Structural characteristics of *in situ* undisturbed human oral biofilm and activity of antimicrobial agents

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The scientific community considers that the analysis of undisturbed human plaque-like biofilm (PL-biofilm), using confocal laser scanning microscopy (CLSM), is the most suitable approach to the study of the *in situ* of PL-biofilm.

Although some evidence on the structural characteristics of *in situ* early PL-biofilm has been described, there are few studies on the antibacterial effects of agents on this microbial structure. A major application of advanced microbiological and/or microscopy techniques, in combination with CLSM, needs to be exploited in future research in order to increase knowledge of the global characterisation of *in situ* PL-biofilms, as well as of the antibacterial effects of agents frequently used in Dentistry on biofilm structure.

Keywords: *in situ* studies; undisturbed human plaque-like-biofilm; confocal laser scanning microscopy; structural characteristics; antimicrobial agents.

1. Introduction

Dental plaque is considered a specialized microbial biofilm [1,2], in which more than 6000 species-level phylotypes have been identified [3]. The adherent biofilm that forms on the hard and soft tissues of the mouth constitutes the principal aetiological agent in the origin of caries, gingivitis and periodontal disease [4-6].

The *in vitro* development of biofilm models has led to significant advances in the study of oral biofilms [7]. However, *in vitro* oral biofilm models tend to involve limited numbers of species and, in addition, they are created under conditions that still cannot adequately reflect the physiological situation in the oral cavity [8-10]. Factors related to the oral cavity, such as the turnover rate of saliva, the ability of antibacterial substances to adhere to the pellicle of the tooth or the surface of soft tissues in order to achieve their effects, and the interaction with unculturable bacteria, cannot be modelled *in vitro* experiments [11]. Consequently, at the present time, the scientific community recognizes that *in vitro* models cannot guarantee the creation of oral biofilms whose composition and structure is comparable with those that form *in situ* [8-10,12]. For this reason, there is a special interest in exploring the early stages of the *in situ* formation of the plaque-like biofilm (PL-biofilm) on undisturbed samples, as an understanding of the processes involved may open new avenues for acting on the pathogenic properties of biofilm [13]. This review summarises the existing literature on *in situ* studies of undisturbed PL-biofilm by using confocal laser scanning microscopy (CLSM), differentiating 2 sections: structural characteristics of biofilm, and effects of antimicrobial agents on biofilm.

2. Structural characteristics of *in situ* undisturbed oral biofilm

It has been recognized that the structure of *in situ* undisturbed PL-biofilm itself is likely to be an important factor in the modulation of microbial physiology, and in determining the ecology of the site [14]. In this regard, microbial behaviour will depend upon parameters such as biofilm thickness, density and the openness of the architecture**,** as well as the cell: matrix ratio [14].

As of now, there is some evidence concerning the three-dimensional structure of undisturbed *in situ* PL-biofilm applying CLSM in relation to thickness, architecture, vitality state and bacterial topography.

2.1 Thickness

Netuschil et al [15], in 1998, were the first to observe that PL-biofilm thickness on enamel and glass slabs inserted in acrylic appliances depended on the subjects and the plaque age (1-day-old ranged from 0-10 μm; 2-day-old ranged from 8-35 μm; 3-day-old ranged from 6-45 μm).

Accordingly, the majority of authors who subsequently analysed *in situ* PL-biofilm emphasised the great variation detected in biofilm thickness between the different individuals [8,16,17]. In this regard, Auschill et al [16] stated that "*the height of the oral biofilms formed depended on the plaque-forming rate of the individual donors*". Hovewer, Zaura-Arite et al [18] found no differences in 2-day biofilm thickness between heavy and light plaque formers (on the basis of the average protein amount in early smooth-surface plaque samples); although these authors applied a groove model for PL-biofilm growth. With respect to the influence of other factors, it has also been demonstrated that the following factors do not show any correlation with the PL-biofilm thickness in different time periods (after 48 hours and 5 days,

respectively): caries risk (assessed by the number of mutans streptococci), the number of decayed, missing and filled teeth, salivary flow rate and lactic acid formation rate [19,20].

On the other hand, Al-Ahmad et al [11] found that the biofilm thickness formed on bovine enamel slabs fixed in an individual acrylic appliance increased from 14.9 ± 5.0 μm after 1 day to 49.3 ± 11.6 μm after 7 days. However, interestingly, it has been detected that this increase in biofilm thickness is not progressive throughout each day [11,21]. The significant influence of "plaque age" factor has been corroborated in other more recent studies, in which shorter oral exposure times were analysed, such as those published by Dige et al (PL-biofilms of 6 and 12 hours, 1 and 2 days) and Jung et al (PL-biofilms of 30 minutes, 2 and 6 hours) [17,22]. Nevertheless, an important detail reported on PL-biofilm is that the microbial deposits observed after 2 days (as at previous stages) are not uniform in thickness [23].

Some authors have described *in situ* PL-biofilm formation on dental restorative materials, using various substrates: amalgam, gold, ceramic, resin composite, compomer, glass-ionomer cement, and polymethyl methacrylate [24-26]. Recently, Bremer et al [27] observed that the 1-day PL-biofilm formation on various types of dental ceramics differed significantly; in particular, zirconia exhibited the lowest surface coating and biofilm thickness (19.0%, 1.9 μm) and lithium disilicate glass-ceramic the highest mean values (46.8%, 12.6 μm). Over the last few years, some researchers have studied the *in situ* formation of PL-biofilm on various implant surfaces with varying degrees of roughness [12,28]. Al-Ahmad et al [12] evaluated PL-biofilms of 3 and 5 days on different titanium and zirconia implants and concluded that the influence of roughness and material on biofilm formation was compensated by biofilm maturation.

2.2 Architecture

Some authors have analysed the architectural characteristics of *in situ* PL-biofilms of less than 24 hours [22,23,29]. In 30-minute PL-biofilms, Jung et al [22] found that bacteria on the enamel and dentine were distributed randomly, although on dentine specimens they were detected preferentially at the openings of the dentine tubules. Within the first 2 hours of oral exposure, mainly single bacteria and small aggregates were observed, whereas after 6 hours, accumulated colonies or mono-layered chains were detectable. Dige et al [23], after studying 6-hour PL-biofilms, described how the pattern of microbial colonization had showed 3 distinct appearances: single bacteria, bacteria in pairs, and clumps of bacteria of varying sizes.

Palmer et al [29], on 8-hour PL-biofilms, observed that although solitary cells were detected, the majority of biomass was found in cell aggregates heterogeneously stained (so these may consist of more than one cell type), not as single cells as in the 4-hour biofilms. These authors estimated that the increase in cell numbers between 4 and 8 hours was 2- to 40-fold. Consequently, the mixed-species microcolonies seen at 8 hours were formed by a combination of adherence of planktonic cells to already attached cells (for example, those observed at 4 hours) and growth of the cells in the microcolony [29]. On the one hand, coadhesion of bacteria from saliva has been considered to be a continuing process adding to the biomass of the developing PL-biofilm, which is further supported by the discovery of genotypically different bacteria colonizing at the outer surface of the biofilm [30].

On the other hand, the implication of a "bacterial growth mechanism" has also been proved in other *in situ* series on 6-hour and 12-hour PL-biofilms, due to detection of many bacteria that appeared in a stage of cell division reflected by their pair-wise or branching arrangement [23]. Consequently, cell division is a significant contributor to the biomass increase [30]. This fact, combined with the exponential growth of the bacteria during the first and second days [31], supports the view that the increase in mass of the initial PL-biofilm occurs predominantly by multiplication [32,33].

One interesting detail reported by Dige et al [23], after studying 12-hour PL-biofilms, was that "*most of the planes analysed were closed to the glass surface*". For these authors, the reason for this is that in monolayer colonies rod-shaped and filamentous bacteria were mainly oriented with their long axis parallel to the surface [23]. In contrast, in multilayer colonies pleomorphic bacteria were frequently oriented with their long axis perpendicular to the surface [34-36], which makes it more difficult to distinguish between coccoid and pleomorphic species in the x–y plane. In addition, large 1–2-μm coccoid bacteria of unknown identity, which were typically seen in pairs, tetrads or clusters, were observed in the majority of individuals. This growth pattern was confirmed by the presence of protruding microcolonies forming "chimney- like" structures. Some authors stated that "*this particular growth pattern might reflect a constrained physical environment during expansion of the biofilm*" [23].

To our knowledge, only 2 papers have investigated night/day differences *in situ* PL-biofilm accumulation [37,38]. Recently, Dige et al [38] studied *in situ* PL-biofilm from healthy individuals, which were collected for 12 hours during the day and night, respectively. After analysing 12-hour PL-biofilms, using steorological methods and digital image analysis by CLSM, the results revealed a statistically significant difference between both the total number of bacteria and the biovolume in the 2 groups, with the highest accumulation of bacteria during daytime. For these authors, these data provided firm evidence that initial PL-biofilm formation decreases during the night, which may reflect differences in the availability of salivary nutrients [38].

In several *in situ* studies on PL-biofilms of 24 hours or more, images generated previously by electron microscopy suggested that biofilm has a compact architecture [39,40]. In contrast to these observations, a high number of authors have agreed that *in situ* PL-biofilm analysed by CLSM has an open complex, and a heterogeneous architecture model, and is characterised by the presence of a complex system of channels and voids described as an integral part of biofilm structure [8,14,16,18,19]. The first descriptions of *in situ* PL-biofilm architecture were made by Wood et al in 2000

[14], who demonstrated that 4-day PL-biofilms present a highly heterogeneous architecture in terms of distribution of cells, matrix, and fluid-filled spaces. Strikingly, all of the samples examined contained fluid-filled pores and channels, some of which were throughout the entire thickness of the biofilm [14]. These channels were surrounded by cellular/matrix aggregates, some of which exhibited inverted biomass. At higher magnification, Dige et al [23] even observed that these channels were colonized by scattered bacteria. Auschill et al [16], after analysing 5-day PL-biofilms, also localised the presence of "non-stained (black) bubble-like structures" within the biofilm architecture. These voids may be filled with biological substances such as exopolysaccharides and glycoproteins, which are not stainable by different fluorochromes. In this regard, Robinson et al [41] reported 2 interesting aspects of the architecture of 7-day PL-biofilms: the presence of a fairly consistent pattern in biomass distribution (biomass density measured as intensity of reflection increased from saliva plaque interface inwards); and the frequency of channels and voids also seemingly decreasing in this direction.

It has been suggested that the presence of an open architecture with such channels and voids *in situ* PL-biofilm would presumably provide direct communication between the oral environment and the enamel surface [14]. These "circulatory" channels and voids could have important implications for the movement of tooth damaging organic acids, bacterial toxins, and other antigens, as well as for the delivery of antimicrobial agents to the desired targets within the PL-biofilm [14]. Dige et al [23] recognized that further *in situ* studies, using fluorescent probes for specific matrix components, are needed to analyse the structure of these channels and voids, as well as to clarify their function *in situ* PL-biofilms.

In accordance with findings described in younger PL-biofilms (12 hours old), some researchers agreed to state that the pattern and degree of microbial coverage in 1-day and 2-day PL-biofilms varied greatly, not only among the subjects, but also across the individual surfaces [17,18,23,30]. Dige et al [17] estimated there was a 12.5-fold difference between individuals in the total number of bacteria colonizing the surfaces after 2 days, and they described from incomplete to complete surface coverage by bacteria, where areas of monolayer were intermingled with areas of multilayers. Some authors also found that the complexity of the PL-biofilm structure increased with increasing plaque age and thickness [18,23].

With the aim of investigating the relationship between PL-biofilm structure and physiology, von Ohle et al [42] examined the metabolic activity on the 3-day PL-biofilms during exposure to sucrose. The sucrose addition resulted in a strong pH decrease, particularly at the base of the biofilm, with the lowest values ranging from 3.97 to 4.66 (in contrast to pH 7). These authors stated that the degree of acidification in PL-biofilms would be expected to vary between individuals, as a function of both host and bacterial factors such as biofilm thickness, concentration of acidophilic bacteria, and saliva chemistry [42].

2.3 Bacterial vitality

In early studies, in which conventional microbiological culturing techniques as well as vital fluorescence were applied, it was shown that young and sparse dental plaque consisted of more dead material than living microorganisms [43-45]. Accordingly, in 1998, Netuschil et al [15] reported, after analysing *in situ* 1, 2 and 3-day PL-biofilms by CLSM, that living microorganisms were located on top of a dense layer of dead material. However, Zaura-Arite et al [18] observed that in 2-day PL-biofilms, clusters of vital bacteria were intermingled with dead material.

Vital microorganisms located on, and embedded in, these dead layers may be responsible for further plaque growth [15]. Dead bacteria may supplement living flora with all the materials needed for rapid growth [15,46,47], and protect them against antibacterial influences in the oral environment [16]. Because of this, it has been stated that dead cellular material is a major component of the biomass during the initial stages of PL-biofilm accumulation and development [15,16]. Void-like structures ("black holes") were seen in these PL-biofilms surrounded by vital bacteria, which could mean that these bacteria have direct access to the nutrients diffusing through the pores and voids [48-50].

The *in* situ studies published by Arweiler et al [19], Auschill et al [9] and von Ohle et al [42] on PL-biofilm vitality over 2 and 3 day periods respectively, reported mean bacterial vitality values between 60 and 77%. In other series, the 5-day PL-biofilm vitality detected *in situ* ranged from 57% to 63% [20]. However, other authors found an increase in the PL-biofilm vitality associated with an increase in the biofilm age, and consequently in the thickness [16,18].

In some studies, large inter-individual differences were found among the subjects in their PL-biofilm vitality distribution [18], and consequently no general pattern for bacterial vitality distribution could be described [18,42]. In contrast, it has been suggested that a relatively constant ecological environment exists in each volunteer, which obviously leads to a microbial identity pattern [19]. On this point, Arweiler et al [19] detected a great variation in the bacterial vitality values in the 2-day PL-biofilms for the different biofilm layers, identifying 3 vitality patterns: first, patterns where a high number of dead bacteria (low vitality values) were found in layers nearest the substrate, increasing in higher layers, and then ending with low values at the outermost surface of the PL-biofilm; second, patterns where dead bacteria are then superposed by new, vital bacteria, or some still vital or cultivable bacteria proliferate, forming a new layer of vital PL-biofilm; and third, patterns where PL-biofilms that start with high vitality values adjacent to the substrate surface, then decrease at their external aspect. Auschill et al [16] detected that the bacterial vitality distribution in the PL-biofilms accumulated on smooth-surfaces for 5 days exhibited a similar slope: the percentage of vital bacteria was lower adjacent to the enamel surface, increased in the z-axis towards the central parts,

and decreased again towards the outer layers. In contrast, Arweiler et al [20] showed a very similar vitality pattern in all 5-day PL-biofilms, with lower values in the bottom and higher values in the top layers (bottom= 51% ant top= 65%), which is in accordance with the knowledge that bacteria in deeper biofilm layers are rather metabolically inactive (dormant zones) [15,51].

2.4 Bacterial topography

Despite the spatiotemporal variability in species composition, consensus exists that supra- and subgingival PL-biofilm develops according to sequential and reproducible patterns [29]. The initial microbial colonization of tooth surfaces is a repeatable and selective process, with certain bacterial species predominating in the nascent biofilm, and then being followed by secondary colonizers [52,53].

One important factor in the initial colonization during the formation of the PL-biofilm is the salivary acquired pellicle, which is the basis for further bacterial adhesion and colonization of the tooth surface [54]. In this regard, Jung et al [22] showed no direct adherence of the bacteria to bare dentine and enamel surfaces *in situ* PL-biofilm, but there were always pellicle structures between the bacteria and the dental hard tissues (the pellicle layer analysed by TEM was 10–20 nm in thickness on the surfaces of dentine and up to 100 nm on enamel globular and granular structures).

Diaz et al [55], using molecular methods and a retrievable enamel chip model, described the microbial diversity of *in situ* early PL-biofilms (at 4 and 8 hours). In all subjects (n= 3), 4 and 8-hour communities were dominated by *Streptococcus* spp. belonging to the *Streptococcus oralis*/*Streptococcus mitis* group. Other frequently observed genera (comprising at least 5% of clone sequences in at least 1 of the 6 clone libraries) were *Actinomyces*, *Gemella*, *Granulicatella*, *Neisseria*, *Prevotella*, *Rothia*, and *Veillonella.* Dige et al [17], applying a stereological method for bacterial quantification *in situ* PL-biofilms as a function of time (for 6 and 12 hours, 1 and 2 days), observed a relatively constant relationship in the majority of individuals between streptococci and non-streptococci over the examination period (the number of streptococci was about 15 times higher than the number of non-streptococci). Al-Ahmad et al [11] applied a multiplex FISH in combination with CLSM, to analyse the levels of important bacterial members (all eubacteria, *Streptococcus* spp., *Actinomyces naeslundii, Fusobacterium nucleatum* and *Veillonella* spp.) of *in situ* PL-biofilms formed after different periods of time (1, 2, 3, 5 and 7 days). The results revealed that all of the investigated bacterial targets were detected in each biofilm over the course of 7 days, representing up to 35.5%-59.8% of the total amount of bacteria in PL-biofilm. Communities of streptococci were detected, representing 41% of bacteria at 1 day *vs* 13% at 7 days. Equally, Al-Ahmad et al [12], who examined the biofilm formation and composition on different zirconia and titanium implant materials, detected *Streptococcus* spp. as the primary component of the biofilm after 3 and 5 days (10.67%-26.13%). Consequently, streptococci may play a significant role in the total mass increase of developing young biofilms *in situ*. Two important characteristics of streptococci have to be considered in this context: firstly, there is a shorter doubling time of streptococci of only 1.4 h as compared with other bacterial species [56], and secondly, streptococci possess numerous adhesins that recognize receptors in the acquired pellicle that coats the enamel [57-61].

Also, the presence of other aerobic and aerotolerant bacteria might provide a favourable biofilm niche for subsequent or concomitant colonization of facultative and obligate anaerobes, which allows the aerobe-to-anaerobe transition for oral bacterial climax community development [62]. Al-Ahmad et al [11] found that *A. naeslundii* constituted up to 18% of the microbiota within the first days of *in situ* PL-biofilm formation, with a notable decrease over the 7 day observation period. In contrast, there was an increased presence of *F. nucleatum* in older and thicker PL-biofilms (representing 10% of bacteria at 1 day *vs* 22% at 7 days). These findings are in accordance with those described by von Ohle et al [42], who demonstrated the existence of an anaerobic zone (with oxygen saturation values of <0.5%) in deep layers of *in situ* 3-day PL-biofilms.

Interestingly, Diaz et al [55] showed that the initial PL-biofilm of each subject is unique in terms of diversity and composition, which suggests that the spatiotemporal interactions and ecological shifts that accompany biofilm maturation occur in a subject-dependent manner. Specifically, Dige et al [17] found a 12.5-fold difference between individuals in the total number of streptococci colonizing the surfaces for 2 days.

Two important processes in biofilm development in the oral cavity are coadhesion and coaggregation. Coadhesion consists of recognition between planktonic cells and surface-attached cells, and coaggregation is the cell–cell recognition between genetically distinct bacteria [63]. Despite extensive description of bacterial coaggregation compiled by using oral bacteria *in vitro* [64-67], it has been difficult to investigate the occurrence, and thus the significance, of coaggregation *in situ* [29]. In the majority of *in situ* studies, researchers have mainly focused on analysis of streptococcal interactions with other bacterial genera during the initial stages of the PL-biofilm formation [23,29,55,68] .

With respect to *in situ* PL-biofilms of less than 24 hours, Dige et al [23] performed a systematic description of initial biofilm formation on glass surfaces using FISH and CLSM. After 6-12 hours, these authors observed that the bacteria in the small monolayered clusters were composed of streptococci only, non-streptococci only, or mixtures of streptococci and non-streptococci [23]. Palmer et al [29] assessed the role of intrageneric coaggregation between streptococci, and the role of intergeneric coaggregation between streptococci and actinomyces *in situ* PL-biofilm formation at 4- and 8-hours on retrievable enamel chips. The data obtained in this study clearly indicated the omnipresence of intimate

interactions between different coccoid cell types, which could represent intrageneric interactions (e.g., *S. oralis* 34 with *S. gordonii* DL1) or interactions within a species (e.g., *S. sanguinis* SK163 with *S. sanguinis* SK1). Although actinomyces represented a small percentage of early PL-biofilm (these cells were easier to find at 8-hours), the juxtaposition of cells reactive with antibody against type 2 fimbriae of *Actinomyces naeslundii* T14V (anti-type-2) with cells reactive with antibody against receptor polysaccharides of streptococci (anti-RPS) in biofilm provided direct evidence that intergeneric coaggregation begins early in PL-biofilm development [29].

It has been speculated that cell-cell recognition between streptococci and veillonellae may be important in supragingival plaque formation, and consequently some authors have examined this bacterial relationship within the *in situ* human model system [69,70]. Specifically, on 6-hour PL-biofilms, Chalmers et al [70] detected several antiveillonella-reactive cells positioned within clusters of RPS-bearing streptococci, which were revealed as mixed-species communities of initial PL-biofilms. Palmer et al [69] demonstrated that between 4 and 8 hours into PL-biofilm development the dominant strains of *Veillonella* change in their phenotypic (coaggregation and antibody reactivity) and genotypic characteristics (16S rDNA gene sequences as well as strain level fingerprint patterns), confirming that the microdiversity within the veillonella population is subject-dependent.

As regards *in situ* PL-biofilms equal to or older than 24 hours, Dige et al [23] observed that in all individuals the PL-biofilms were dominated by streptococci intermingled with single nonstreptococci or groups of non-streptococci, including the previously described large cocci and rod-shaped/filamentous bacteria. When biofilms were examined in the z-plane (x–y sections parallel to the surface), the presence of protruding microcolonies forming "chimney like" structures**,** consisting of non-streptococci surrounded by streptococci, were detected. Analysis of the biofilm at various depths of the chimney-like microcolonies revealed differences in structural characteristics. When the central part of these complex microcolonies contained non-streptococci, these bacteria, in most cases, extended all the way from the supporting surface to the exterior of the biofilm. However, when the central part of the microcolonies was sparsely colonized, x–y sections in the z-plane showed an apparent unstained region with a larger diameter next to the supporting surface. Furthermore, most of the chimneys that were sparsely colonized in the centre were covered by a "lid" of bacteria. In consequence, Dige et al [23] speculated that "*these structures may constitute the result of a specific nutritional inter-relationship between different microbial species or specific coadhesion/coaggregation processes*".

Gu et al [71] explored the possibility of using 3 well-characterized monoclonal antibodies (MAbs) against *Streptococcus mutans*, *Actinomyces naeslundii* and *Lactobacillus casei* to identify by CLSM these important members of the oral microbiota *in situ* PL-biofilms. *S. mutans* turned out to be the predominant species in the 2-day PL-biofilms examined, whereas *L. casei* and *A. naeslundii* were often found to colocalize with *S. mutans*. Subsequently, Dige et al [30] described the pattern of dynamic colonization of *A. naeslundii* as compared to that of streptococci and other bacteria, during the initial 6 hours–2 days of PL-biofilm formation. The results showed a notable increase in the number of streptococci and *A. naeslundii* over time. However, there was a tendency towards a slower growth rate compared to that of streptococci and other members of the microbiota. This was a direct result of slower cell division of *A. naeslundii*, which formed branching filaments or 'spider colonies'. In this series, *A. naeslundii* represented a large spectrum of morphotypes, ranging from coccoid form predominating during the early stages to rod-shaped or filamentous forms becoming prominent after 1–2 days. *A. naeslundii* was located mainly in the inner part of the multilayered PL-biofilm, indicating that it is one of the species that attaches directly to the acquired pellicle, which may have interesting ecological implications in oral infections [30].

Recently, Al-Ahmad et al [72] investigated whether different enterococci from food (cheese containing enterococci) are able to reside *in situ* PL-biofilms. The study was completed by applying culture techniques, 16S rDNA gene sequencing, FISH and CLSM. *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus avium* and *Enterococcus durans* were detected in the initial PL-biofilm after 2 hours, as well as in the 5-day PL-biofilm. On the basis on these results, these authors concluded that food-borne enterococci, particularly *E. faecalis*, might not only be transient but could also survive in the PL-biofilm and become a source for endodontic infections [72]. In this context, the relatedness of streptococci and enterococci should be kept in mind; both groups belong to the same family of lactobacilli (*Streptoccaceae*).

In relation to *in situ* subgingival biofilms, to our knowledge, the paper published by Wecke et al [73] is the only research where the number and the spatial arrangement of spirochetes within the *in situ* subgingival biofilm were evaluated. At higher magnification, different morphotypes of, for example, cocci or rods-some organized as microcolonies, were clearly differentiated, with large spirochetes interspersed between other bacteria. In some parts of the biofilm, treponemes were rather separately localized, whereas in other areas high numbers of group I treponemes were spread over the biofilm [73]. Nevertheless, detailed information on PL-biofilm structure in subgingival habitat is still lacking.

3. Effects of antimicrobial agents on *in situ* undisturbed oral biofilm

It has been stated that antimicrobial agents can interfere in several ways with the mechanism of PL-biofilm formation: (a) they can prevent the constitution of a biofilm; (b) they can destroy the existing biofilm; (c) they can prevent growth processes in the biofilm, or (d) they can destroy individual microorganisms in the biofilm [74]. Numerous authors have

demonstrated that bacteria growing *in vitro* structured communities on a surface differ phenotypically from their counterparts growing in planktonic phase, behaving like cells in a multicellular organism [75-77]. In fact, bacteria in biofilms can be 10–1,000 times more resistant to antimicrobial treatment than those grown in the planktonic phase [76,78-84]. Pratten and Wilson [85] observed that oral microorganisms grown *in vitro* biofilms are up to 250 times more resistant to Chlorhexidine (CHX) than their planktonic counterparts. Even after a 60-minute exposure to 0.2% CHX, substantial numbers of bacteria in the biofilm remained vital [86]. This condition might be related to the slower growth rate of biofilm [87-89], to problems of antimicrobial agent penetration into the biofilm, or to inactivation of the agent in the biofilm [90].

An ideal prerequisite for a successful antimicrobial agent is that all bacteria within the biofilm should be exposed at an adequate concentration for an adequate time in order to achieve a clinically relevant reduction in pathogenesis [42]. The rate and extent of antimicrobial agent penetration depends on factors such as the biofilm structure and composition [10,14], the physicochemical properties of the solute [10,14] and, perhaps most importantly, the biofilm thickness [91]. On the other hand, although another very interesting aspect is based on solute penetration during brief exposure periods (<2 minutes), it is relatively unexplored [10]. Up to date, it has been evaluated exposure periods of antimicrobial agents *in vitro* biofilm models ranging from several minutes [92] to hours [93], which has little relevance to the oral cavity, where biofilm exposure to antimicrobial agents may be 30 seconds-1 minute [94-96].

Von Ohle et al [42] used a simple diffusion model to calculate the CHX concentration as a function of depth and time of application on *in situ* 3-day PL-biofilms, and they decided that a concentration of 0.1% CHX would be a clinically relevant concentration. The model predicts that it would take 17.5 minutes to achieve 0.1% CHX at the base of a 300 μm-thick biofilm (e.g., interproximal or at other retention sites). However, if the thickness of the biofilm were reduced to 100 μm, the time would be reduced to <2 minutes; if it were reduced to 30 μm, then it would only take 12 seconds. On the basis on these observations, these authors emphasised the clinical importance of limiting the PL-biofilm thickness by mechanical means (e.g. brushing or scaling) prior to exposure to rinses, and they also stressed that PL-biofilms are unlikely to be controlled by rinsing alone, unless the penetration time can be increased, or the activity is more aggressive than CHX [42].

In relation to the influence of physicochemical properties of the solute, Robinson et al [41] observed that *in situ* 7-day PL-biofilms, at least in the outer layers, seemed to be robust in terms of both mechanical and chemical assault when the biofilm is exposed to 0.9% and 9% sodium chloride (as well as to low pH), but it can change dramatically when detergents are present.

Despite the in-depth knowledge of the antimicrobial activity of different agents on *in vitro* biofilms [97-99], there are few studies published in the literature in which the effects of antimicrobial agents on *in situ* PL-biofilms at defined time points have been investigated applying CLSM together with bacterial vitality techniques. These studies could be analysed, distinguishing between those based on the antimicrobial effect derived from a single application, and those based on antimicrobial effect derived from multiple applications.

3.1. Effects of antimicrobial agents derived from a single application

In 2001, Zaura-Arite et al [18] were the first authors to visualize the antimicrobial effect of a single *exvivo* CHX treatment on PL-biofilm remaining on bovine dentin discs. These discs were removed at 6 hours, 1 and 2 days and then broken into halves along the middle groove. One of the halves was covered with 50 μl of 0.2% CHX digluconate and treated for 1 minute; the other half served as a control covered with 50 μl of saline. The mean bacterial vitality values of control and CHX-treated biofilms were at 6 hours (35% *vs* 18%), at 1 day (outer layer 49% *vs* 34%; middle layer 67% *vs* 42%; inner layer 50% *vs* 48%) and at 2 days (outer layer 52% *vs* 33%; middle layer 63% *vs* 42%; inner layer 54% *vs* 48%). Consequently, these authors concluded that only minor and superficial bactericidal effects of CHX were obtained on the PL-biofilm, with a thickness less than 65 μm. However, it is very interesting to note that the subjects brushed their teeth twice a day (without the presence of intraoral appliances) with NaF toothpastes [18], which could have conditioned the results obtained. Recently, von Ohle et al [42] examined the antimicrobial effect of CHX treatment on the physiology of PL-biofilms during exposure to sucrose, using a 0.2% CHX formulation, and biofilms were *exvivo* exposed to 10 ml of the CHX solution for either 1 or 10 minutes. In contrast to the results reported by Zaura-Arite [18], CHX treatment significantly reduced the bacterial vitality (67% in control biofilm compared to 2% and 0.7% in CHX-treated biofilms at 1 and 10 minutes, respectively). Hovewer, CHX treatment did not significantly reduce the overall oxygen metabolism, since even after a 10 minute exposure the biofilm was still physiologically active, demonstrating incomplete killing, and there was a relocation of activities deeper in the biofilm. CHX inactivated bacteria from the top down and layer by layer of PL-biofilms were killed with the highest efficacy in the outer regions. CHX had a more pronounced effect on acidification than respiration, thereby creating a more aerated and less acidogenic environment, and consequently the microecology could be changed to a less pathogenic one. [42].

Recently, Gosau et al [100] evaluated the efficacy of 6 antimicrobial agents (sodium hypochlorite, hydrogen peroxide 3%, 0.2% CHX, Plax, Listerine and citric acid 40%) on the surface decontamination of *in situ* PL-biofilms attached to titanium implants. The 12-hour PL-biofilms were then treated with these antimicrobial agents *exvivo* for 1 minute. Afterwards, the authors quantified the total bacterial load and the vitality of adhering bacteria by live or dead cell labelling in combination with fluorescence microscopy. All tested antiseptics seemed capable of reducing the total

amount of microorganisms accumulating on titanium surfaces. Furthermore, sodium hypochlorite, hydrogen peroxide, CHX, and Listerine showed a significant bactericidal effect against adhering bacteria (the ratio of dead bacteria to total ranged from 42%-98% *vs* 9%-36% after incubation in control solution) [100].

Tomás et al [101] stated that fluorescence assays could be particularly useful for simultaneous analysis of the effect of antimicrobials that alter the cytoplasmic membrane integrity on different oral ecosystems. However, there are no studies published in the literature in which the *in situ* activity of antibacterial agents on saliva and PL-biofilms were compared. Recently, we have evaluated the *in situ* antibacterial activity of a mouthrinse with 0.2% CHX on undisturbed *de novo* PL-biofilm and on salivary flora up to 7 hours after its application (data unpublished). A special acrylic appliance was designed, with 3 glass discs inserted on each buccal side, which allowed for PL-biofilm growth. Fifteen healthy volunteers wore the appliance during 48 hours and then performed a mouthrinse with 0.2% CHX; discs were removed at 30 seconds and at 1, 3, 5 and 7 hours after the mouthrinsing. Applying a washout period, saliva samples were collected from each volunteer at 30 seconds and at 1, 3, 5 and 7 hours after performing a mouthrinse with 0.2% CHX (M-0.2% CHX). At 30 seconds after the M-0.2% CHX, the levels of vital bacteria detected in saliva were significantly lower than those observed in PL-biofilm (1% *vs* 5%). At 1 and 3 hours after the M-0.2% CHX, the levels of vital bacteria detected in saliva and PL-biofilm were similar. The difference in the percentage of live bacteria detected in saliva was significantly higher than that observed in PL-biofilm at 5 (55% *vs* 25%) and 7 hours after the M-0.2% CHX (77% *vs* 32%). The 0.2% CHX solution showed a higher substantivity on the PL-biofilm than on salivary flora at 5 and 7 hours after mouthrinsing, which could be related to the slower growth rate of PL-biofilm [89,102] and the possible reservoir function associated with *de novo* PL-biofilm [103]. Figure 1 shows the bacterial vitality of *in situ* undisturbed 2 day PL-biofilms at baseline, as well as the antibacterial activity of M-0.2% CHX on the biofilm structure at 30 seconds and 7 hours after the mouthrinsing.

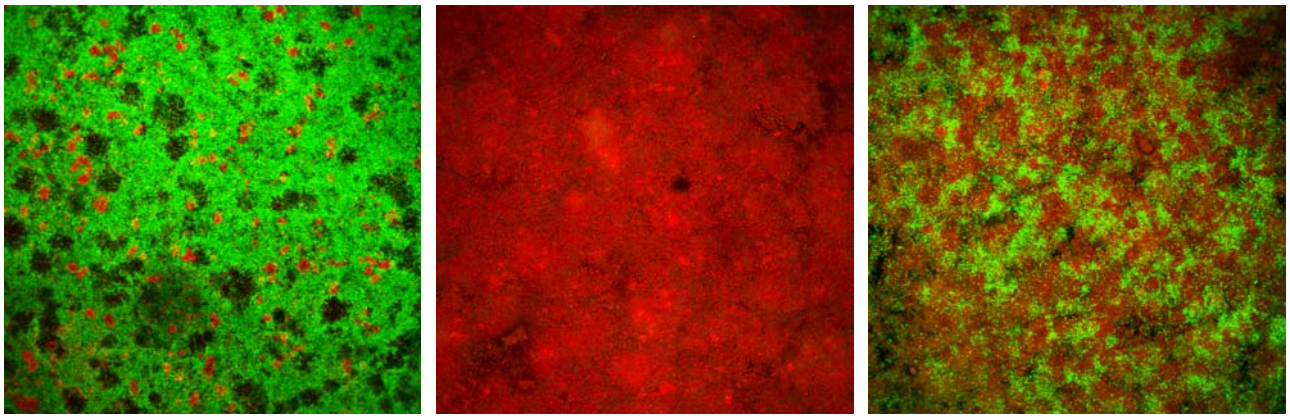

Fig 1. Structure and bacterial vitality of *in situ* undisturbed 2-day PL-biofilms at baseline (left image), as well as the antibacterial activity of a mouthrinse with 0.2% chlorhexidine on the biofilm structure at 30 seconds (image in the middle) and at 7 hours after the mouthrinsing (right image).

To date, there are no studies published in the literature, which examine the *in situ* antibacterial activity of a single mouthrinse with CHX on different oral ecosystems, analysing the influence of factors intrinsic to the mouthrinse such as the moment of application (morning *vs* night). Recently, we assessed the *in situ* substantivity of a single mouthrinse with 0.2% CHX in saliva and undisturbed *de novo* PL-biofilm, differentiating between 2 times of application: CHX mouthrinse in the morning and CHX mouthrinse at night (data unpublished). Ten health volunteers wore an individual splint with glass discs for 48 hours to boost the growth of the PL-biofilm. Saliva samples were collected and 2 discs were removed from each volunteers´ splint at 8, 10 and 12 hours after performing a mouthrinse of 0.2% CHX at 7 AM (M-0.2% CHX-diurnal) and 1 AM (M-0.2% CHX-nocturnal). In this study, the M-0.2% CHX-diurnal was associated with a significantly shorter duration of antimicrobial activity on the salivary flora than that observed with the M-0.2% CHX-nocturnal (the differences in the percentage of bacterial vitality varied from 37% at 8 hours after the mouthrinse, to 26% at 12 hours after the mouthrinse). In our opinion, the greater substantivity of M-0.2% CHX-nocturnal on the salivary flora could be attributed to the circadian rhythm of saliva secretion; it has been demonstrated that during sleep there is a significant reduction in salivary flow and a change in its composition [104,105]. As a result, there is a reduction in the protective effects of the saliva of the oral cavity and, simultaneously, a fall in nutrient supply, which has a direct effect on bacterial growth [105,106]. On the other hand, in our study with the M-0.2% CHX-diurnal, the percentage of vital bacteria present in the salivary flora was significantly higher than that observed in the bacterial plaque at 8 hours (59.66% *vs* 39.76%), 10 hours (71.44% *vs* 35.85%), and 12 hours (82.88% *vs* 36.95%) after the mouthrinse. With the M-0.2% CHX-nocturnal, the percentage of vital bacteria present in the salivary flora was significantly lower than that observed in the bacterial plaque at 8 hours (22.22% *vs* 36.46%), and significantly higher at 12 hours (57.22% *vs* 35.32%) after the mouthrinse. Again, these results support the greater physiological dynamics of the salivary flora and the possible reservoir function associated with the structure of *de novo* PL-biofilm [103].

3.2. Effects of antimicrobial agents derived from multiple applications

It has been stated that the concept of "penetration" plays a very important role in *in situ* PL-biofilms, where a single application of an antimicrobial agent is tested [20]. However, this concept is not too important when antimicrobial agents are continuously applied on the developing biofilm (several times over the course of several days) [20].

The paper by Auschill et al, published in 2005 [9] represented the first examination, under real clinical conditions, of biofilms grown directly in the oral cavity under the influence of an antibacterial mouthrinse (specifically, amine fluoride/stannous fluoride –ASF- and 0.2% CHX compared with water). The volunteers had to rinse twice daily for 1 minute with 10 ml of the allocated mouthrinse. After 2 days of application, both mouthrinses (ASF and CHX) showed antibacterial and plaque-reducing properties against the *in situ* PL-biofilm. Biofilm thickness was significantly reduced with the ASF and CHX mouthrinses when compared with the negative control (a thickness reduction of 80% and 89% respectively as compared with water). Equally, the mean bacterial vitality was significantly reduced from 66.1% ± 20.4% to 23.9% ± 12.4% and 23.3% ± 11.6% using ASF and CHX respectively (which corresponds to a 64% reduction by both solutions compared with the negative control). Although it has been assumed that dead bacteria and exopolymeric substances produced impede fast penetration of the antimicrobial through the biofilm, in contrast, other authors have stated that PL-biofilm may also perform a reservoir function for antimicrobial agents [103].

Arweiler et al [20] evaluated the influence of food preservatives on *in situ* PL- biofilm growth in a group of 24 volunteers who wore appliances with bovine enamel discs. During 3 test cycles, the subjects had to put one half of the appliance in one of the assigned active solutions twice a day (0.1% benzoate, BA; 0.1% sorbate, SA or 0.2% CHX) and the other into NaCl. After 5 days, the use of SA, BA and CHX resulted in a significant reduction in the biofilm thickness of 17%, 21% and 57% in comparison with the negative control. The mean bacterial vitality under the influence of SA, BA and CHX was significantly reduced by 26%, 29% and 62% when compared to the negative control. Biofilm thickness and bacterial vitality differences between SA and BA were not significant, while CHX showed significantly lower values. With respect to the distribution of biofilm vitality of all subjects divided into 3 layers, the 3 test products (SA, BA and CHX) showed a very similar vitality pattern with lower values in the bottom and higher values in the top layers (bottom= 19-40% ant top= 29-49%), although no statistically significant differences between the different layers (top, middle and bottom) were found. Consequently, the increased usage of preservatives and their availability in foods could be contributing to the decline in prevalence of dental caries [20].

We recently examined the influence of CHX and amoxicillin/clavulanic acid (AMOXICLAV) on *in situ* PL-biofilm growth (data unpublished). After a professional toothcleaning, 10 healthy volunteers had to wear a special acrylic appliance, in which 6 specimens were inserted to allow the build-up of oral biofilms. The volunteers didn't receive any antimicrobial agent (negative control), they had to take AMOXICLAV 875/125 mg in 3 doses each day, and they had to rinse twice daily with 0.2% CHX. After a 4 week wash-out period, a new test cycle was started. After 4 days of wearing, the specimens with the adhering biofilms were removed from the splints and stained with 2 fluorescent dyes. Under the CLSM, the biofilm thickness (μm), area occupied by biofilm (%) and bacterial vitality (%) were evaluated. The mean values of the biofilm thickness, area occupied by biofilm and bacterial vitality of the 4-day PL-biofilm under basal conditions were 25 μm, 79% and 50% respectively. The use of 0.2% CHX resulted in a biofilm thickness of 6 μm, an area occupied by biofilm of 21% and a bacterial vitality of 14%; these differences were statistically significant compared with baseline. The use of AMOXICLAV resulted in similar results when compared with baseline (biofilm thickness= 21 μm, area occupied by biofilm= 68% and bacterial vitality= 56%). Differences between 0.2% CHX and AMOXICLAV were statistically significant. In contrast to CHX, the AMOXICLAV regimen applied under clinical conditions didn't show antibacterial and plaque-reducing properties against the *in situ* PL-biofilm. Figure 2 shows the structure and bacterial vitality of *in situ* undisturbed 4-day PL-biofilms at baseline, as well as the antibacterial and plaque-reducing properties after using 0.2% CHX and taking AMOXICLAV.

Recently, Beyth et al [107] speculated that quaternary ammonium polyethylenimine (QPEI) nanoparticles incorporated into a resin composite have a potent antibacterial effect *in situ,* and that this stress condition triggers a suicide module in the PL-biofilm. To analyse this, 10 volunteers wore a removable acrylic appliance, in which 2 control resin composite specimens and 2 resin composite specimens, incorporating 1% w*t*/wt QPEI nanoparticles, were inserted to allow the build up of PL-biofilms. After 4 hours, the mean percentage of bacterial vitality in specimens incorporating QPEI was significantly reduced by >50%, whereas, interestingly, the biofilm thickness was significantly increased. The *in vitro* tests showed a 70% decrease in viable bacteria. These results strongly suggest that QPEI nanoparticles incorporated at a low concentration in resin composite exert a significant antibiofilm activity and exhibit a potent broad spectrum antibacterial activity against salivary bacteria [107].

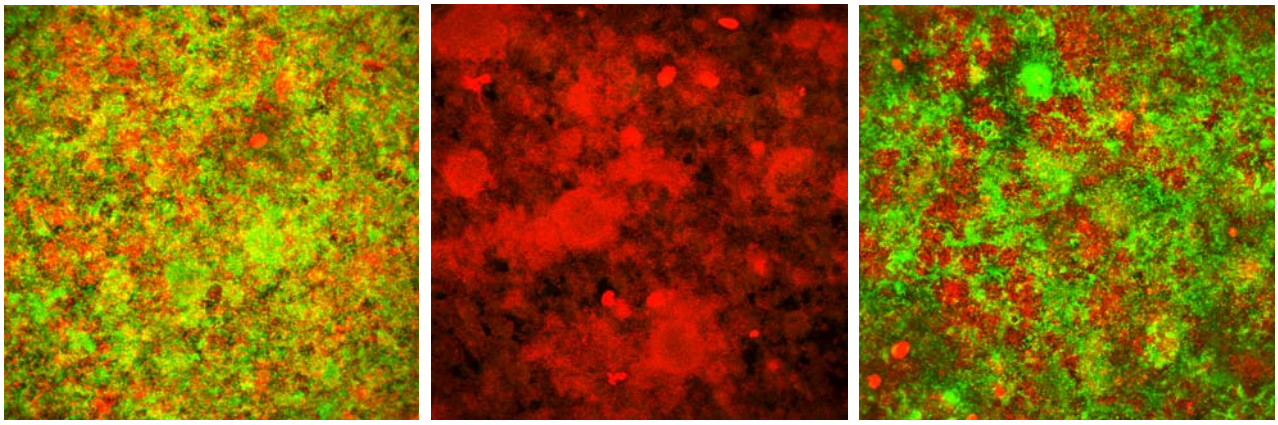

Fig 2. Structure and bacterial vitality of *in situ* undisturbed 4-day PL-biofilms at baseline (left image), as well as the antibacterial and plaque-reducing properties after using 0.2% CHX (image in the middle) and taking AMOXICLAV (right image).

In conclusion, the scientific community considers that the analysis of undisturbed PL-biofilm using CLSM is the most suitable approach to the study of the *in situ* of PL-biofilm. Although some evidence on the structural characteristics of *in situ* early PL-biofilm has been described, there are few studies on the activity of antimicrobial agents on this microbial structure. Consequently, a major application of advanced microbiological and/or microscopy techniques in combination with CLSM needs to be exploited in future research in order to increase knowledge of the global characterisation of *in situ* PL-biofilms (e.g. adding older biofilm and subgingival biofilm samples). Similarly, further in-depth work needs to be done on the antibacterial effects of agents frequently used in Dentistry on the *in situ* biofilm structure (e.g. comparing this effect with that obtained in other oral ecosystems).

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