In vitro modelling of Streptococcus intermedius biofilm on titanium dental implants

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Abstract

Streptococcus intermedius is part of healthy oral flora; it is an opportunist pathogen in endogenous/systemic infections. This strain exhibits a tropism toward different typical biofilm diseases, e.g. periodontititis and peri-implantis. The aim of this study was to determine the biofilm colonization kinetics produced in vitro on titanium dental implants. Biofilm formation was evaluated by a PCR real-time protocol in order to determine the number of S. intermedius cells on the titanium implant surface.

The in vitro model showed a striking fast progression of biofilm formation. Coating of salivary proteins on the implant surface peaked after 4 hours. The highest concentration of bacteria on the implant surface was at its highest after 4 hours as well. This indicates that the biofilm reached maturation within 5 hours.

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Introduction

Biofilms play an important role in the spread of antimicrobic or antibiotic resistance. It is able to extend bacteria's virulence products on human tissues [1]. In these biofilms no single microbial species have been identified which satisfies Koch's postulates of an unique infectious agent in either periodontitis or peri-implantitis [2, 3]. There is strong evidence that both diseases have the same multi-bacterial etiology [4]. Because bacteria proliferate in this well-developed structure, it is irrelevant to expect eradicating dental implant failure due to infection. The same applies to periodontitis where the costs related to maintenance and prosthetic work replacement are high [5]. Infected

implants are colonized by mostly sub-gingival species; this includes strict Gram negative anaerobes, micro-aerophiles and some aerophiles species [6, 7]. In this context, distinct patients may be colonized by dissimilar microbial complexes. This indicates that optimal treatment should be directed specifically to multi-infection processes [8, 9].

Streptococcus intermedius and others Gram positive bacteria are recognized to be pioneer colonizers in these biofilms [10]. *S. intermedius* is a gram-positive, micro-aerophilic streptococcus that is part of the "anginous group" and the Socransky yellow complex [11]. This pathogen is implicated in numerous serious pyogenic human infections such as periodontitis, pneumonia, endocarditis, abdominal cerebral and liver abscesses [12]. The primary ecological niches of this bacterium are the mouth, the upper respiratory and the intestinal tract. The type and growth of *S. intermedius* biofilm in the oral cavity follows the typical Gram positive regulation [13]. Biofilm maturation and expression of virulence factors are some of the several identified *quorum sensing*-controlled behaviors [14].

S. intermedius interaction with the host self and not-self components are most often associated with surface proteins. Generally, a salivary proteins pellicle play a significant role in the initial adhesion of oral streptococci to different surfaces exposed to the oral cavity, e.g. orthodontic brackets and dental implants [15]. Subsequently the growth of the biofilm is regulated by different and complex signaling that have different roles in bacterial interspecies and interkingdom communication [13]. Bacteria in biofilms exhibit an increased resistance to usual antibiotic or antimicrobic therapies. This is the reason why non-surgical periodontal treatment such as scaling and root surfacing is so appropriate to the treatment of periodontal disease [16]. To have a better understanding of the way biofilms are functioning, it is relevant to investigate the time required for the pathogens to reach biofilm maturation. This could generate crucial information when developing a new antibiofilm therapy against peri-implantitis is aimed at, because the time of prophylactic management is critical [17].

This paper investigates the kinetics of biofilm formation on the surface of dental implants in an *in vitro* biofilm structure composed by a salivary acquired pellicle and *S. intermedius* considered as an initial Gram positive biofilm colonizer.

Materials and methods

Strain and cultural procedures

Streptococcus intermedius strain DSMZ 20573 (German Collections of Microorganisms and Cell Cultures) was inoculated in a brain heart infusion broth, BHI (Microbiol Uta, Cagliari) with 5% CO_2. In the middle of log phase (after ca. 8 hours) an aliquot was used as starter and was inoculated in a shake culture reactor with a final title of 10^6 CFU/ml.

Saliva/Shaedler Cultural medium

Saliva samples were collected from five healthy subjects. Prior to use, the saliva was thawed and centrifuged at 4000 rpm to remove any possible precipitate. The samples were pooled and then centrifuged at 4000 rpm for 10 min. The supernatant was diluted with Shaedler Broth (Microbiol Uta, Cagliari) to produce a growth medium with 80% saliva. It was then sterilized by filtration through a 0.22 μm pore-size filter (Millipore, MA-USA), The samples were then stored at -20°C. Five milliliters of this medium was distributed in each of the glass tubes contained in the bioreactor (Dynamic shake culture).

Dynamic shake culture model, DIB reactor

The experiments in this work rebuilding the oral ecosystem have been performed in a bioreactor. This dynamic culture model (fig. 1) was composed of:

(i)   a shaking support (Continental Instruments, Italy).

(ii)   a thermostatic 37°C chamber (Vivacar diagnostic, Vivadent, Italy) containing 4 sterile flasks of 5 ml of saliva medium and 2 dental titanium implant each (Leone, Sesto Fiorentino, Italy).

(iii)   a flask containing 5 ml of saliva medium not inoculated with the pathogen was used as a negative control.

The cultural medium in these apparatus was maintained in constant shaking for 12 hours. Every 30 min an implant from the positive and one from the negative controls were sampled and used for DNA/RNA extraction for bacteria enumeration and for *luxS* expression respectively. In order to oppose non-specific bacterial adhesion and to reproduce a mean *in vivo* salivary flow the bioreactor was generating 60 oscillations/min (fig. 1).
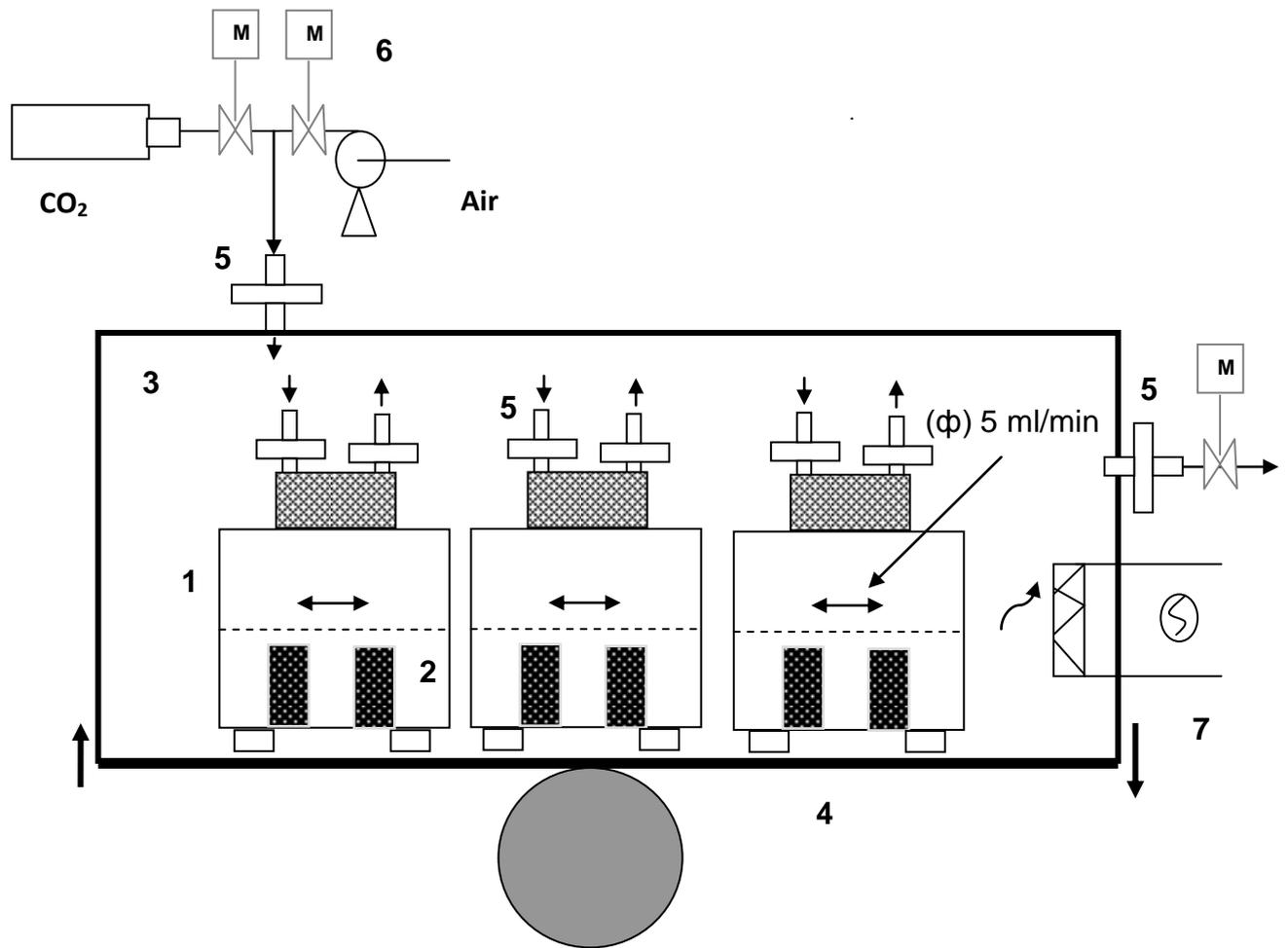
Fig. 1. Schematic representation of the customized bascule culture system DIB (*Dental Implant Bioreactor*) used in this work: (1) glass flask containing the culture medium [Saliva/Shaedler 80/20], (2) titanium implant in the tube ,(3) thermostatic 37°C chamber, (4) shaking bascule support, (5) air Millipore filter 0.22 µm, (6) CO_2/air mix, (7) electrical resistance. The medium flow (ф) was 5 ml/min.

RNA/DNA/Protein extraction

At each implant, the simultaneous extraction of RNA, DNA and protein was performed using a modified TRIzol reagent method described by Xiong *et al.,* [18] (TRIzol®, Life Technologies, Carlsbad, CA-USA). After extraction, the 3 extracts (RNA, DNA, Proteins) were maintained at a temperature of -80°C prior to analysis.

S. intermedius growth curve

The dynamics of *S. intermedius* growth inside the bioreactor was studied by plotting the pathogen cell growth and measuring the 550 nm absorbance *vs.* incubation time. In practice, 1 ml of medium sampled from positive controls was put in a cuvette (Corning 1 cm) and was read by a spectrophotometer (DMS90 Varian). The specific growth rate (µ) of the pathogen in the culture conditions described above was calculated by the following formula:

$$\mu = 2.303 \, (\lg OD_2 - \lg OD_1) / (t_2 - t_1)$$

where, OD_i represent the absorbance at 550 nm at the initial time (t_1) and at the measurement time (t_2).

The experimental start time (t_2) for biofilm measurement was calculated when the growth was in the middle of the exponential phase or rather assuming $t_1 = 0$ and $OD_1 = 0$ at the start of the exponential phase:

$$2 = 2.303 \ (lg \ OD_2 - 1) \ / \ (t_2)$$
$$t_2 = 2.303 \ (lg \ OD_2 - 1) \ / \ 2$$

Protein quantification

A comparison of total proteins distributes at different times on the implant surface was obtained from the protein extracts with the Warburg method [19]. The absorbance of the protein extracts was measured with a Coleman 124 Perkin-Elmer spectrophotometer at A260 nm and A280 nm with a light path of 1 cm. The suitable sample cuvettes were compared vs. a suitable blank (bi-distilled water, Gibco). In order to obtain a valid result, the A280 nm had to be greater than the A260 nm.

The protein amount was calculated with the following formula:

mg protein/ml = [(1.31 x A280) - (0.57 x A260)] x dilution factor

A serial 1/10 standard with albumin bovine (Sigma) from 100 mg/ml to 1 was performed for calculate the standard error and the sensitivity of the procedure.

Total bacteria count

The total mass of *S. intermedius* on implant surface was evaluated through the method described by Denotti *et al.* using a real time PCR [20]. Briefly, the real time PCR reaction was performed by using the light-Cycler instrument and Light-Cycler DNA Master SYBR Green I kit (Roche Diagnostics, Mannheim, Germany) following the manufacturer's instructions. PCR reaction has been considered on a region of the 16S rRNA gene. The primers for the PCR (OG347 and OG348) were designed to a flank sequence of 177 bp, GenBank accession n. AF104671.

Results

Dental implant surfaces coated with salivary proteins

Different authors have indicated the crucial role of the salivary components, in particular the proteins, in the binding of the oral Streptococci to surfaces and in the formation of a biofilm on the surface of dental implants. The maximum amount of protein slime was observed after 4 hours of incubation time (Max) (fig. 2). At this point, the microorganisms were inoculated inside the DIB reactor.

Kinetics of *S. intermedius* adhesion

The number of *S. intermedius* bound to the implant surface was calculated by real time PCR trough the rrs gene enumeration in real time PCR by absolute quantization (described above). The process was very rapid and the first measurable colonization was observed 20-30 min after *inoculum* ($1*10^7$ genomes/cm^2) (fig.3a).
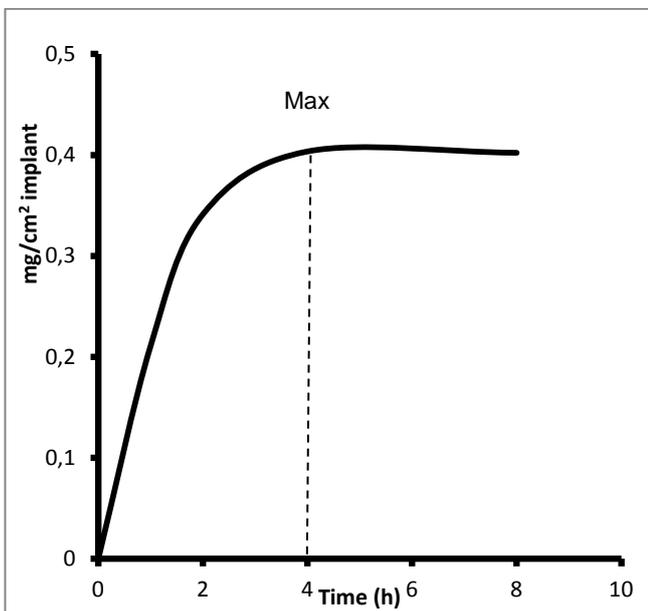

Fig. 2. Kinetics of the coating protein formation on the dental implant.

The maximum number of bacteria per cm^2 of implant surface was observed after 5 hours from *inoculum* $6.3*10^7$ genomes. Figure 3b shows the effect of the salivary medium proteins on the adhesion rate (*genomes/min*). Different regression curves were obtained with the bacterial genome count on a cm^2 of implant surface over a 0 to 60 min period. The addition of medium proteins, and more specifically saliva, resulted in a dramatic increase of

bacterial adhesion. In comparison to water, the increase was a 445 fold when H_2O/SH was added. Increase was 885 fold when saliva/SH was added (fig. 3b). This adhesion process could be due to an electrostatic bound between the bacteria and the coated proteins surfaces.
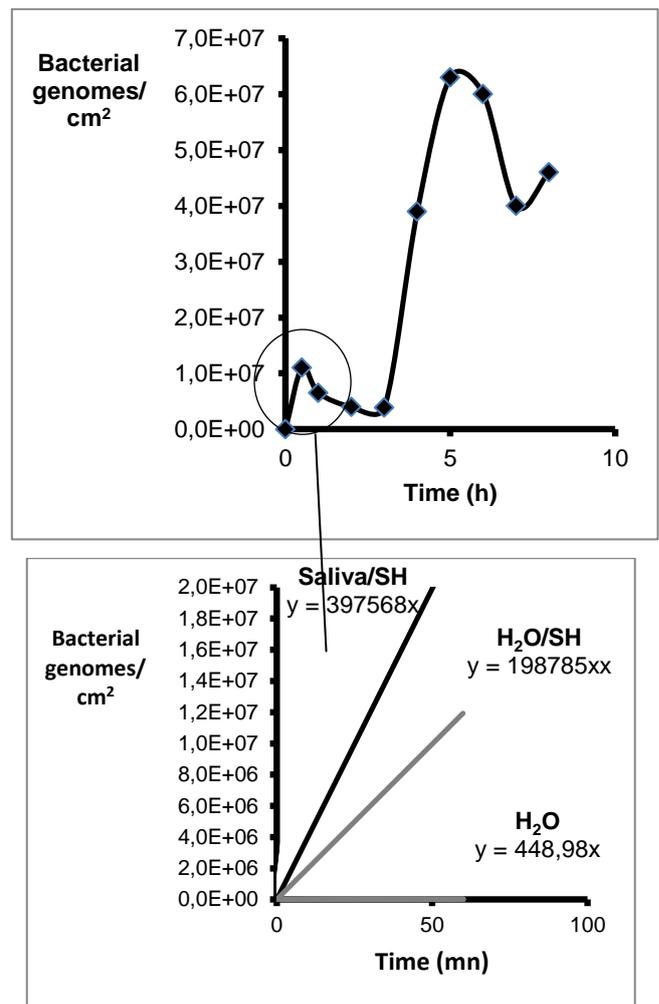

Fig. 3. Kinetics of *S. intermedius* adhesion on the dental implants: (i) numbers of *S. intermedius* genomes bound on a cm^2 of implant surface; (ii) adhesion velocity during the first 60 min in liquid media with distinct protein contents.

Growth of *S. intermedius* in the bioreactor and comparison with biofilm parameters

Figure 4 shows the growth curve of *S. intermedius* in the bioreactor involving the culture conditions described above. The exponential phase of the planktonic phase *ep* was observed 2.5 to 7 hours after the inoculum, *(*ABS$_{550}$ 0.25-0.84 respectively). The graph indicates the values of max implant surfaces coated with salivary proteins and max *S. intermedius* biofilm mass.

Discussion and Conclusion

This *in vitro* work simulated the initial colonization of an implant surface by the human pathogen *S. intermedius*. This bacteria has been chosen because it is known in classical microbiology to change the saprophytic phase in human mucosa. It can induce a variety of infections including meningitis, endocarditis and abscesses [21-23]. It is a constitutive flora of different biofilm types engaged in periodontitis and perimplantitis [24-28]. As described by other authors, the dental implant infection follows a classical biofilm structuration event.

This preliminary work suggests, i) an early infection/colonization during biofilm formation, ii) a structured biofilm is obtained within 5 hours after infection, iii) any prophylactic action involving antibacterial tools should be implemented at a very early stage of biofilm formation.
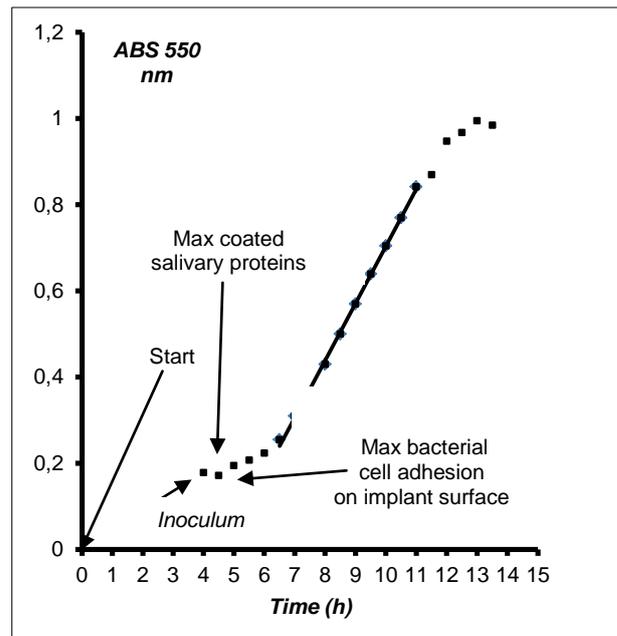

Fig. 4. Representation of the crucial time points for *S. intermedius* biofilm formation of S. intermedius on dental implant, in comparison to the growth curve of pathogen planktonic form in the same culture medium in the bioreactor.

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