosal implant surfaces *in vitro"*
2005 - 2007	*Tallinn University of Technology, Tallinn, Estonia* MSc in Natural Sciences, Gene Technology Title of thesis: "Potato virus A as a model system plant virus coat protein based vaccine design"
2002 – 2005	*Tallinn University of Technology, Tallinn, Estonia* BSc in Natural Sciences, Gene Technology Title of thesis: “Detection of specific markers for rheumatoid arthritis diagnostics”

WORK EXPERIENCE

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PUBLICATIONS

1) Astasov-Frauenhoffer M, Braissant O, Hauser-Gerspach I, Daniels AU, Wirz D, Weiger R, Waltimo T. "Quantification of vital adherent *Streptococcus sanguinis* cells on protein-coated titanium after disinfectant treatment", *J Mater Sci Mater Med. 2011 Sep;22(9):2045-51*.
2) Astasov-Frauenhoffer M, Braissant O, Hauser-Gerspach I, Daniels AU, Weiger R, Waltimo T. "Isothermal microcalorimetry provides new insights into biofilm variability and dynamics", *FEMS Microbiol. Lett. 2012; 337:31–7.*
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4) Gerber L.C., Mohn D., Fortunato G., Astasov-Frauenhoffer M., Imfeld T., Waltimo T., Zehnder M., Stark W.J. „Incorporation of reactive silver-tricalcium phosphate nanoparticles into polyamide 6 allows preparation of self-disinfecting fibers", *Polym Eng Sci. 2011, 51(1):71-7*.

PRESENTATIONS

03. 2009 M. Astasov-Frauenhoffer, I. Hauser-Gerspach, N. U. Zitzmann & T. Waltimo. "Susceptibility of adherent *Streptococcus sanguinis* to antibiotic agents applied against peri-implantitis". German Association for General and Applied Microbiology conference "How dead is dead? – Survival and final inactivation of microorganisms", Bochum, Germany (Poster presentation)

06. 2010 M. Astasov-Frauenhoffer, O. Braissant, I. Hauser-Gerspach, R.

Weiger & T. Waltimo. "Characterization of a three-species biofilm *in vitro*"
Annual meeting of Swiss Society for Microbiology, Zurich, Switzerland (Poster presentation)

05. 2011 M. Astasov-Frauenhoffer, O. Braissant, I. Hauser-Gerspach, A. U. Daniels, R.Weiger & T. Waltimo. "Quantification of vital adherent *Streptococcus sanguinis* cells on protein-coated titanium after disinfectant treatment"
European Oral Microbiology Workshop, Zeist, The Netherlands (Oral presentation)

06. 2011 M. Astasov-Frauenhoffer, O. Braissant, I. Hauser-Gerspach, A. U. Daniels, R. Weiger & T. Waltimo. "Characterization of a three-species biofilm *in vitro*"
eCM XII: Implant Infection Meeting, Davos, Switzerland (Oral presentation)

06. 2012 M. Astasov-Frauenhoffer, O. Braissant, I. Hauser-Gerspach, R. Weiger & T. Waltimo. "Optimized susceptibility testing of adherent bacterial cells"
Annual meeting of Swiss Society for Microbiology, St. Gallen, Switzerland (Poster presentation)