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Studies on innovative tools against oral pathogens: *in vitro* and *ex vivo* models

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TABLE OF CONTENTS

ABSTRACT

During recent years, novel compounds/tools are being proposed to maintain oral health and/or treat dental/periodontal problems. As well known, dental caries is among the most diffused infections and their improper management leads to relevant disease(s) and eventually tooth extraction. Extensive literature documents the pathogenic role of certain microorganisms and their ability to persist in the oral cavity, as a complex microbial community, including bacteria, viruses and fungi, tightly enclosed in a polymeric matrix of polysaccharide origin. Such sessile community, and particularly dental plaque, the first deeply studied human-associated biofilm, is notoriously refractory not only to common cleaning procedures by mouthwashes and tooth-pastes/brushes, but also to antimicrobial drugs and host immune defenses. This scenario becomes further complicated considering that the widely diffused orthodontic treatments, with fixed or removal brackets, extend the clinical challenge, being such devices an additional good habitat for microbial adhesion, growth and biofilm formation. To a similar extent, patients with dental implants may locally develop biofilm-related diseases, allowing clinical progression toward pathogen-related peri-mucositis or peri-implantitis. From here, the need arises for innovative tools/compounds to facilitate microbial removal and maintenance of oral cavity homeostasis. Besides the most investigated oral pathogens, including *Streptococcus mutans*-group and the "red complex" Gram-negative anaerobe bacilli [including *Porphyromonas gingivalis* (*P. gingivalis*), *Treponema denticola* (*T. denticola*) and *Tannerella forsythia* (*T. forsythia*)], also other microorganisms such as *Candida albicans (C. albicans*), *Staphylococcus aureus (S. aureus*) and *Pseudomonas aeruginosa* (*P. aeruginosa*)may occur as causative agent of oral diseases. Often harbored as commensal of healthy mucosae, *C. albicans* is the main fungal pathogen involved in oral mucositis, while *S. aureus* and *P. aeruginosa* are two subtle bacterial pathogens, responsible of wide-spectrum diseases; they are extensively used for *in vitro* studies, because of their numerous virulence factors and wide-spectrum antimicrobial resistance.

The aim of the present thesis was to evaluate *in vitro* and *ex vivo*, the antimicrobial and antibiofilm efficacy of innovative approaches against oral pathogens.

Firstly, we evaluated the efficacy of a commercially available copper-calcium-hydroxide-based endodontic paste, named Cupral, against both bacterial and fungal pathogens. We found that such compound significantly reduced growth of *S. aureus*,

P. aeruginosa and *C. albicans*; also, biofilm formation and persistence were affected and, in parallel, the production of pyoverdines, considered a relevant bacterial virulence factor, was deeply affected. Based on these *in vitro* data, we performed *ex vivo* studies to establish the ability of Cupral to remove microbial plaque, naturally produced onto orthodontic devices. Thus, clear aligners, regularly used during standard orthodontic therapy, were exposed or not to Cupral and assessed for total and residual microbial population, by conventional/confocal microscopy and colony-forming unit (CFU) assay. We found that, following Cupral treatment, microbial load dropped to undetectable levels, irrespectively of the experimental conditions tested. Overall, we provided the first evidence on effectiveness of an endodontic paste against both *in vitro* and *ex vivo* produced bacterial and fungal biofilms, strengthening the use of such compound in clinical dentistry practice.

Secondly, we investigated the efficacy of different decontamination systems with the aim to identify the optimal approach capable of removing microbial biofilm without modifying implant surface properties or affecting reparative cell behavior. Two different systems, Ni-Ti Brushes (Brush) and Air-Polishing with 40 μm bicarbonate powder (Bic-40), were compared against experimentally contaminated titanium surfaces [machined (MCH) and Ca^{++} nanostructured (NCA)]; also, their potential damage on the human STRO-1^{+}/c-Kit^{+} dental pulp stem cells (hDPSCs) was evaluated. Our findings highlighted the high Bic-40 treatment performance, in that it maintained the implant surface characteristics and allowed hDPSC proliferation preserving their stemness properties; also, a vigorous removal of bacterial biofilm from both titanium surfaces was observed as well as a consistent limitation in biofilm re-growth.

Thirdly, propolis, a natural product endowed with numerous protective and curative properties, was investigated for its ability to counteract *P. aeruginosa;* in particular, the effects of 3 propolis extracts were assessed against several virulence-related factors, such as growth rate, biofilm formation, extracellular DNA (eDNA) release and phenazine production. In parallel, by high-performance liquid chromatography (HPLC) coupled with mass spectrometry (MS), the profiles of propolis extracts were characterized, focusing on some specific polyphenols, before and after exposure to *P. aeruginosa* cells. Among the tested extracts, the propolis ethanol extract was the most effective in inhibiting both microbial growth and biofilm formation; also, this

phenomenon was accompanied to a decrease in eDNA release and phenazine production. Finally, we were able to provide the first evidence that, upon propolis exposure to bacteria, certain components, namely caffeic acid phenethyl ester (CAPE) and quercetin, were consistently consumed, thus suggesting their direct role as antimicrobial compounds.

Fourthly, we assessed the efficacy of new commercially products recently proposed, as novel tools for oral care and hygiene. Thus, by an *in vitro* pilot study performed upon approval by the local Ethical Committee (AVEN), we tested the effects of two products, a toothpaste and a gum containing probiotics (Biorepair Peribioma), on the behavior of oral microorganisms, obtained from saliva from healthy subjects. We found that microbial growth as well as biofilm formation and persistence onto orthodontic elastics were significantly affected; a change in the relative abundance of different microbial species was also detected by time, suggesting a precious displacement of potentially relevant pathogens. These findings provide initial evidence on the impact that certain oral health-care products may have on oral microbiota, underlying the need of careful and conscious choice by clinicians also with respect to over-the-counter products.

Overall, our data provided novel *in vitro* and *ex vivo* evidence on the antimicrobial efficacy of several dental-care compounds. Using a basic science-approach, we have contributed to increase our knowledge on how to counteract biofilm-producing pathogens; in turn, this will facilitate prevention and/or treatment of dental and oral biofilm-associated infections, making a huge difference in terms of health promotion. The potential implications of these original findings will be detailed in the respective chapters.

RIASSUNTO

Negli ultimi anni, sono stati proposti nuovi composti/strumenti per mantenere la salute orale e/o per trattare diversi problemi dentali/parodontali. Come è noto, la carie dentale si pone tra le infezioni più diffuse ed una sua gestione impropria comporta lo sviluppo di malattie rilevanti ed eventualmente all'estrazione dell'elemento dentale. Una vasta letteratura documenta il ruolo patogenetico di diversi microorganismi che sono in grado di persistere nel cavo orale, in quanto capaci di organizzandosi come comunità microbica eterogenea (comprendente batteri, virus e funghi), adesa alle diverse superfici, strettamente racchiusa in una matrice polimerica di origine polisaccaridica. Tale comunità sessile, che se adesa ai denti è detta placca dentale, è notoriamente refrattaria non solo alle comuni procedure di pulizia con collutori e dentifrici/spazzolini, ma anche ai farmaci antimicrobici e alle difese immunitarie dell'ospite. Questo scenario si complica ulteriormente considerando che l'ampio uso di attacchi fissi o rimovibili nei trattamenti ortodontici espande la problematica e la conseguente sfida clinica, essendo tali dispositivi un ulteriore habitat utile per l'adesione microbica, la crescita e la formazione di biofilm. In misura simile, i pazienti con impianti dentali possono sviluppare localmente malattie legate alla produzione di biofilm impianto-associato, consentendo la progressione clinica verso quadri di perimucosite o perimplantite infettiva. Da qui, sorge la necessità di strumenti/composti innovativi per facilitare la rimozione di microrganismi potenzialmente patogeni e il mantenimento dell'omeostasi del cavo orale. Oltre ai patogeni orali più noti, tra cui il gruppo *Streptococcus mutans* e il "complesso rosso" dei bacilli anaerobi Gram-negativi [includendo *Porphyromonas gingivalis* (*P. gingivalis*), *Treponema denticola* (*T. denticola*), e *Tannerella forsythia* (*T. forsythia*)], anche altri microrganismi come *Candida albicans* (C. albicans), *Staphylococcus aureus* (*S. aureus*) e *Pseudomonas aeruginosa* (*P. aeruginosa*) possono essere agenti eziologici di malattie orali. Spesso ospitato come commensale delle mucose sane, *C. albicans* è il principale patogeno fungino coinvolto nella mucosite orale; *S. aureus* e *P. aeruginosa* sono patogeni batterici molto subdoli, responsabili di malattie ad ampio spettro; considerati i loro numerosi fattori di virulenza e l'ampia farmaco-resistenza, *S. aureus* e *P. aeruginosa* sono ampiamente utilizzati per studi *in vitro* come preziosi prototipi di patogeni Gram-positivi e Gram-negativi.

Lo scopo della presente tesi era di valutare *in vitro* ed *ex vivo* l'efficacia antimicrobica e antibiofilm di approcci innovativi contro i patogeni orali. Per questo, il lavoro è stato articolato in linee di indagine distinte.

Inizialmente, abbiamo valutato l'efficacia di una pasta endodontica a base di idrossido di rame-calcio disponibile in commercio, denominata Cupral, contro i patogeni batterici e fungini. Abbiamo trovato che tale composto riduceva significativamente sia la crescita di *S. aureus*, *P. aeruginosa* e *C. albicans* sia la loro capacità di formare biofilm; allo stesso modo, la produzione di pioverdine, un tratto di virulenza importante *di P. aeruginosa*, è stata profondamente ridotta. Sulla base di questi dati, abbiamo eseguito studi *ex vivo* per stabilire la capacità di Cupral di rimuovere la placca microbica, prodotta naturalmente sui dispositivi ortodontici. Pertanto, allineatori trasparenti, utilizzati da un soggetto sano durante la terapia ortodontica standard, sono stati recuperati a fine trattamento, esposti o meno a Cupral e valutati per la popolazione microbica totale e residua, mediante microscopia convenzionale/confocale e analisi dell'unità formante di colonie (CFU). Abbiamo dimostrato che, dopo il trattamento con Cupral, la carica microbica è scesa a livelli non rilevabili, indipendentemente dalle condizioni sperimentali saggiate. Nel complesso, questa prima linea di indagine ha fornito le prime prove sull'efficacia di una pasta, attualmente in uso in endodonzia, contro i biofilm batterici e fungini, prodotti sia *in vitro* che *ex vivo*, rafforzando l'uso di tale composto nella pratica clinica.

In secondo luogo, abbiamo studiato l'efficacia di diversi sistemi di decontaminazione con l'obiettivo di identificare l'approccio ottimale in grado di rimuovere il biofilm microbico senza modificare le proprietà della superficie dell'impianto o alterare il potenziale replicativo di cellule staminali. Due diversi sistemi, Spazzole in Ni-Ti (Brush) e Air-Polishing con 40 µm di polvere di bicarbonato (Bic-40), sono stati saggiati su superfici di titanio contaminate sperimentalmente [lavorate (MCH) e Ca^{++} nanostrutturate (NCA)]; inoltre, è stato valutato il loro potenziale danno su cellule staminali della polpa dentale umana, utilizzando la linea STRO-1^{+}/c-Kit^{+} (hDPSC). I nostri risultati hanno evidenziato una miglior prestazione da parte del trattamento Bic-40, in quanto non ha modificato le caratteristiche della superficie dell'impianto e non ha alterato la capacità proliferativa delle cellule hDPSC; inoltre, ha rimosso

efficacemente il biofilm batterico, riducendone anche la possibilità di ricrescita, indipendentemente dalla superficie considerata.

In terzo luogo, abbiamo valutato la composizione chimica di un prodotto naturale, come la propoli, e la sua capacità di contrastare *P. aeruginosa*, influenzandone l'espressione di diversi fattori correlati alla virulenza, come la capacità di crescita, la formazione di biofilm, il rilascio di DNA extracellulare (eDNA) e la produzione di fenazine. In particolare, mediante cromatografia liquida ad alta prestazione accoppiata con spettrometria di massa (HPLC-MS), tre estratti di propoli ottenuti con solventi diversi sono stati comparati tra loro e saggiati per livelli relativi di specifici polifenoli nell'estratto di propoli, prima e dopo l'esposizione a *P. aeruginosa*. I risultati ottenuti dimostrano che l'estratto di propoli in etanolo è particolarmente efficace nell'inibire tutti i parametri saggiati, ovvero la crescita microbica, la formazione di biofilm, il rilascio di eDNA e la produzione di fenazine. Infine, siamo stati in grado di fornire la prima evidenza che propoli esposto a *P. aeruginosa* mostra un calo sensibile nei livelli di estere fenetilico dell'acido caffeico (CAPE) e quercetina, suggerendo così un ruolo antimicrobico diretto di questi composti.

Infine, abbiamo valutato l'efficacia di alcuni prodotti, recentemente proposti dal mercato, come strumenti innovativi per la cura e l'igiene orale. In particolare, con uno studio pilota approvato dal Comitato Etico locale (AVEN), abbiamo dimostrato che due specifici prodotti, un dentifricio e una gomma contenente probiotici (Biorepair Peribioma), alterano il comportamento dei microorganismi orali, ottenuti dalla saliva da soggetti sani. In particolare, la crescita microbica così come la formazione e la persistenza del biofilm su elastici ortodontici, sono significativamente ridotte dai due prodotti; inoltre, è stato riscontrato un cambiamento tempo-dipendente nell'abbondanza relativa di alcune specie microbiche, suggerendo un interessante contenimento di germi potenzialmente patogeni. Pertanto, questo studio fornisce una prima evidenza sull'impatto che prodotti per la cura della salute orale possono avere sul microbiota della bocca; da qui, l'importanza di effettuare attentamente scelte consapevoli anche nell'uso di prodotti da banco.

Nel complesso, questo lavoro di tesi ha fornito evidenze *in vitro* ed *ex vivo* sull'efficacia antimicrobica di composti nuovi e tradizionali per la cura e l'igiene del cavo orale. Da questi dati potranno derivare in prospettiva scelte più razionali e consapevoli, utili a disegnare strategie efficaci per la prevenzione e/o il trattamento

delle infezioni dentali e orali associate a biofilm. Le potenziali implicazioni di questi risultati originali e le possibili ricadute in termini di promozione della salute saranno descritte nei rispettivi capitoli.

INTRODUCTION

1. The oral cavity

In the human body, the oral cavity, also known as mouth or buccal cavity, has a key position as the first portion of the digestive system. This organ consists of different anatomical units that work together effectively and harmoniously to perform different functions for the benefit of our body. These units include the cheeks, lips, tongue, palate, and teeth. The oral cavity has also a complex and unique structure with several nerves and blood vessels within it. It is important to note that this complex network is necessary for its diverse role in human life (Fig. 1) [1].

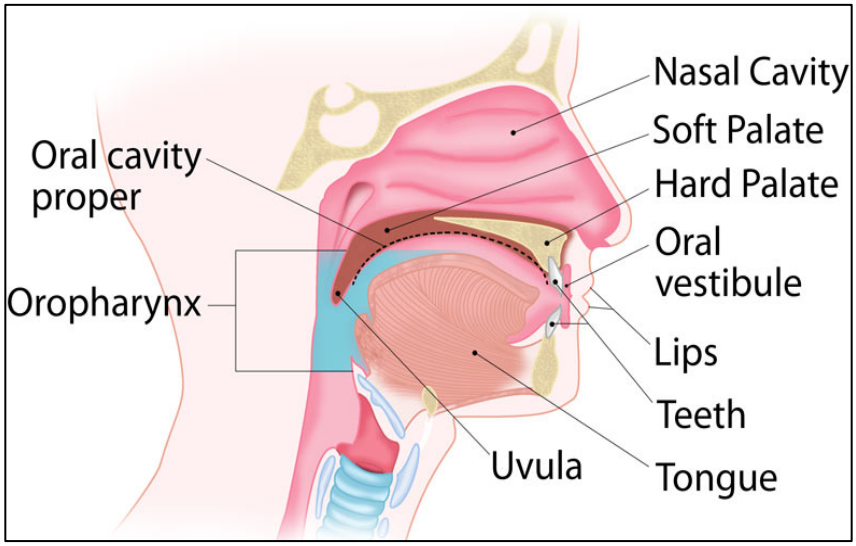


Figure 1. Anatomy of the oral cavity [1]

1.1 Oral niches and resident microbiota

Because of its anatomical diversity, the oral cavity provides diverse niches (lips, cheeks, palate, teeth, gingival sulcus, etc.) suitable for different kinds of microbial biofilms/populations [2]. In 1683, Antony van Leeuwenhoek, through his homemade microscope, examined the biofilm grown into his mouth. Dr. Leeuwenhoek, on September of the same year, reported his findings to the Royal Society, becoming the first scientific publication on the matter [3]. It took a very long time before having the description of an oral microbiota with high numbers of bacteria, archaea and fungi. Our body is a habitat of trillions of microbes, especially the oral cavity is one of the heavily colonized sites, with around 700 to 1000 microbial species [4]. Nowadays, more than 900 different microbial species have been isolated

from the oral cavity [5]; by the Human Oral Microbe Database (HOMD), it is possible to obtain taxonomic information about them [6]. It is noteworthy that the most predominant microbes of the oral cavity are *Firmicutes* and *Streptococcus* species [7]. The buccal mucosa is distinguished by an aerobic environment with a high epithelial turn-over, constituted mostly by *Streptococcus* spp. On the other hand, a complex microbiota is found on the dorsum of the tongue, because of its lower degree of epithelial desquamation and crypts with anaerobic conditions [8]. Indeed, the identified species can be classified on the bases of their oxygen requirements, distinguishing among obligate aerobes (*Rothia mucilaginosa*, *Rothia aeria*) and anaerobes (*Veillonella* and *Fusobacterium*), facultative anaerobes *(Streptococcus* spp and *Actinomyce*s), microaerophiles (species grown better in low concentrations of O_2, from 2% to 10%), and capnophiles (species such as *Neisseria* that grow better at high CO_2 concentrations, from 5% to 10%) [9].

Biofilm formation can occur on a variety of oral cavity surfaces, such as dental surfaces, saliva-coated enamel, epithelial cells, dental devices, etc., commonly generating environments favorable to the establishment of mixed-species biofilms [10]. *In primis*, biofilm formation occurs with the adhesion of primary colonizers to the surface, thus creating a microbial monolayer of so called “pioneers”. Subsequently, attachment of other microorganisms as secondary colonizers directs towards a multilayered micro-colonies formation, which determines the establishment of a multispecies community, tightly embedded in a polymeric extracellular matrix [10]. Impressive is how bacteria can communicate with each other within the biofilm, using quorum sensing (QS), a microbial interplay mediated by molecules secreted by bacteria themselves [11]. QS modulates growth and biofilm formation as well; additionally, different QS systems have been identified in bacteria and fungi, where the best-known examples are the acyl homoserine lactones, the auto inducers-2 (AI-2) and other systems [11]. In the oral microbiota, the primary colonizers for both mucosal and dental surfaces are usually streptococci that produce approximately 80% of early biofilms. Such bacterial species also produce oral adhesins, such as antigen I/II (AgI/II), a family of adhesins, amylase-binding proteins (ABPs), and type 1 fimbriae-associated proteins [9,10]. Many studies describe the dual role of certain bacteria or fungi in the oral cavity, underlining their relevance as commensal and also their critical contribution towards disease once biofilm in produced. As an example,

Candida albicans (*C. albicans*) is a fungus, commonly present as commensal in the oral cavity of healthy subjects [12]. Yet, *C. albicans* can form biofilms on oral epithelia or solid surfaces, such as implanted medical devices (dental prostheses or orthodontic appliances); thus, it may exert pathogenic relevance also because of its high levels of tolerance to detergents and antifungal drug resistance [13].

A crucial element tightly controlling the oral cavity homeostasis is saliva. Its numerous compounds deeply influence microbial growth and biofilm formation [14]. When secreted, saliva is sterile [15], but, rapidly, it is enriched in bacteria coming from the different oral surfaces [14,15]. Various other factors can affect the oral biofilm including temperature, pH, atmospheric conditions, redox potential, salinity, water activity and flow of saliva. Moreover, saliva is used by oral biofilms as a delivery system, bringing nutrients, peptides, and carbohydrates to epithelial cells; it performs also other functions, such as food lubrication, temperature regulation, host defense, etc., [16]. Furthermore, saliva flow is not the same on the different locations of the oral cavity [15].

The presence of a resident microbiota onto human mucosae is not essential for life, but it is crucial to promote and maintain a health condition. The resident microbiota composition is the result of a long-term evolution conditioning the symbiotic coexistence between the human host and microbes; today, the gathering of these two players is referred to a unit called holobiont [17]. Data on the beneficial role of the resident microbiota originate from studies on germ-free or conventional laboratory animals or humans, where normal microbiota has been disrupted by long-term administration of antibiotics or because of systemic diseases. Consequently, some microbial species, usually behaving as commensal, then become invaders, avoid host immune defense and produce biofilms where potential pathogens may find an insidious shelter inside the host [17].

Oral health is the result of a complex and continuous balance between the resident oral microbiota and the host immune system. Changes in any of these components, can create an imbalance or a dysbiosis, which in turn can lead to either local or disseminated diseases. It is important to consider that the oral microbiota has great plasticity over time, and the colonized surfaces are various and related to well-known oral microorganisms [18]. An unbalanced oral microbiota may compromise also

general health in several ways. This is referred as the theory of focal infection, which states that oral microbes are capable to induce disease in distant parts of the human body. Oral microorganisms can enter the blood stream through a dental infection or a damaged oral mucosa, thus increasing the risk for a variety of systemic diseases [19]. It is not surprising that, in patients with untreated periodontitis, bacterial DNA has been detected in multiple areas of the cardiovascular system, such as heart valves and atherosclerotic plaques [20].

1.2 Dental plaque

The mouth is an open highly dynamic ecosystem, where microorganisms are always present and tend to form mixed biofilms. Dental plaque is one of the first studied human-associated biofilms; generally, it originates as supra-gingival plaque and then develops gradually invading the nearby tissues, producing the well-described damage (Fig. 2) [21].

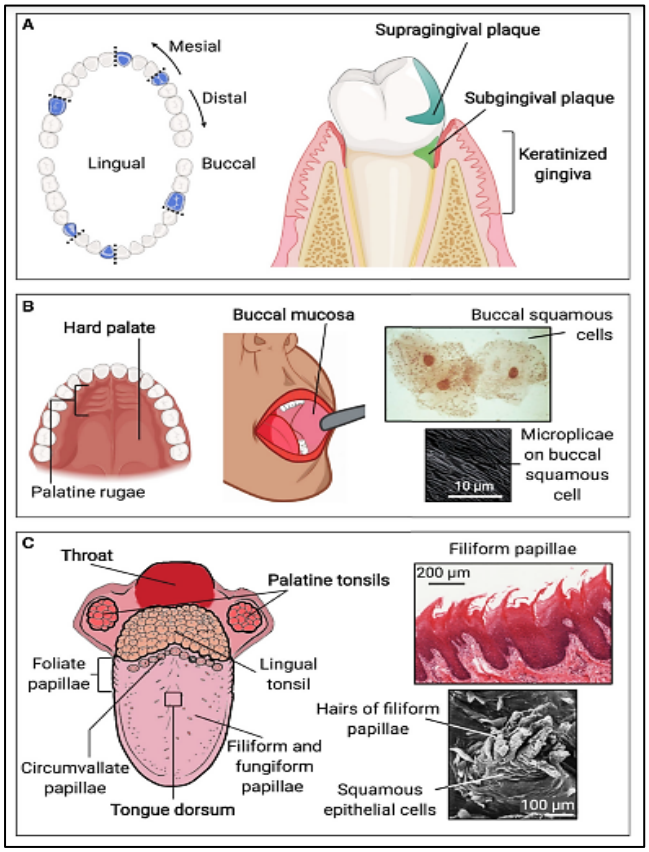


Figure 2. The mouth contains a range of topographies and substrates that present varied habitats for diverse microbes [21]

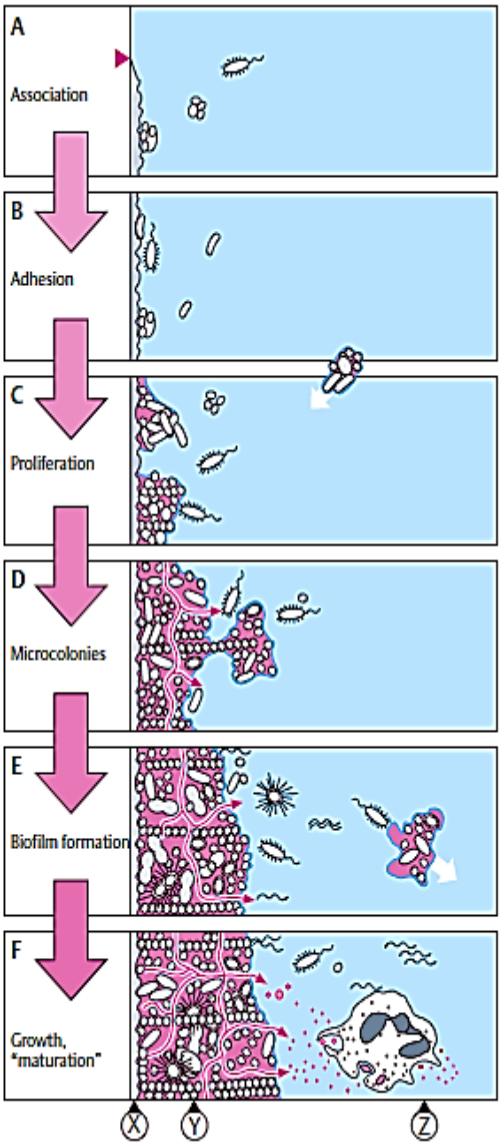
Dental plaque is composed by various bacterial populations localized in distinct anatomical surfaces such as dental fissures, proximal and smooth surfaces, gingival crevice or hard substances (tooth and root surfaces, restorative materials, implants, prostheses, orthodontic devices, etc.). Macroscopically, dental plaque appears as soft accumulation, adherent to the tooth surface or other hard surfaces, including removable and/or fixed restorations. Then, it may evolve as a hard deposition, known as dental calculus, covered by a non-mineralized plaque layer.

Anatomically, dental plaque is classified as: a) **supragingival plaque,** which is located above the gingival margin and when in direct contact with the gingival margin it is quotes as a marginal plaque; and b) as **subgingival plaque** located below the gingival margin, between the tooth and the gingival sulcular tissue. Interestingly, the different location of dental plaque predisposes to the occurrence of different dental or periodontal diseases. For example, marginal plaque gives gingivitis, supragingival plaque associated with subgingival plaque produce calculus and root caries, while subgingival plaque gives several forms of periodontitis. Actually, calculus is not pathogenic *per se*, but its rough surface represents a retention area for pathogenic bacteria, as well as carious lesions represent a huge bacterial reservoir [21].

Supragingivally, Gram-positive cocci accumulate as first colonizers (*Streptococcus* spp, *Actinomyces* spp); then, Gram-negative cocci, Gram-positive and gram-negative rods, as well as the first filamentous forms gradually colonize the tooth surfaces over the time. Moving apically from the supragingival area, a subgingival microbial biofilm, described as “adherent” plaque, is responsible for the time-dependent formation of a gingival pocket. Besides Gram-positive bacteria, such as streptococci, *Actinomyce*s, etc., the number of anaerobic Gram-negative bacteria proceeds with increasing probing depth. Periodontal bacteria, often increasing significantly in the acute phases of the disease, include *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythia*, spirochetes etc., [21,22].

When a healthy dental-gingival relationship exists, there is a balance between support mechanisms within the biofilm and corrosive forces that tend to reduce its formation; in this context, it is worth mentioning also the self-cleaning effects by the cheeks and tongue, diet and mechanical oral hygiene. Despite such a good cleaning of the mouth, the production of a biofilm occurs within a few hours or days and this is

inevitable as a phenomenon [23]. Below the dental plaque formation in phases is shown (Fig. 3).



A: *Association* → through physical forces, bacteria vaguely associate with the pellicle.

B: *Adhesion* → because they possess special surface molecules (adhesins) that bind to pellicle receptors, some bacteria become the "primary colonizers," particularly streptococci and actinomyces. Subsequently, other microorganisms will adhere to such primary colonizers.

C: Bacterial *proliferation* ensues.

D: *Micro-colonies* are formed → many streptococci secrete protective extracellular polysaccharides (e.g., dextrans, levans).

E: *Biofilm ("attached plaque") formation* → micro-colonies form complex groups with metabolic advantages for the constituents.

F: *Plaque growth-maturation* → the biofilm is characterized by a primitive "circulatory system". The plaque begins to "behave" as a *complex organism*! Anaerobic species increase. Metabolic products and cell wall constituents (e.g., lipopolysaccharides, vesicles) serve to activate the host immune response. Bacteria within the biofilm are protected from phagocytic cells (PMN) and against exogenous bactericidal agents.

X → Pellicle
Y → Biofilm - "Attached Plaque"
Z → Planktonic Phase

Figure 3. Schematic representation of the various phases characterizing dental plaque formation [23]

1.2.1 Microbial biofilm composition (cariogenic bacteria)

Once organized as biofilm, microorganisms are embedded in a matrix containing extracellular polymeric substances (EPS). The production of such EPS instantly mediates microbial adhesion to a surface and cell-to-cell adhesion; also, the mechanical stability of biofilm greatly improves [24]. The physical-chemical properties of EPS control diffusion of micro-molecules and cause the formation of

chemical/nutrient gradients, thus creating microenvironments within the biofilm, such as pH, redox and nutrient availability, that can affect microbial behavior. In this way, the matrix lets the cells to organize themselves into cohesive multicellular ecosystems, in which these interactions take place within a heterogeneous and fluctuant physical-chemical environment; likely, differently localized niches are created and are capable of hosting different potential pathogens [24]. Overall, the polymicrobial cell-to-cell interactions as well as the microenvironments locally produced within the biofilm play an essential role in modulating health conditions and disease, too [25]. Matrix production plays a key role also in microbial virulence, tolerance to external stresses, ensuring persistence and resilience; its diffusion modulating properties help to form intra-biofilm micro-environments protected from antimicrobials [26]. Moreover, it is known that changes occurring in the host (e.g., increased sugar intake or changes in his immunological status) may push pathogens to reshape the local micro-environment, remodeling microbial community. For instance, the matrix may become the key connecting structure for the production of cariogenic oral biofilms that "stick" to the teeth, despite exposure to shear forces and "washing out" by the salivary swab [27].

Over the years, two species of "mutans streptococci (MS)", i.e., *Streptococcus mutans* (*S. mutans*) and *Streptococcus sobrinus* (*S. sobrinus*) have been identified as the main agents of enamel caries [26]. Other bacteria such as *Lactobacillu*s and *Actinomyces* spp. have also been associated with caries. In particular, *Actinomyces odontolyticus* colonizes infants before erupting teeth, while root carious lesions are mainly related to *A. naeslundii*, *A. israelii* and *A. gerencseriae*[28]. Caries also includes other significant species such as *Streptococcus mitis* and *Bifidobacterium* spp., likely because of "low pH" aciduric isolates present in the dental white spot lesions [29].

A study conducted in 2011 has identified a new bacterial species, *Scardovia wiggsiae* (*S. wiggsiae*), upon culture of dental plaque under anaerobic conditions, demonstrating a significant association between *S. wiggsiae* and severe early childhood caries (ECC). Importantly, this species has also been identified in children with carious lesions in the absence of *S. mutans* presence, thus providing evidence on a direct role of *S. wiggsiae* in the cariogenic process [30]. Further literature has

highlighted as well an important link between the *S. wiggsiae* presence and early stages of caries in pediatric subjects undergoing orthodontic therapy [31].

1.2.2 Dental caries

Dental caries is known to be a classic biofilm-induced disease that causes the destruction of mineralized dental tissue [32]. Unlike general microbiota, the cariogenic bacteria are supported by the host diet, in that a sugar-rich diet promotes the EPS matrix production and accumulation of acidogenic and acidophil species (Fig. 4) [33].

Studies on fossil records show that, during Neolithic and Mesolithic periods, *S. mutans* was not detected in dental plaque samples; conversely, *S. mutans* appears as a cariogenic bacterium when humans changed their habits and enriched their diet with sugars. In modern times, with the prevalence of diet with refined sugars, the dental caries incidence further increases [34].

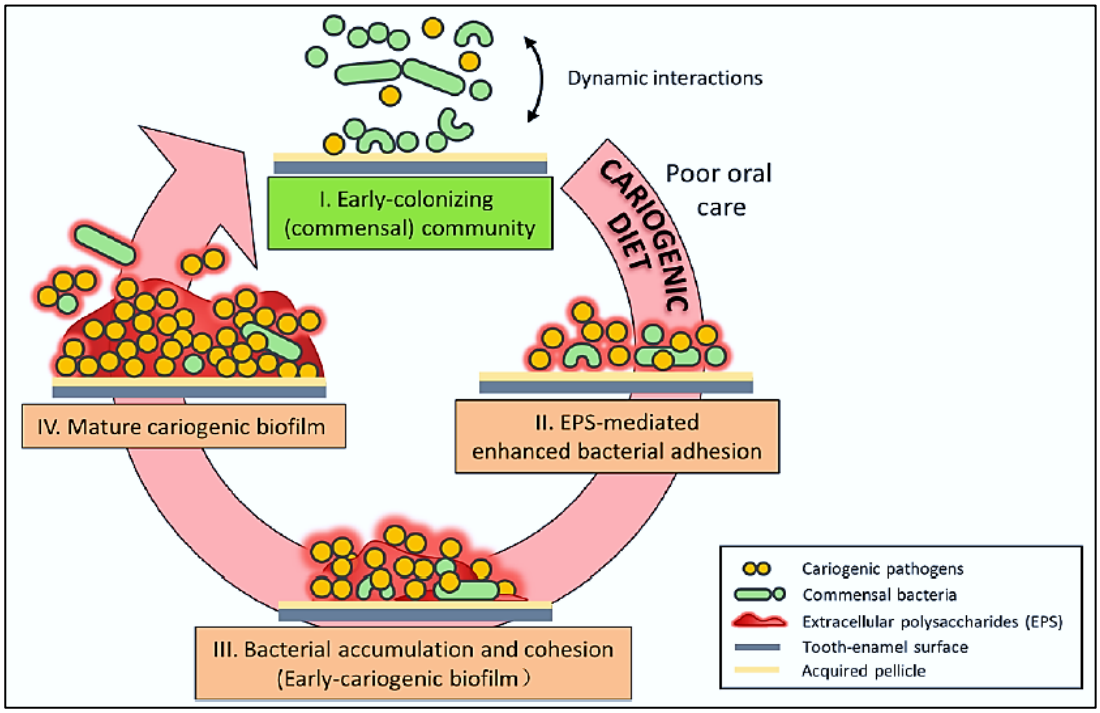


Figure 4. The matrix is a "multifunctional harbor", essential to biofilm life-cycle that starts with early-colonization (I), followed by bacterial adhesion (II), microbial accumulation (III) and biofilm maturation (IV) [33]

Numerous clinical, epidemiological, and experimental studies in animals have demonstrated that MS is strongly associated with the disease, particularly in ECC [35].

Particularly, sucrose, is known to be the historical sweetener in cooking, the most cariogenic as part of hexoses (glucose and fructose), that by fermentation causes pH lowering. Sucrose is also converted into insoluble extracellular glucans that in turn will increase bacterial adhesion and EPS matrix formation [33]. Furthermore, *S. mutans* does not act alone in causing dental caries, but it interacts with other microorganisms in a dynamic and coordinated attempt to form a cariogenic biofilm [26, 33-35].

Against dental caries, saliva plays a precious role being capable of neutralizing the acids produced in the mouth, where its heterogeneous distribution and pH in the structure of the oral biofilm has long been evaluated [26]. Notoriously, viscous saliva is less effective than watery one in cleaning carbohydrates, which provide physical protection; differently, chemical protection involves calcium, phosphate, fluoride, buffers, bicarbonate, and small proteins that neutralize the acids after consumption of fermentable carbohydrates. Also, several antibacterial substances are present in saliva, that directly act against microbial cells [36]. Initial data suggest that saliva does not have any effect on the acid production in the depths of dental plaque; rather dietary sugars can be easily dispersed throughout the biofilm [37]. When, in cases of systemic diseases, medications or radiation therapy impair salivary production, oral health is consequently affected and in particular teeth are at high risk for dental caries [38].

1.3 Other common oral pathogens: an overall picture

One of the most common pathologies of the alveolar bone is the apical periodontitis. It results from necrotic dental pulp, bacterial exposure to host immune system and extensive inflammatory reaction in periradicular tissues [39]. Periodontitis has a polymicrobial etiology, where the primary endodontic bacteria include *Bacteroides, Streptococcus, Actinomycetes, Treponema, Prevotella, Porphyromonas, Peptostreptococcus, Fusobacterium, Eubacterium* spp. and etc. A frequent complication of periodontitis is the occurrence of periradicular abscesses, mostly showing *Porphyromonas, Prevotella, Fusobacterium, Treponema, Bacteroides, Streptococcus* and *Peptostreptococcus*[40]. Several factors can influence the persistence of apical periodontitis, especially when the root infection has not been

adequately eliminated, probably by an insufficient aseptic control, improper tooth cavity access and accessory canals, inadequate instrumentation, micro-leakage due to temporary restorations [41]. Consequently, different microbial species are commonly isolated in the secondary and/or persistent endodontic infections; the most frequently isolated pathogens belong to *Enterococcus, Streptococcus, Staphylococcus, Pseudomonas, Candida, Actinomyces, Propionibacterium* species (Fig. 5) [39-41].

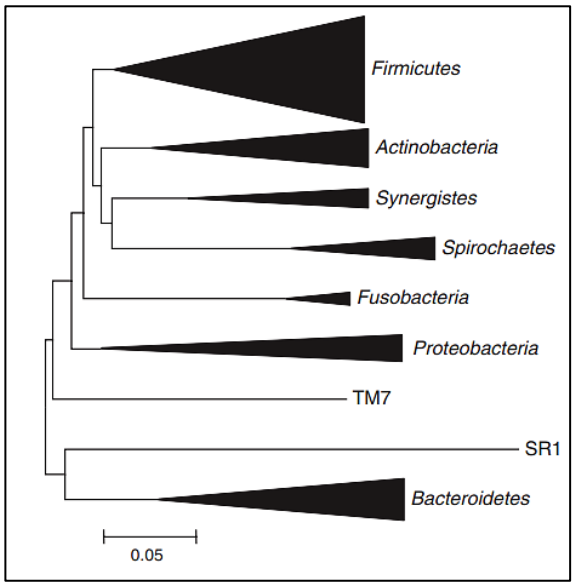


Figure 5. Bacterial phyla that have representatives in endodontic infections [40]

1.3.1 Staphylococcus aureus

Staphylococcus aureus (*S. aureus*), is a facultative anaerobic, Gram-positive coccal (round) bacterium also known as "golden staph". *S. aureus* is non-motile, does not form spores and can commonly reside as a commensal microorganism in human mucosa and skin [42]. In culture, *S. aureus* produces round, golden-yellow colonies, often surrounded by a hemolysis halo, when grown onto blood agar plates. It reproduces by binary division. After the complete division of the daughter cells mediated by *S. aureus* autolysin and, in its absence or targeted inhibition, daughter cells remain fused together and emerge as grape-like clusters [42].

Sometimes, to distinguish staphylococci from enterococci and streptococci, catalase-activity tests are used; catalase converts hydrogen peroxide (H_2O_2) to water and

oxygen. In this case, *S. aureus* is catalase-positive (so it can produce the enzyme catalase) [43]. *S. aureus* is a versatile bacterium as demonstrated by its ability to gain rapid resistance to new antibiotics; also, its many virulence factors have evolved to counteract host's immune defenses, allowing disease spread (Fig. 6) [44]. It has the ability to induce lysis of host cells and promote tissue invasion and destruction, or to specifically manipulate immune responses, including inhibition of complement activation, preventing function or recruiting neutrophils and inhibiting phagocyte function [45].

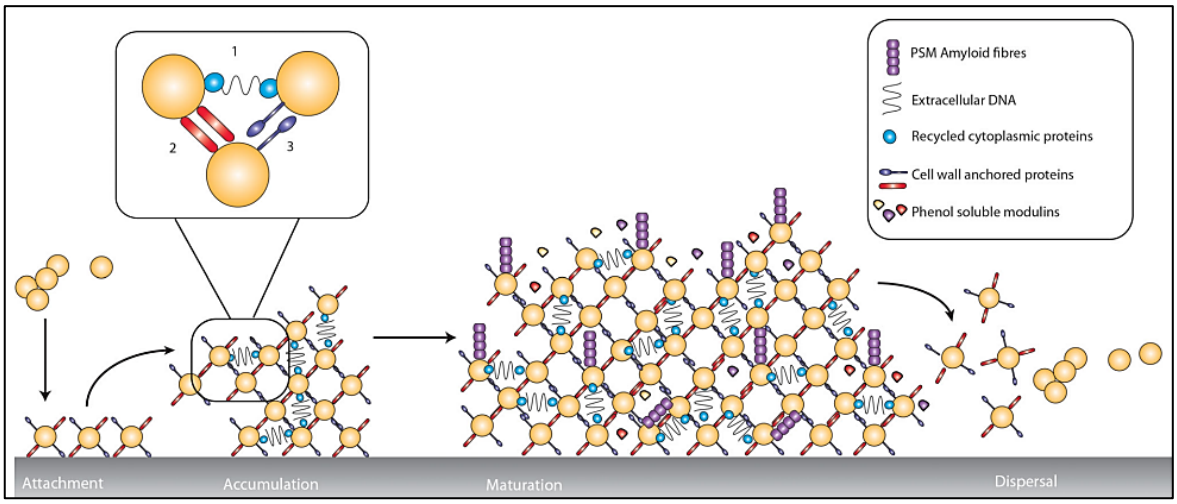


Figure 6. *S. aureus* biofilm formation [44]

As known, most of healthy individuals (30%) harbor *S. aureus*, often carrying it on their skin and mucosae. Such carriers serve as a source of infection for themselves and others [44,45]. Staphylococci have long been recognized as constituents of the oral microbiota; yet, their role in oral health and disease remains unclear. The isolation rates for *S. aureus* vary depending on the sample studied, with values ranging from 24% to 84% in the oral cavities of healthy adults [46]. Additionally, several oral infections, such as angular cheilitis [47], parotitis [48] or staphylococcal mucositis [49] are caused by *S. aureus*. Furthermore, it has been suggested that *S. aureus* may be involved in dental implant failure [50]. In general, consistently with infections caused by *S. aureus* elsewhere in the body, a number of oral staphylococcal infections are likely the result of mixed infections. Indeed, *S. aureus* has been associated with *C. albicans*; interestingly, invasive hyphal elements happen to facilitate *S. aureus* invasion of mucosal barriers, leading to underlying tissue damage or systemic spread [51].

1.3.2 Pseudomonas aeruginosa

Pseudomonas aeruginosa (*P. aeruginosa*) is a motile, non-fermenting, Gram-negative organism belonging to the *Pseudomonadaceae* family [52]. In 1850s, Sédillot observed that a blue-green discharge was frequently present and associated with infection in surgical wound dressings [52]. It is an opportunistic pathogen, capable of causing a wide-range of life-threatening acute and chronic diseases, particularly in patients with compromised immune defense. *P. aeruginosa* is the main cause of morbidity and mortality in cystic fibrosis (CF) patients and one of the leading nosocomial pathogens affecting hospitalized patients, while being intrinsically resistant to a lot of antibiotics [53]. For decades, the anti-pseudomonas activity has been one of the goals in novel drug breakthroughs; yet, *P. aeruginosa* remains one of the most refractory and difficult organisms to be treated. It is also tolerant to high temperatures (50 °C) and is capable to grow under either aerobic or anaerobic conditions [53].

P. aeruginosa pathogenesis is mediated by numerous virulence factors, mediating motility, adhesion, tissue damage, immune cell avoidance, etc. It is important to emphasize the role of quorum sensing (QS), a mechanism of bacterial “cell-to-cell” communication via diffusible chemical compounds. A critical number of bacteria (quorum) is required to produce secreted signal molecules (called auto-inducers) that in turn activate the expression of a large number of genes (more than 300) and the production of selected regulatory molecules [54]. QS is also known to determine tolerance of *P. aeruginosa* biofilms to antibiotic treatment [55]. Overall, *P. aeruginosa* is regarded as a good "model organism" for studies in the QS field. The most common class of autoinducers used by Gram-negative bacteria, including *P. aeruginosa,* is acyl-homoserine lactones (AHL), which diffuse freely across bacterial membranes. When the autoinducer amount reaches a particular threshold, such molecules bind to a transcriptional activator (LasR/RhlR) forming a complex that in turn activates genes coding for different virulence factors, some of which are also involved in biofilm formation. Other virulence factors, such as extracellular enzymes and lytic toxins, are important for the pathogenesis of the infection as a protective bacterial shield against phagocyte-mediated defenses [53,54].

P. aeruginosa produces a mature biofilm in a few days (Fig. 7). The initial step involves planktonic (individual freely moving) bacteria, that reversibly attach to a surface. The next step is irreversible binding to the surface, multiplication of the bacteria and microcolony formation, and production of a polymer matrix around such microcolonies. The biofilm becomes thick (up to 50 μm) and mushroom-like structures are often observed as a characteristic feature of the mature *in vitro* biofilm. The subsequent biofilm stage involves focal dissolution, liberation of planktonic bacterial cells that can then spread to other locations where new biofilms can be formed. This process may in part be mediated by bacteriophage activity within the biofilm. Motile planktonic bacteria can use type-IV pili to adhere to a biofilm formed by other bacteria, colonize the top of such biofilm, rendering it a mixed community [56].

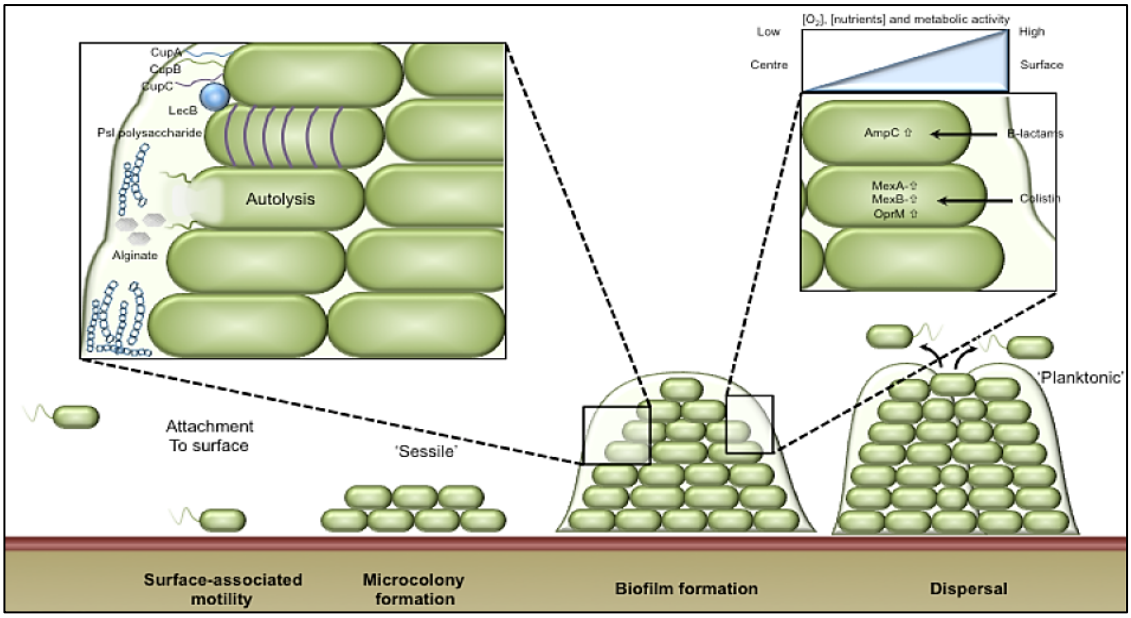


Figure 7. Development of a *P. aeruginosa* biofilm [56]

P. aeruginosa biofilm can be formed on abiotic and biotic surfaces; its matrix may account for over 90% of the whole biofilm mass dry weight. In fact, this dense matrix creates a niche favorable for intense cell-cell interaction and communication as well as a reservoir of metabolic substances, nutrients and energy that promote bacterial growth and eventually cell shielding once unfavorable conditions would happen. The matrix is mainly formed by extracellular substances (EPS), which are mainly polysaccharides, proteins, extracellular DNA (eDNA) and lipids [57]. In particular, the

exopolysaccharides Psl, Pel, and alginate are the major constituents of *P. aeruginosa* biofilm matrix; together with eDNA, they determine the sessile community architecture. Also, these EPS play an important role in biofilm resistance to host immune reaction and antibiotic treatments [57]. The differential role of each EPS has been analyzed at each stage of biofilm development. The various exopolysaccharides and eDNA were shown to interactively contribute to the biofilm architecture [57]. The presence of various EPS exhibiting different physiochemical properties confers a survival strategy, increasing the flexibility and stability of biofilm under various conditions [57]. The type 4 pili are important for *P. aeruginosa* adhesion and promote initial attachment of cells to surfaces at early stage of biofilm formation. Together with eDNA, flagella and the type 4 pili mediate migration required for the formation of the stalk and the cap in the mushroom-shaped microcolonies in the mature biofilm [58].

1.3.3 Candida albicans

C. albicans can cause two principal types of infections in humans: a) mucosal infections, such as oral and/or vaginal candidiasis, and b) life-threatening diseases, namely deep-seated or systemic infections [59]. *C. albicans* and other *Candida* species are present in the oral cavity, accounting up to 75% of all candidal infections [59,60]. In healthy individuals, this colonization generally remains benign. Under certain circumstances, *Candida* may shift its role from commensal to pathogenic and causes clinical pictures termed “oral candidiasis” (OC). *Candida*s pecies have been isolated from a variety of oral habitats, involving both soft and hard tissues of biological and non-biological origin; this underlines the high plasticity and adaptability of *Candida*. *C. albicans* has been isolated from enamel, periodontal pockets, root canals, orthodontic appliances, dentures and mucosal surfaces (Fig. 8) [60].

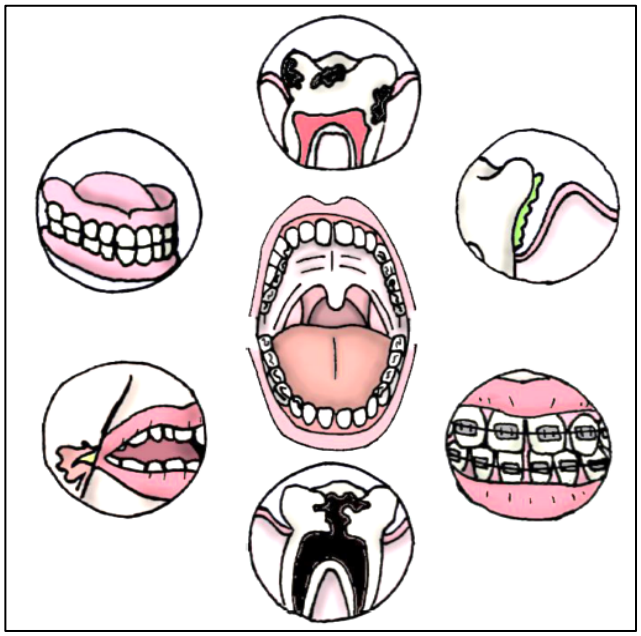


Figure 8. Oral sites of polymicrobial biofilm-associated diseases. The schematic diagram illustrates site within the oral cavity where typically Candida-bacterial polymicrobial biofilms are observed: caries, periodontitis, orthodontic, endodontic, angular cheilitis, denture stomatitis [60]

C. albicans is a pleomorphic fungus that can grow either as ovoid-shaped budding yeast, as elongated ellipsoid cells with constrictions at the septa (pseudohyphae) or as parallel-walled true hyphae [61]. Further morphologies include white and opaque cells, formed during switching, and chlamydospores, which are thick-walled spore-like structures. A range of environmental cues affect *C. albicans* morphology. For example, at low pH (<6) *C. albicans* cells predominantly grow in the yeast form, while at a high pH (>7) hyphal growth is induced. Indeed, a number of conditions, including starvation, the presence of serum or N-acetylglucosamine, physiological temperature and CO_2 promote the formation of hyphae [62].

The transition between yeast and hyphal forms is termed dimorphism. Both growth forms are important for *Candida* pathogenicity. The hyphal form has been shown to be more invasive than the yeast form. On the other hand the small-size yeast forms are primarily involved in dissemination. *Candida* morphogenesis has also been shown to be regulated by QS, a mechanism of microbial communication, where the main QS molecules include farnesol, tyrosol and dodecanol. High cell densities ($>10^7$ cells mL^{-1}) promote yeast growth, while low cell densities ($<10^7$ cells mL^{-1}) favor hyphal formation [62,63].

Finally, a better understanding of *C. albicans* pathogenic mechanisms is crucial for the development of new antifungal therapies and diagnostics. Several virulence factors, such as dimorphism, the secretion of proteases and the expression of adhesins and invasins, have been suggested as attractive anti-*Candida* targets,and recent investigations have further broadened the understanding of *C. albicans* factors and activities which contribute to virulence [64].

"Prevention is better than cure" — Desiderius Erasmus

1.4 Oral hygiene

Let's start this subsection with a question, which we often ask ourselves: "**What is a good oral hygiene?!**" As response, we may find: "The perfect condition of the mouth!", but, in the reality, this is not easily or often achieved. At a first glance, by good oral hygiene we may indicate a healthy looking and smelling mouth. This means that teeth are cleaned and free of debris; the gums are pink and do not hurt or bleed when teeth are brushed or flossed; bad breath (halitosis) is not a constant problem. Mainly, dental health is a matter of good dental hygiene, healthy eating and controlled drinking consumption, fluoride use and regular dental check-ups. Therefore, oral hygiene and clean teeth are necessary for the maintenance of the overall good health status.

The definition of oral health by the American Dental Association (ADA) claims: "Oral health is a state of functional, structural, aesthetic, physiological and psychosocial well-being and is essential for general health and quality of life of an individual" [65]. Furthermore, the World Health Organization (WHO) has also evaluated the effects of various risk factors, which may affect the overall health status and deteriorate the quality of life [66].

As detailed above, dental biofilm is the result of microorganisms' accumulation on the surface of the teeth, thus forming a community wrapped in an extracellular matrix composed mainly of glycoproteins and polysaccharides (Fig. 9). An efficacious oral

hygiene that controls of dental plaque is a well-established clinical strategy for the prevention of caries and various periodontal diseases [67].

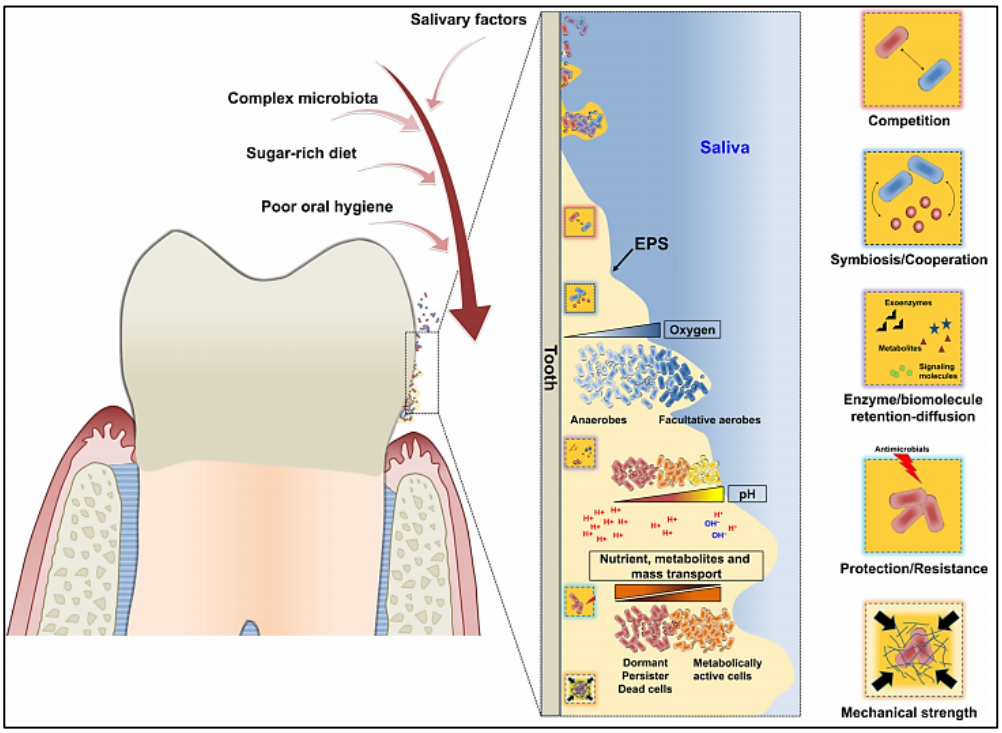


Figure 9. A cariogenic biofilm [67]

Many studies have shown that oral hygiene habits and oral health consciousness are related to demographic characteristics of the individuals. According to Rajab et al, parents with a high level of education are more concerned with their children's oral health [68]. Other authors point out that, unlike individuals with a higher socioeconomic status, people with low socioeconomic status visit a dentist more frequently only at the time when pain or discomfort appear [69].

Epidemiological studies show that caries levels can be successfully controlled by improving oral hygiene [70]. Above all, oral health care programs are fundamental and effective in protecting and maintaining dental health in children and adults. The main lines of dental plaque treatment include its mechanical removal, as well as systemic and/or topical antibiotics. To prevent and reduce oral microbial overload, a wide-spectrum search for non-toxic, clinically efficacious and cost-effective alternatives (novel toothpastes, mouthwashes or gum gels), is highly recommended to avoid repetitive and painful cleaning procedures and counteract antimicrobial drug-resistance phenomenon [71]. Besides the significant impact on general health, a poor

oral hygiene is also associated with systemic diseases (Fig. 10), significant morbidity and eventually mortality [72-74].

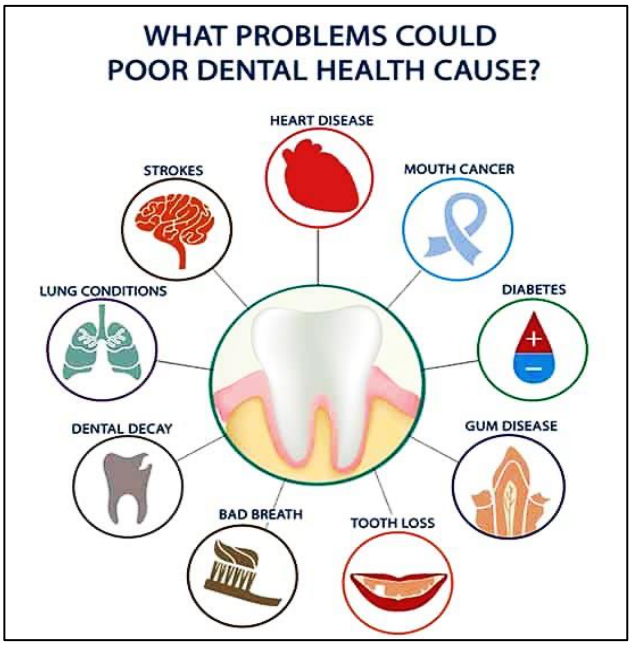


Figure 10. Oral health problems and overall health: How connected are they? [73]

Individual oral hygiene habits, such as tooth brushing used to protect the oral and dental health may vary depending on the level of oral health knowledge. Also, the attitudes and skills of parents about oral hygiene affect the prevalence of oral and dental diseases as well as the development of oral hygiene habits in children [75].

Daily tooth-brushing at regular intervals is the most popular mechanical method for biofilm control. It is important to spend about 2-3 minutes of brushing each day to improve dental care associated with mouth rinses as well (Fig. 11) [76]. Toothbrushes are the most common, effective, and inexpensive tools for removing dental biofilm. Above all, effective brushing depends on numerous factors, including patient motivation, dexterity, product association, usage time and bristle wears [77].

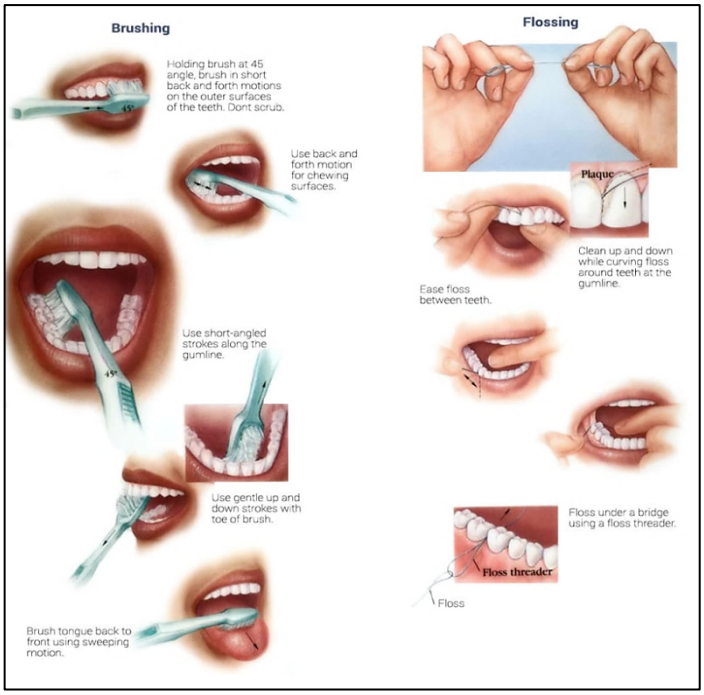


Figure 11. Don't rush when you brush! Techniques of hygiene [76]

Another important aspect is halitosis; the latter is a condition defined as the presence of unpleasant breath originates mainly from breathing through the lungs, or that related to the mouth. Halitosis is due to a number of malodorous substances, including here volatile sulfur compounds in the oral cavity. These gases are produced by microbial biofilm as metabolic products, originated from the deep periodontal pocket, tongue, tonsils, pharynx, as well as gastrointestinal tract [78]. Therefore, the mechanical removal of dental biofilm by daily oral hygiene practice is essential in reducing microbial load in the oral cavity and, in turn, halitosis. Yet, in many cases, tooth-brushing alone does not eliminate such a problem [79].

Increasing evidence indicates that regular use of suitable toothpastes (such as those with ingredients that possess antibacterial, anti-inflammatory, antioxidant and regenerative properties), is one of the most effective strategies against halitosis, prevention and treatment of cavities, gingivitis and periodontal diseases [80]. Importantly, the antibacterial properties of several compounds for oral hygiene have been related to the addition of chlorhexidine, fluorides [81], xylitol [82], triclosan [81] and their combinations [83]; indeed, a decrease in bacterial load has been documented in *in vitro* systems, including bacterial biofilms, as well as in *in vivo* studies showing antibacterial, anti-caries, and anti-inflammatory effects. To date, concerns about the

absorption, retention and multi-organ toxicity of such additives are steadily increasing. For example, fluoride-containing substances, that are virtually omnipresent in modern toothpastes and mouthwashes have a low safety profile [84].

2. Novel antimicrobial tools/compounds

Several studies on oral microbiota have focused on different microbial species associated with socially relevant diseases, such as caries, periodontal inflammatory diseases, peri-implantitis or other oral mucosal diseases [85]. Because of the well-known ability of most of such pathogens to produce biofilm and given the biofilm low sensitivity to detergents, antimicrobial drugs and host defenses, the therapeutic protocols have to be carefully defined and sometimes may be ineffective against oral diseases [86].

In general, a large number of different classes and formulations of drugs are available to manage human infections. Yet, the microorganisms that cause odontogenic or periodontal infections belong to a restricted group; thus, a relatively small number of antimicrobial agents are commonly selected and used to manage oral diseases [87].

2.1 A copper-calcium-hydroxide based endodontic paste

As known, the enamel is the hardest tissue of the human body that covers the entire clinical crown of the tooth. It acts as a protective barrier for dentin and pulp against chemical or thermal insults and biological agents as well. The enamel resistance to these external *noxae* depends on its chemical composition and its structure, formed during the odontogenesis process [88]. Several oligoelements present in tooth enamel can affect the composition of the enamel before and after tooth eruption. It is hypothesized that the main components of the enamel may influence the susceptibility of the enamel to erosive conditions in the oral environment [89]. Furthermore, calcium and magnesium are well known building minerals (hydroxyapatite and magnesium phosphate) which are essential for the structure and strength of the enamel, but oligoelements such as zinc (Zn) and copper (Cu), are important for its integrity and elasticity [90]. Zn and Cu are potential

components linked to the formation and changes within the enamel matrix; where Zn is a potent inhibitor of serine proteases, including here also kallikrein 4 [91]. Mostly, Zn accumulates in the teeth after their eruption and protein binding is a mechanism that determines the level of Zn. The latter also interacts with hydroxyapatite through absorption on the crystalline surface and its incorporation into the crystal lattice. Low concentrations of this element modify or inhibit remineralization; however, it significantly reduces enamel dissolution [92]. Meanwhile, Cu has a significant impact on the acid solubility of enamel, a process that takes part in the development of tooth decay and erosion [89,93]. According to Abdullach et al [94], the Cu content in the enamel is 2-6 µg/g. Another study reports that the average concentration of Cu in the enamel surface without pathological changes is 36.67 µg/L [89]. Considering the mechanism of action of Cu ions (Cu^{2+}) and their influence on enamel, this effect is certainly desirable [95]. Additionally, Cu^{2+} exerts a direct cariostatic effect, inhibiting bacterial growth and impairing bacterial enzymes [93]. Clinical and experimental data document that bacterial invasion of dental tubules, root-canal ramifications, isthmuses or apical deltas are responsible for persistent endodontic infections; accordingly, the success of root canal treatment depends on the complete removal of bacteria from these root canal spaces [96]. Overall, the main task of a successful endodontic treatment is the complete elimination of all the microorganisms and the protection of the treated dental element from a potential reinfection. Primarily, this goal is achieved using various antimicrobial agents through various methods, like irrigation, ultrasonic preparation, ionophoresis, electrophoresis, etc.; in any case, the important point is to exert a wide spectrum antimicrobial effect, given the polymicrobial nature of these infections [97]. A variety of intracanal drugs are also used that, besides their antibacterial properties, may act as a mechanical barrier by reducing marginal infiltration, ensuring canal drying, neutralizing tissue debris, and reducing inflammation of the periapical tissue [97].

Innovative endodontic techniques have become widely available in recent years [98]; in particular, a copper-calcium-hydroxide (Cupral)-based treatment has been carefully considered given its potent antimicrobial properties [99]. The now commercially available endodontic paste, named Cupral (Humanchemie, Alfeld, Germany), contains highly dispersed calcium hydroxide [$Ca(OH)_2$], copper sulphate (II) ($CuSO_4$), calcium sulphate ($CaSO_4$), copper hydroxide (II) [$Cu(OH)_2$],

methylcellulose $[C_6H_7O_2(OH)x(OCH_3)_y]_n$ and distilled water. This copper-calcium-hydroxide containing paste and its use in endodontic treatment had been proposed for the first time by Prof. Adolf Knappwost in 1993. He described a novel method of disinfecting root canals, by inserting this paste into the coronary third of the root canal system using an electrophoresis system to mobilize ions [100]. As reported by Knappwost [100], when in contact with irrorated tissues, Cupral reacts with the buffer solution of carbonic acid/hydrogen carbonate of the blood forming a calcium carbonate mineral membrane that increases overtime in thickness along the borderline of blood-supplied tissues. On the other hand, this membrane prevents the entry of OH^- ions into the tissues, neutralizing the acidic environment at the inflamed site and providing analgesic effects as well [100]. It is relevant to underline, that during clinical Cupral-electrophoresis treatment, the patient does not feel any discomfort or pain. This is because of the very weak current, caused by a low voltage, and only focally used in the endodontic cavity. According to different authors [101,102], the amount of copper, a physiological compound in human body, used during electrophoresis does not show negative or toxic effects. Upon treatment, high proteolytic activities of hydroxy-cuprate and hydroxyl ions occur in the endodontic cavities. This allows decontamination of the radicular canal and periapical tissue, and in turn will fasten debris absorption from radicular canal, by the organism. Upon such treatment, exudate production (sterile proteolysis) is facilitated in the endodontic environment, decreasing in this manner the pain during chewing process. Furthermore, to support the growing interest in Cupral, not only German groups have published, but also other groups such as Polish, Russian or Bulgarian, demonstrating the advantage of Cupral clinical use [103]. Many *in vitro* studies have included *Enterococcus faecalis* or various *Streptococcus* spp. demonstrating the wide-spectrum antimicrobial effects of Cupral [104]. As undiluted paste, Cupral is also applied in gingivitis as well as in indirect dental pulp coverage. Particularly, the effect of electrophoresis with Cupral, coupled to transcanalar current, guarantees antibacterial and long-term action when applied to dental roots affected by problematic curves and periapical destructive damages. Not only vegetative forms, but also bacterial spores and viruses have been eliminated, supporting the efficiency of such a complete system [100].

2.2 Decontamination techniques applicable to titanium implants

The implant dentistry is evolving rapidly with an increase in survival at 5-10 years, while plaque-induced inflammation around implants is still the leading cause of implant failure [105]. The peri-implant disease is an inflammatory process characterized by bleeding, suppuration and bone loss; it affects both the soft and hard tissues surrounding functional implants [106]. Among several risk factors, poor oral hygiene, smoking and history of periodontitis are relevant for the development of peri-implantitis [106]. As well known, these factors lead to inflammation and degradation of the surface properties of dental implants; also, their biocompatibility is greatly affected [107].

Whereas peri-implantitis and periodontitis have similar characteristics, they differ in some aspects and the treatment of these two diseases is also different [108]. In periodontitis, is possible to restore the infected cementum surface cleaning and detoxifying it by means of mechanical or chemical methods; in this respect, it is worth noting that implant surface detoxification is more challenging due to the roughness of such material. Meantime, biofilm removal is not sufficient to restore the biocompatibility of titanium surfaces; thus, various methods of implant surface decontamination have been described [109]. When treating peri-implantitis, the decontamination procedure can modify the microstructure, roughness and biocompatibility properties of the implant surface. Notoriously, implant surface characteristics significantly influence host cellular response [110].

The commonly used decontamination methods are mainly mechanical (including curettes made of metal, titanium or carbon, ultrasonic scalers, an airborne-particle abrasion system), chemical (involving different chemicals, citric acid, chlorhexidine or hydrogen peroxide), and laser treatments such as Er: YAG and Er, Cr: YSCG. Unfortunately, most of these lasers operate in evaporation mode e produce high temperatures that in turn can alter or damage the implant surface, making them unsuitable for the peri-implantitis treatment. They can also cause tissue charring or clotting, thereby delaying the repair cascade [111,112].

The interaction of the dental implant with its surrounding cells is critical in order to stabilize and maintain the implant. A crucial step in osseointegration is early host cell

adhesion to the implant. Also, platelets are relevant; becoming activated they allow fibrin formation around the implant within minutes [113]. Moreover, human gingival fibroblasts (HGFs), which are the major cellular components of oral soft tissue, are essential for maintaining oral implants in good condition through numerous functions, including repairment of tissue damage and isolation of implants from the oral microbial environment [114].

2.3 Propolis and its wide-spectrum beneficial properties

The use of natural products for the treatment of a wide range of diseases is becoming a trend, in many industrialized countries. The search of new products of natural origin is encouraged, as only 15% of the approximately 300,000 plant species worldwide have been evaluated for their pharmacological potential [115]. Propolis is one of these natural products that is being experimented on a large scale and used in various fields of medicine. It is known as a balsamic and resin product with a complex chemical composition, considered also as a natural matrix with high biotechnological potential.

The word propolis originates from the Ancient Greek ("pro" - "in front" or "at the entrance" and "polis" - "community" or "city"), referring to its main function in protecting the hive [116]. Interestingly Egyptians, Greeks, and Romans have already reported the multiple properties of propolis that has been used for lesion healing and corpse embalming [116]. Others, like Aristotle, Pliny, and Galen have described some of the medicinal properties of propolis and its use as an antiseptic and wound-healing agent; then, in the medieval period, propolis has mainly been used by Arabian physicians. Subsequently, the Incas used propolis as an antipyretic. In the 18th century, the London pharmacopoeia has listed propolis as an official medicine and since that time, propolis has become very popular in Europe because of its antibacterial activity. While, during World War II, propolis has been used as an antimicrobial and anti-inflammatory agent [116].

It is known that not all the species of the genus *Apis* (honey bee) produce and use propolis to the same extent; for example, the Apis mellifera species produce and use propolis more than the others [116,117]. Indeed, the propolis production starts when the

bees take parts of vegetables or plants with their mandibles and chew them till, they obtain a resin. After, the bees manipulate this resin with their forelegs and pack it with the hind legs. Then, once to the hive, the resin is mixed with bee saliva and partially hydrolyzed by its enzymes. In this way, this mass is cemented in the hives and mixed with beeswax. This complex compound is then used by bees to protect them from other insects and microorganisms and to repair their hives [118].

The chemical composition of propolis varies considerably according to the geographical and botanical origin, i.e., of impact are climatic factors, plant sources and harvest seasons [118]. Because of its complex and variable composition, standardization of propolis is required in order to release it as a commercial product for medical purposes [118]. From the analysis of various propolis extracts, data on its chemical composition can be obtained. In particular, for its dissolution and extraction of biologically active compounds, different solvents have been used: water, alcohols (e.g., ethanol and methanol), glycols (e.g., propylene glycol), oils (e.g., olive oil); for analytical purposes, other organic solvents, such as hexane, acetone, dichloromethane and chloroform, have also been used [118].

Propolis is mainly composed of: resin and balsam (50%), wax (30%), essential oils (10%), pollen (5%) and other organic compounds including sugars, amino acids, vitamins and minerals (5%) [119]. More in-depth analyses have revealed that the main groups of chemical compounds in propolis are: phenols (e.g., flavonoids, polyphenols, phenolic acids and other phenolic compounds) and their esters, aromatic esters, terpenes and terpenoids, steroids, aromatic acids, aldehydes, alcohols, sugars, sugar alcohols and acids, amino acids, vitamins, fatty acids, hydrocarbons, mineral elements and alcohols [119]. The main group of phenolic compounds is the flavonoid group that contributes greatly to the biological and pharmacological activities of propolis. According to the chemical structure, the flavonoids in propolis are classified into subclasses: flavans, isoflavans, flavanones, flavanonols, flavones, isoflavones, isodihydroflavones, flavonols, chalcones, dihydrochalcones and neoflavonoids [118,119].

The flavonoid group in propolis mainly includes the following substances: chrysin, pinocembrin, apigenin, rutin, luteolin, galangin, kaempferol, myricetin, catechin, naringenin, quercetin, tectochrysin, pinostrobin, acacetin and others [120].

Another important group of compounds present in propolis are phenolic acids, among which the most common are ferulic, cinnamic, caffeic, benzoic, salicylic and p-coumaric acids [120]. Volatile compounds such as terpenes and terpenoids are present for only about 10%; yet, they contribute significantly to the biological and pharmacological activities of propolis and are responsible for its characteristic aroma [119]. Many different minerals (macro and microelements) are found in different propolis samples, such as Ca, K, Na, Mg, Mn, Fe, Si, Zn, Se, Cu, Ni, Al, B, C, E, Ba, Cr and Sr [119,120].

Propolis exerts its beneficial effects against infectious diseases acting at two levels: a) directly as anti-microbial agent and b) indirectly by stimulating the immune system such as host innate defense mechanisms [121]. Particularly, it exerts antimicrobial activity, against both Gram-positive and Gram-negative bacteria, the latter being susceptible to a lower extent [121]. Numerous studies describe other multiple biological properties of propolis extracts, such as antioxidant [122], antimicrobial [123], antiviral [124], antiparasitic [125], antitumor [126], immunomodulatory [126], anti-inflammatory [127], and hepatoprotective properties [128]. Given this wide range of biological properties, its potential for the development of products to be used in human and animal health is strongly suggested.

Also, propolis is widely used in cosmetics, food and formulations of pharmaceutical products and is one of the most popular and used natural products. To facilitate its handling and use, propolis is proposed in various forms, capsules, throat pastilles, mouthwashes, lotions, and wax-free products [129].

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AIM OF THE STUDY

A variety of microorganisms colonize dental structures and oral mucosae of healthy subjects, behaving as harmless commensal. Endogenous and exogenous factors may affect such homeostasis, driving towards oral infections that may affect and deeply damage teeth and mucosae, especially once microorganisms succeed in producing biofilm onto biotic and/or abiotic surfaces. From here, the need to identify and investigate new compounds/tools capable of preventing or limiting dental/oral biofilm formation.

The aim of the present PhD project was to evaluate *in vitro* and *ex vivo* the effects of innovative antimicrobial compounds against oral biofilms. In line with previous studies, *Staphylococcus aureus, Pseudomonas aeruginosa and Candida albicans* were chosen, as representative members of different groups of pathogens, namely Gram positive, Gram negative and fungi; because of their high degree of tolerance to chemical and physical stresses, such reference strains represent indeed useful prototypes for *in vitro* investigations.

The results obtained during the 3-years PhD, which were published in peer-reviews journals, are herebelow organized in six main chapters, as follows:

- **Chapter 1** – Antimicrobial and antibiofilm efficacy of a copper/calcium hydroxide-based endodontic paste against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans*.
- **Chapter 2** – Efficacy of a copper–calcium–hydroxide solution in reducing microbial plaque on orthodontic clear aligners: a case report.
- **Chapter 3** – Evaluation of biological response of STRO-1/c-Kit enriched human dental pulp stem cells to titanium surfaces treated with two different cleaning systems.
- **Chapter 4** – Differential efficacy of two dental implant decontamination techniques in reducing microbial biofilm and re-growth onto titanium disks *in vitro*.
- **Chapter 5** – Propolis affects *Pseudomonas aeruginosa* growth, biofilm formation, eDNA release and phenazine production: potential involvement of polyphenols.
- **Chapter 6** – Antibacterial effects of MicroRepair®BIOMA-based toothpaste and chewing gum on orthodontic elastics contaminated *in vitro* with saliva from healthy donors: a pilot study.

CHAPTER 1

Dental Materials Journal 2019; 38(4): 591–603. doi:10.4012/dmj.2018-252

RESEARCH ARTICLE

Antimicrobial and antibiofilm efficacy of a copper/calcium hydroxide-based endodontic paste against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans*

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Abstract

Endodontic biofilm is a microbial community, enclosed in a polymeric matrix of polysaccharide origin where are found pathogens, like bacteria and opportunistic fungi responsible for various endodontic pathologies. As clinical importance is the fact, that biofilm is extremely resistant to common intracanal irrigants, antimicrobial drugs and host immune responses. The aim of this study was to evaluate the *in vitro* efficacy of a Cu/$CaOH_2$-based endodontic paste, against bacteria and fungi, such as *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans*. We found that such compound significantly reduced microbial replication time and cell growth. Moreover, biofilm formation and persistence were also affected; treated biofilms showed both a reduced number of cells and levels of released pyoverdine. This study provides the first evidence on effectiveness of this endodontic compound against microbial biofilms. Given its wide range of action, its use in prevention and treatment of the main oral biofilm-associated infections will be discussed.

INTRODUCTION

In human body, one of the most complex and heterogeneous microbial communities occurs in the oral cavity, where adhesion of planktonic microbes to a surface, either biotic or abiotic, is followed by coaggregation, growth, production of an extracellular matrix and maturation of a sessile structure, the so call oral biofilm[1)]. The relevance of such biofilm in health and disease, both locally and distally, is receiving increasing attention.

The main etiological factor in the emergence of apical periodontitis is an infection by oral microbes and their products, that once reaching the pulp and periapical tissues, locally produce tissue damage and necrosis in about 90% of cases[2)]. Accordingly, special efforts are aimed at counteracting the microbial agents present in an infected pulp system[3)]; nevertheless, it is very difficult to guarantee their complete elimination. Increasing literature documents the presence of facultative anaerobic bacteria, such as Gram-positive cocci (*Enterococcus faecalis* and *Staphylococcus aureus*) and Gram-negative bacteria (*Pseudomonas aeruginosa*) as pathogenic for dental pulp condition[4-6)]. Another opportunistic fungal pathogen, *Candida* spp has occasionally been isolated from root canal periapical lesions, granulomas and necrotic pulp tissue as well[7,8)].

Microbial agents tend to penetrate tooth structure and accumulate in dentinal canals, at a considerable depth, where they likely produce endodontic biofilms and are hardly reached by endodontic instruments and irrigants[9,10)], especially in cases of complicated anatomy, lateral canals and apical ramifications[11-13)]. *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans* (the most commonly isolated fungal specie in the oral cavity)[8,14)], are frequently observed inside pulp system[4,15)] and they are one of the multiple factors responsible for the failure of endodontic therapy. An indispensable condition for success in endodoncy is not only the treatment of the main root canal, but also the elimination of microbial cells commonly localized in lateral canals, apical deltas and dentinal tubules; only in this way, a permanent sterility of the treated dental element may be guaranteed[16)]. Despite improvement in endodontic techniques, the percentage of failure remains high, because of intra-radicular bacteria persistence[17)].

For this reason, it is necessary to use antimicrobial agents that besides being not toxic for the patients are capable to deeply penetrate into dentinal system, and, in turn, efficiently eliminate bacteria, possibly even when structured in biofilm. Notoriously, once structured as biofilm, microbial agents have enhanced resistance to antibiotics and disinfectants, given their complex and heterogeneous arrangement as a microbial sessile population embedded into an extracellular, minimally permeable polymeric matrix[18-20]. Among the numerous soluble factors involved in biofilm formation/maturation, quorum sensing molecule, including pyoverdine[21,22] and eDNA[23,24], have been described. Because of such a complex scenario, antibiotic therapy as well as intracanal irrigants treatment have to be focused towards the eradication of root canals infections and possibly endodontic biofilm disruption[19].

Innovative endodontic techniques have become widely available over the last years[25,26] in particular, a copper-calcium-hydroxide (Cupral) treatment has been carefully regarded given its potent antimicrobial properties[27]. Not only vegetative forms, but also bacterial spores and viruses were affected, underlying the widerange efficacy such system[27]. Initial evidence exists on its performance in clinical practice, where it is used as undiluted paste; in particular, histological analysis of teeth, endodontically treated with Cupral and then extracted for prosthetic reasons 3 to 6 months later, revealed the presence of copper crystals inside the intracanal system and absence of bacteria, thus implying a Cupral-mediated long lasting endodontic sterility[28]. Recently, Cupral is being used also in gingivitis and in the indirect covering of dental pulp.

The aims of this *in vitro* study were: 1) to evaluate the efficacy of Cupral against planktonic forms of selected pathogens, belonging to different categories: Gram+ (*Staphylococcus aureus*) and Gram− (*Pseudomonas aeruginosa*) bacteria and fungal cells (*Candida albicans*); 2) to assess the ability of Cupral to prevent microbial biofilm formation and persistence.

MATERIALS AND METHODS

Microbial strains

The following American Type Culture Collection (ATCC) strains were used in this study: *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 9027 and *Candida albicans* SC5314.

Bacterial and fungal cultures conditions

Bacteria from frozen stocks were placed into Tryptic Soy Broth (TSB: OXOID, Milan, Italy) and placed to grow at 37°C overnight. Then, by sterile loops, 10 µL of each broth were seeded onto Tryptic Soy Agar (TSA) plates (OXOID) and allowed to grow for 24 h. Isolated colonies were then collected (1 or 2 for each species), added to 10 mL of TSB and allowed to grow overnight at 37 °C with gentle shaking. Bacterial concentrations were then assessed by a McFarland standard curve and diluted to the working strength concentration of 1×10^5 Colony Forming Units (CFU)/mL. *C. albicans* cultures were maintained by biweekly passages onto Sabouraud Dextrose Agar (SDA) plates (OXOID). The day before each experiment, fresh cultures were seeded onto SDA plates and incubated at 37 °C. After the overnight incubation, fungal cells were harvested by a sterile inoculating loop, suspended in phosphate buffered saline (PBS; EuroClone, Whethereby, UK), washed twice by centrifugation at 3,500 rpm for 10 min, counted by Burker's chamber and suspended at 1×10^5 yeast cells/ mL in YPD (OXOID) prior to be used in the experiments. For long-term storage, *C. albicans* was maintained as frozen stocks at −80 °C, in glycerol solution 20% (v/v).

Endodontic paste

A commercially available compound (Cupral, HUMANCHEMIE, Alfeld, Germany), containing highly dispersed calcium hydroxide [$Ca(OH)_2$], copper sulphate (II) ($CuSO_4$), calcium sulphate ($CaSO_4$), copper hydroxide (II) [$Cu(OH)_2$], methylcellulose [$C_6H_7O_2(OH)\times(OCH_6)$] and distilled water was used. A starting solution of Cupral (weight/volume) was prepared by diluting 1 g of compound in 4 mL of distilled water (25%); serial dilutions (1:10) were then prepared to obtain 2.5% and 0.25% final solutions. Cupral preparations were sterilized by autoclave prior to be used in the

study. Furthermore, in our experiments, Cupral's solutions were diluted 1:1 with culture medium (controls) or microbial cultures; the new concentrations were 12.5, 1.25 and 0.125% into the wells.

Microbial growth assays

Microbial suspensions (1×10^5 CFU/mL; 100 µL/well) in 96-well plates (Sarstedt, Nümbrecht, Germany) were exposed or not to Cupral (100 µL/well, at the concentrations above indicated); blank wells (distilled water or Cupral without bacteria) were also included in the assay. The plates were then incubated at 37 °C in presence or absence of 5% CO_2 for various times, as detailed elsewhere. Then, microbial growth was assessed measuring optical density (OD) or CFU in Cupral-treated and untreated groups, as follows. The absorbance was measured by the SunRise Microplate Reader (Sunrise, Tecan, Salzburg, Austria) at 595 nm wavelength, at the following times: 0, 3, 6, 24 and 48 h. The results were expressed as ΔOD, by subtracting the blank OD values from the OD of the experimental samples. The CFUs were evaluated at 0, 24 and 48 h, according to standard protocols by appropriated diluting and plating microbial 100 µL of each microbial suspension on TSA (bacterial groups) or SDA (fungal groups) plates. After 48 h of incubation, the pH of each microbial culture was measured using the litmus papers. In selected experiments, the doubling time (DT) was determined in control and Cupral-treated microbial cells. Briefly, cultures in exponential growth phase were (90–120 min) exposed or not to Cupral and further incubated at 37 °C; then, the ODs were measured at various time points and converted to CFU, using the McFarland curve for bacterial cells and an in-house reference growth curve for *C. albicans*. The DT was calculated using the following formula, considering time 0 (initial CFU) and various time points (final CFU):

$$\text{Doubling Time} = \frac{\text{time x log (2)}}{\text{log(final CFU) - log(Initial CFU)}}$$

The results of DT were expressed in min.

Disk-diffusion test

The disk-diffusion assay was performed according to "EUCAST disk diffusion antimicrobial susceptibility testing method" (http://www.eucast.org EUCAST European Committee on Antimicrobial Susceptibility Testing Breakpoint tables for interpretation of MICs and zone diameters Version 8.0 2018). Briefly, a sterile cotton swab was dipped in *Staph. aureus* stock suspension (1×10^5 cells/mL) and inoculated on Mueller-Hinton agar (MHA) plates (OXOID). The plates were allowed to dry for 3 to 5 min. Then, sterile and neutral filter paper disks (6 mm) were placed onto the MHA surface and embedded with Cupral 12.5, 1.25 and 0.125% (10 µL/disk) or gentamicin (10 µg/10 µL/disk), used as positive control. The plates were then incubated at 37 °C, examined after 24 or 48 h and the growth inhibition haloes around each disk were measured. The results were expressed as halo diameters in mm.

Confocal microscopy analysis of P. aeruginosa biofilm

Pseudomonas was allowed to produce biofilm onto coverslips inserted into 6-microplate (Sarstedt); then, the fluorescent property[21,22] of *Pseudomonas* spp was exploited to perform confocal microscopy imaging, as detailed elsewhere[20]. Briefly, bacterial suspensions (1×10^5 bacterial cells/mL in TSB) were seeded on coverslips (1,000 µL/well), and treated or not with Cupral 1.25%. The plate was incubated for 24 h at 37 °C and 5% CO_2. After incubation, the coverslips were washed twice with PBS and fixed with 4% paraformaldehyde (PFA: Sigma-Aldrich, Darmstadt, Germany) for 30 min at 4 °C, washed twice with PBS and then analyzed by confocal microscope Leica TCS SP8 (Wetzlar, Germany), at 492/517 nm wavelength excitation/emission as recommended by the distributor.

Pyoverdine production by P. aeruginosa biofilm exposed or not to Cupral

After 24 h of culture with *Pseudomonas*, the supernatants were collected, centrifuged twice at 10,000 rpm for 15 min in order to remove the remaining bacteria. To further, ensure that the supernatants were devoid of bacteria, 50 µL of the supernatants were inoculated onto TSA plates and incubated for 48 h at 37 °C under aerobic conditions. No bacterial colony formation on TSA plates was observed.

Pyoverdine release was quantified in 100 µL of culture supernatants and fluorescence emission was quantified with a multi-well fluorescence plate reader (Synergi HTX, BIOTEK, Winooski, VT, USA) (excitation/emission: 360/460), according to a standard protocol[29]. The measured amounts of pyoverdine were plotted as mean ± SEM (Standard Error Mean) of relative fluorescence units (RFU).

Analysis of Staph.aureus, P. aeruginosa and C. albicans biofilm persistence

The evaluation of biofilm formation was performed by 96-microplate as described elsewhere[30]. Briefly, microbial cell suspensions (1.5×10^8 bacterial cells/mL in TSB and 1×10^6 fungal cells/mL in YPD) were seeded (100 µL/well) and incubated for 24 h at 37 °C in the presence of 5% CO_2 to allow biofilm formation. Then, Cupral was added (3 concentrations) and the incubation further prolonged for additional 24 h; in parallel, control wells, with only Cupral without bacteria or distilled water, were included (blanks). After incubation, crystal violet (CV) assay was performed to quantify the total and residual biofilm biomass. The absorbance was spectrophotometrically measured by the SunRise Microplate Reader. Two wavelengths were used: 570 nm for microbial biofilms and 540 nm for fungal biofilm. The color intensity was proportional to biofilm biomass/well and the values of the respective blanks were subtracted to the OD of experimental samples. The results were expressed as % of biomass (treated cells) compared to 100% of controls biomass (untreated cells). For the morphological studies, the microbial biofilm was prepared as detailed above, on coverslips in 6 well microplate. Then, the coverslips were washed twice with PBS at room temperature (RT) and fixed with 4% PFA for 30 min at 4 °C, washed again twice with PBS and then analyzed by optical microscope. Differential interference contrast (DIC) microscopy was performed using a Nikon Eclipse 90i imaging system equipped with Nomarski DIC optics (Nikon Instruments, Melville, NY, USA). Samples were photographed with a DS-2Mv Nikon digital camera.

Assay for live/dead microbial evaluation

Biofilm formation was performed in a black 96 well microplate. The microbial cells (1×10^8 bacterial cells/mL and 1×10^6 fungal cells/mL) were incubated for 24 h at 37 °C

in presence of CO_2. Biofilms were then treated with diluents (controls) or Cupral 1.25% and incubated for further 6 and 24 h. After incubation, the samples were stained with the "live/dead cells stain kit" (Thermo Fisher Scientific, Waltham, MA, USA), based on 5(6)-carboxyfluorescein diacetate (CFDA) to label alive cells (30 min) and propidium iodide (PI) binds to DNA and labels the dead cells (15 min). The staining was conducted according to manufacturer's instructions. After a total 30 min of incubation at 37 °C, the samples were washed twice with PBS and the fluorescence emission (CFDA excitation/emission: 485/528; PI excitation/emission: 528/645) was analyzed using a multi-well fluorescence plate reader (Synergy HTX, BIOTEK). The results were expressed as RFU.

Statistical analysis

Data depicted in Figs. are the mean ± standard error (SEM) from replicate samples of 2–3 different experiments. Statistical analysis was conducted using GraphPad Prism 7.0 software and carried out with oneway ANOVA with Bonferroni's post-hoc test. Statistical significance was set at $p \leq 0.05$, while $p < 0.0001$ was indicative of highly statistically significant differences.

RESULTS

Evaluation of Cupral pH values under different experimental conditions

The pH value is a crucial parameter for microbial survival and growth. Thus, we initially assessed the pH of Cupralat different working dilutions. Results in Table 1 show that, a Cupral solution in distilled water at 25% (*v/v*) had a pH value of 14, which progressively decreased to 13 and 11, when it was diluted at 2.5 and 0.25%. WhenCupral was diluted in TSB or YPD media (a conditionused throughout the study to set up all the experiments), the pH values remained very high at concentrations of12.5 and 1.25% Cupral, while it decreased to a valueclose to neutrality at 0.125%. When selected samples were maintained in the presence of 5% CO_2 for 48 h, the pH decreased to values between 9 and 7, depending upon Cupral dilutions. Finally, pH values were measured inmicrobial cultures (*Staph. aureus*, *P. aeruginosa*, *C.albicans*) exposed or not to Cupral and then incubated for 48 h in the presence or absence of CO_2. We found that, inthe presence of CO_2, the pH values were lower

than those measured in the absence of CO_2, at all the conditions tested; such reduction was particularly evident at the Cupral 12.5% solution. The culture media alone showed pH values close to 7 regardless of the presence or not of CO_2 (data not shown).

Effect of Cupral on microbial growth

The antimicrobial activity of Cupral was evaluated in *Staph. aureus*, *P. aeruginosa* and *C. albicans*, by the CFU assay and the measurement of OD_{595}. Briefly, microbial cultures were exposed to Cupral at different concentrations (namely, 12.5, 1.25 and 0.125%) or diluent (controls) and then incubated, in the presence or absence of CO_2, up to 48 h. At time zero and at various time points, we measured the OD_{595} and the CFU, as detailed above. The results in Figs. 1 and 2 show the ΔOD kinetic values (left panels) and CFU/mL (right panels) of *Staph. aureus*, *P. aeruginosa* and *C. albicans*, treated with scalar doses of Cupral and incubated in the presence (Fig. 1) or absence (Fig. 2) of CO_2 for different times. We found that Cupral greatly affected both bacterial and fungal cells growth in a concentration-dependent manner; the phenomenon was evident with all the strains tested and mainly occurred at 24–48 h of incubation, both in presence and absence of CO_2. Particularly, in the presence of CO_2 (Fig. 1), *Staph.aureus*, *P. aeruginosa* and *C. albicans*, exposed to Cupral 0.125% behaved similarly to untreated controls, with a time-dependent growth occurring mostly from 24 h of incubation. Moreover, when using the 12.5% Cupral solution, the residual microbial load, evaluated either by OD or CFU assay, dropped to almost undetectable levels, irrespective of the time points assessed. The 1.25% Cupral solution caused intermediate, yet significant, inhibitory effects against all the microbial species tested. Figure 2 shows the results observed in the absence of CO_2. Once again, Cupral 0.125%-treated and untreated controls showed similar trends of growth, although some statistically significant differences were detected. On the other hand, the two Cupral concentrations (12.5 and 1.25%) strongly affected both bacterial and fungal growth, down to values close or even below the control.

According to the “EUCAST disk diffusion antimicrobial susceptibility testing method”, we next assessed the effects of Cupral, by using the halo inhibition test. Figure 3 shows a representative experiment with *Staph. aureus*, in which gentamicin

(10 μg/10 μL/disk) was used as positive control. An inhibition halo of about 13 mm was observed at the highest Cupral concentration (12.5%), while gentamicin returned a halo of 16 mm. No effects were observed with Cupral 1.25 and 0.125%.

Table 1. pH values of Cupral under different experimental conditions

Treatment[a]	pH values		
Distilled H_2O	Cupral 25%	Cupral 2.5%	Cupral 0.25%
	14	13	11

Treatment[b]	Cupral 12.5%	Cupral1.25%	Cupral 0.125%
TSB or YPD + CO_2	9	8.5	7
TSB or YPD - CO_2	13	12	7.5
S. aureus + CO_2	8.5	8.5	8
S. aureus - CO_2	13.5	9	8.5
P. aeruginosa + CO_2	8.5	8	7.5
P. aeruginosa - CO_2	12	9	8
C. albicans + CO_2	8	7.5	6.5
C. albicans - CO_2	12	8	7

[a]pH values of three different concentrations of Cupral in distilled water, [b]pH values of Cupral diluted in the growth media or in the presence of microbial cells and incubated for 48 h in presence or absence of CO_2.

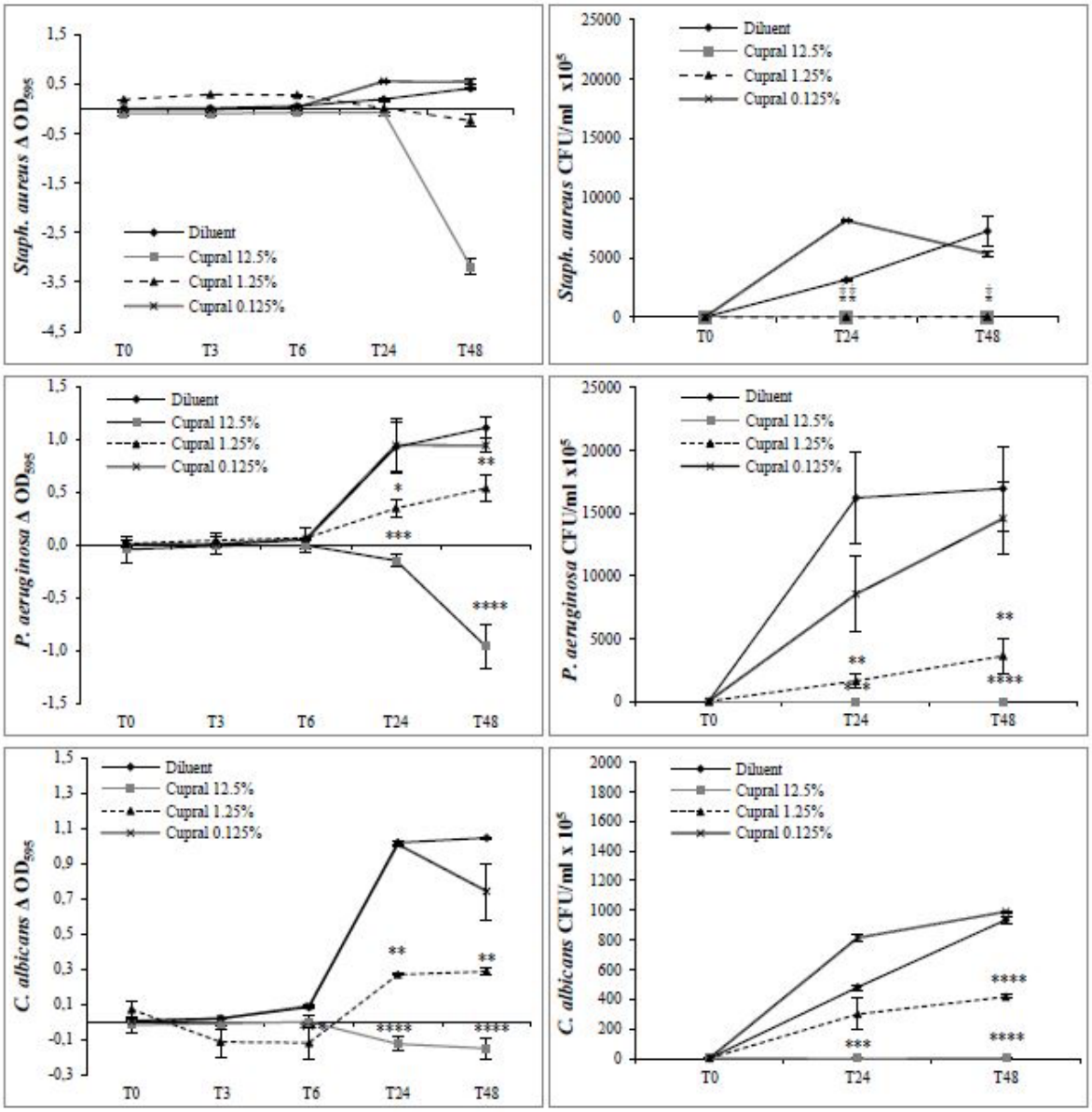


Fig. 1 Cupral effects on microbial growth in the presence of CO_2. Growth curves of *Staph. aureus*, *P. aeruginosa* and *C. albicans* were performed in the absence (diluent ♦) or presence of Cupral at 12.5% (■), 1.25% (▲) and 0.125% (×). Microbial cultures were incubated for 0, 3, 6, 24 and 48 h in the presence of CO_2. Then, the ODs were assessed and the values of ΔOD were calculated as difference between the OD of samples and their respective controls ± SEM (left panels). In parallel groups, microbial cultures were incubated for 0, 24 and 48 h in the presence of CO_2; then, the CFU/ml were evaluated (right panels). The depicted values represent the average of 6 replicates of 2 independent experiments. T: time (h). **p* ≤0.05, ***p* ≤0.005, ****p* ≤0.001, *****p* ≤0.0001.

Finally, the antimicrobial effect of Cupral was assessed by the evaluation of microbial cell duplication time (DT), operationally working during the exponential

growth phase (within 90–120 min). Figure 4 shows the DT values, expressed in minutes, of microorganisms exposed or not to Cupral. Our results show that *Staph. aureus* and *C. albicans* exposed to Cupral 12.5 and 1.25% failed to complete their cell replication cycle; under these same experimental conditions, *P. aeruginosa* DT was approximately 11,000 min. Lastly, the DT values of microbial cultures exposed to Cupral 0.125% returned values comparable to those obtained in their respective controls.

Effect of Cupral on P. aeruginosa biofilm formation

We investigated the ability of Cupral to interfere with biofilm formation through confocal microscopy, exploiting the fluorescent property of *Pseudomonas* spp. Figure 5 (left panel) shows a horizontal view of a control sample (24 h-old biofilm), characterized of cellular aggregates (green fluorescence areas) reaching a medium thickness of 50 ± 11 μm, as established by multiple acquisitions. Lateral views indicate multicellular layers abundantly distributed along the Z-axis. Also, dispersed areas likely consisting of nutrient and water transport channels were detectable (dark areas).

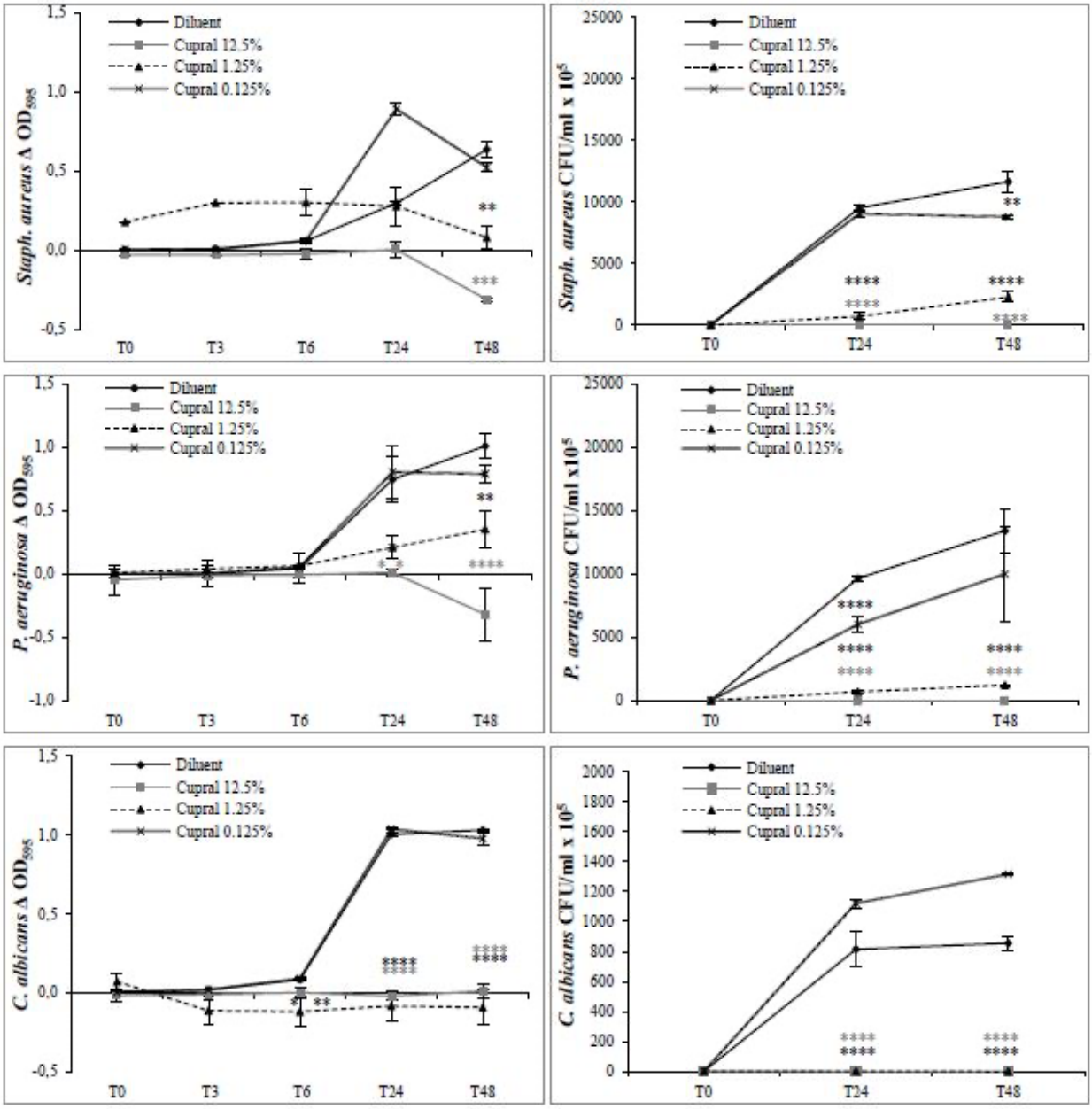


Fig. 2 Cupral effects on microbial growth in the absence of CO_2. Growth curves of *Staph. aureus*, *P. aeruginosa* and *C. albicans* were performed in the absence (diluent ♦) or presence of Cupral at 12.5% (■), 1.25% (▲) and 0.125% (×). Microbial cultures were incubated for 0, 3, 6, 24 and 48 h in the absence of CO_2. Then, the ODs were assessed and the values of ΔOD were calculated as difference between the OD of samples and their respective controls, ± SEM (left panels. In parallel groups, microbial cultures were incubated for 0, 24 and 48 h in the absence of CO_2; then, the CFU/mL were evaluated (right panels). The depicted values represent the average of 6 replicates of 2 independent experiments. T: time (h). *$p \leq 0.05$, **$p \leq 0.005$, ***$p \leq 0.001$, ****$p \leq 0.0001$.

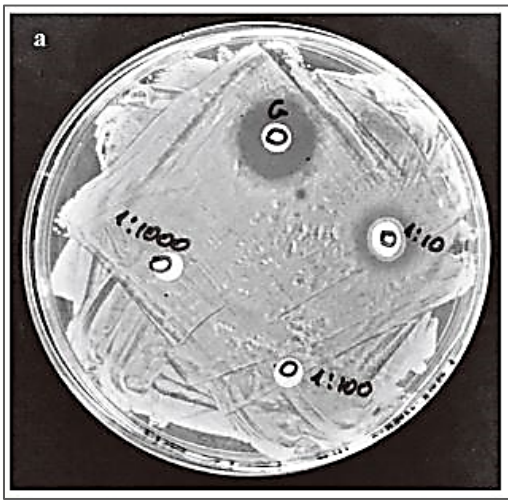
Figure 5 (right panel) is representative of *P. aeruginosa* bacteria exposed to Cupral 1.25% for 24 h; under this condition, only few scattered cells were evident with no tridimensional structure or measurable biofilm thickness.

Pyoverdine production by P. aeruginosa biofilm exposed or not to Cupral

To further investigate Cupral effects on biofilm, we assessed the release of pyoverdine, an important pigment for *in vivo* iron gathering and virulence expression in *P. aeruginosa*[22] and thus promotes the formation of biofilm[31]. As shown in Table 2, Cupral significantly impaired the pyoverdine production at 12.5 and 1.25% concentrations. In contrast, the pyoverdine levels in 0.125% Cupral solution were similar to control sample (biofilm cells); even in planktonic cells the pyoverdine production was very low.

Effect of Cupral on biofilm persistence

We investigated the effect of Cupral on pre-formed biofilm, using the three microbial strains; quantification of total (untreated controls) and residual (following Cupral treatment) biomass was evaluated, by CV assay (Fig. 6) and optical microscope morphological analysis (Fig. 7).



Treatment[b]	Halo diameter (mm)
Gentamicin 10 μg/ml (G)	16
Cupral 12.5% (1:10)	13
Cupral 1.25% (1:100)	no effect
Cupral 0.125% (1:1000)	no effect

Fig. 3 Inhibition haloes in *Staphylococcus aureus* exposed to Cupral. Antibacterial activity of Cupral was assessed by disk diffusion assay. a) *Staph. aureus* cells (1×10^5 cells) were inoculated on plates of Mueller-Hinton agar. Neutral filter paper disks were placed onto the plates surfaces and embedded with Cupral (10 μL/disk: 12.5, 1.25 and 0.125%) or gentamicin, as positive control

Antimicrobial and antibiofilm efficacy of a copper/calcium hydroxide-based endodontic paste against *Staphylococcus aureus, Pseudomonas aeruginosa* and *Candida albicans*
Dental Materials Journal 2019; 38(4): 591–603. doi:10.4012/dmj.2018-252

(10 μg/10 μL/ disk). The plate was examined after 24 h of incubation at 37 °C in absence of CO_2; the disk diffusion haloes were measured and the diameters (mm) reported in lower panel (b).

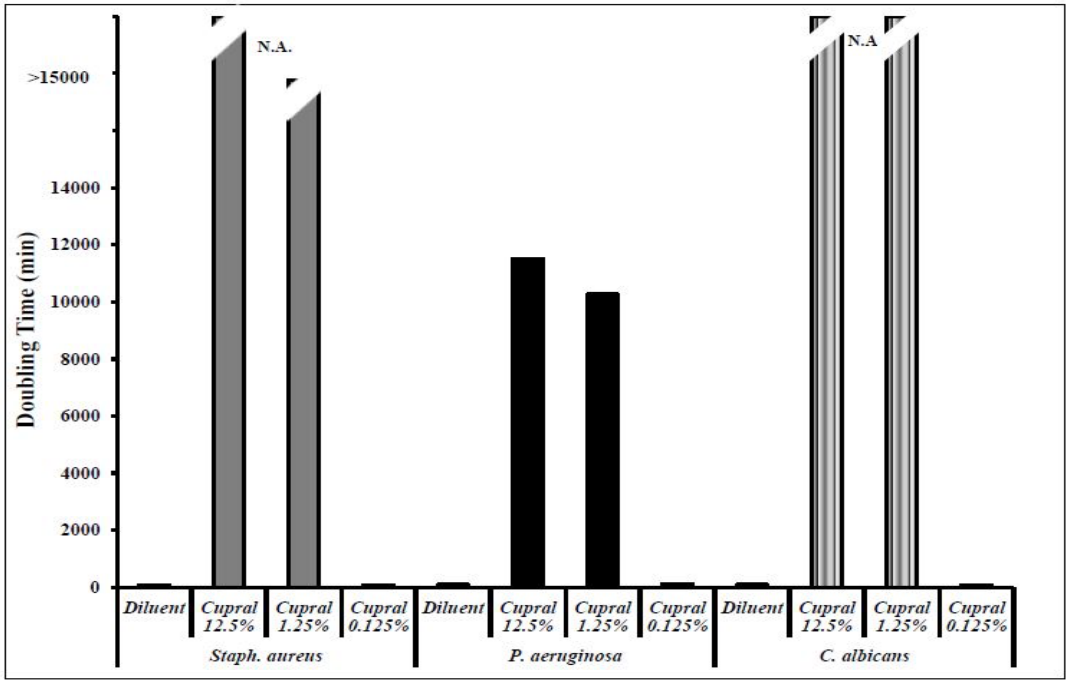


Fig. 4 Cupral effects on microbial doubling time (DT). Inhibitory effects of Cupral on cell growth was calculated as DT of exponentially growing microbial cells exposed or not to Cupral and tested by CFU assay. The DT expressed in minutes were calculated using the formula described in materials and methods. **N.A.:** no assessable.

Briefly, microbial cells were incubated for 24 h in 96 well plates in CO_2 to allow biofilm formation; then, medium or Cupral was added and a further 24 h incubation performed. As shown in Fig. 6, Cupral significantly reduced microbial biomass; the effect was concentration-dependent and similar trends were observed in all the 3 strains tested. In particular, the percentages of biomass reduction ranged from 47 to 94% for *Staph. aureus*, from 28 to 95% for *P. aeruginosa* and from 27 to 75% for *C. albicans*.

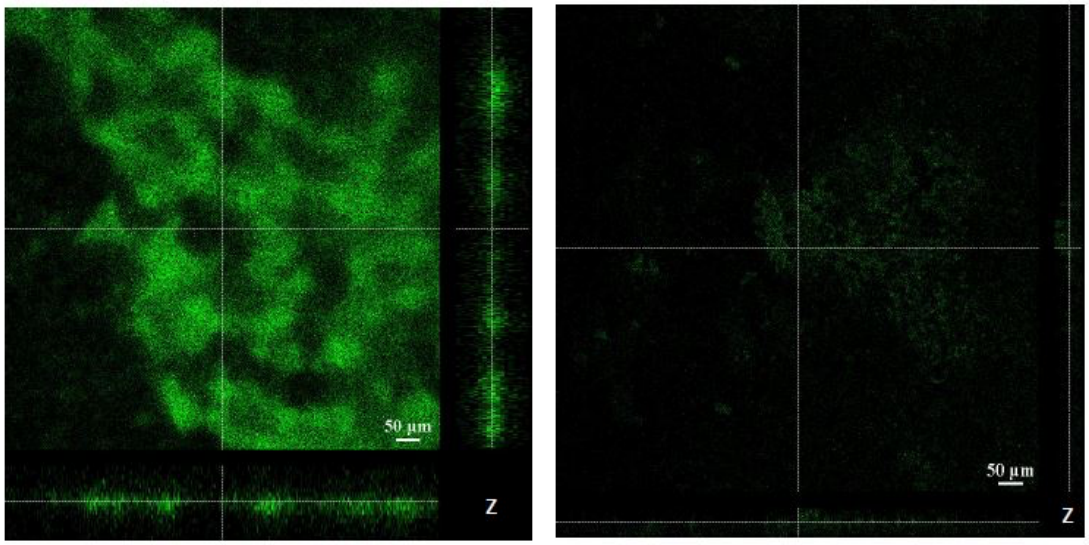


Fig. 5 Confocal microscopy of *P. aeruginosa* exposed or not to Cupral. *P. aeruginosa* biofilm treated or not with Cupral was observed through confocal microscope, at 10× magnification, exploiting the auto-fluorescent property of *Pseudomonas* spp. The main images illustrate two representative horizontal sections (X and Y axes) of the biofilms, while the thin images (laterally and below each main image) indicate biofilms sections observed along the Z axis. The left panel refers to 24 h control biofilm and the right panel to *P. aeruginosa* exposed to Cupral 1.25% for 24 h.

Table 2. Pyoverdine production by *P. aeruginosa* biofilm treated or not with Cupral

	Levels of pyoverdine	
Treatment	RFU ± SEM	*p* values
Untreated biofilm (diluent)	631475 ± 4152	
Cupral 12.5%	202586 ± 7446	≤ 0.00001
Cupral 1.25%	547174 ± 18389	0.06
Cupral 0.125%	764866 ± 1850	Ns
Untreated planktonic cells (diluent)	188963 ± 7211	0.0003

Pyoverdine production (RFU) in *P. aeruginosa* untreated planktonic or biofilm-associated cells exposed for 24 h, to three different concentrations of Cupral. Statistical analysis was performed by comparing the levels of pyoverdine produced by Cupral-treated vs. untreated biofilm cells (diluent). Untreated biofilm *vs* untreated planktonic cells were also statistically evaluated; **Ns:** non-significance

Moreover, Fig. 7 shows representative microscopy images of 24 h-old biofilm of *Staph. aureus*, *P. aeruginosa* and *C. albicans*, in the presence of CO_2, exposed or not to Cupral 1.25% for additional 24 h. As expected, control microbial biofilms (left panels), showed abundant sessile structure; conversely, in parallel samples

exposed to Cupral 1.25%, a thin layer of damaged microbial cells was detectable with scant and heterogeneous debris, probably Cupral derived aggregates (right panels, arrows).

Effect of Cupral on alive/dead cells embedded in microbial biofilm

Finally, we investigated the impact of Cupral on viability of preformed microbial biofilms (Fig. 8). In detail, 24 h-old microbial biofilms were exposed to Cupral 1.25% for additional 6 (Figs. 8a and b) or 24 h (Figs. 8c and d) and then stained with CFDA and PI, to discriminate living from dead cells directly inside biofilms. Cupral treatment reduced the number of alive bacterial cells embedded into biofilms, in all the cases, although reaching significant differences only in *Staph. aureus* at 6 (Fig. 8a) and in *C. albicans* at 24 h (Fig. 8c). Accordingly, when expressing the data as live/dead percentage, we found that the percent of dead cells was consistently higher in Cupral-treated samples than in control groups, at both time points, both in *Staph. aureus* and *C. albicans* biofilms (Figs. 8b and d); as opposed, *P. aeruginosa* biofilm showed higher dead cell percent in the diluent samples than in Cupral-treated samples, at both times (Figs. 8b and d).

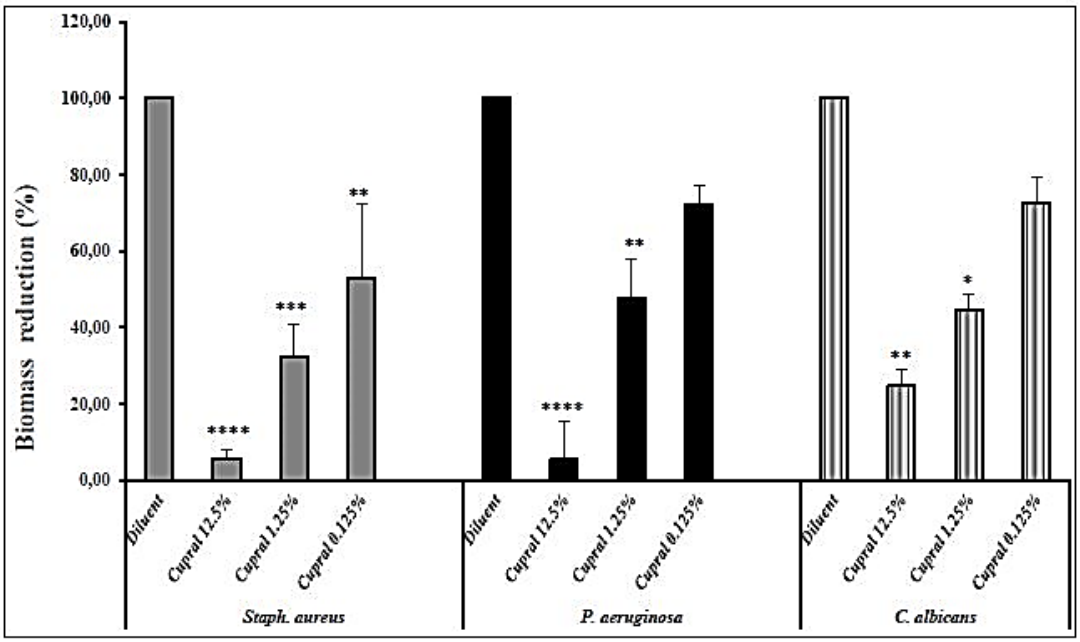


Fig. 6 Cupral effects on biofilm persistence. The effects of Cupral were assessed on pre-formed biofilms by *Staph. aureus*, *P. aeruginosa* and *C. albicans* by CV colorimetric analysis. Two wavelengths were used: 570 nm for microbial biofilms and 540 nm for fungal biofilm. The biomass

reduction in Cupral-treated groups was expressed as % of residual biofilm compared to controls (100%). The values represent the average of 6 replicates of 2 independent experiments ± SEM. **p* ≤0.05, ***p* ≤0.005, ****p* ≤0.001, *****p* ≤0.0001.

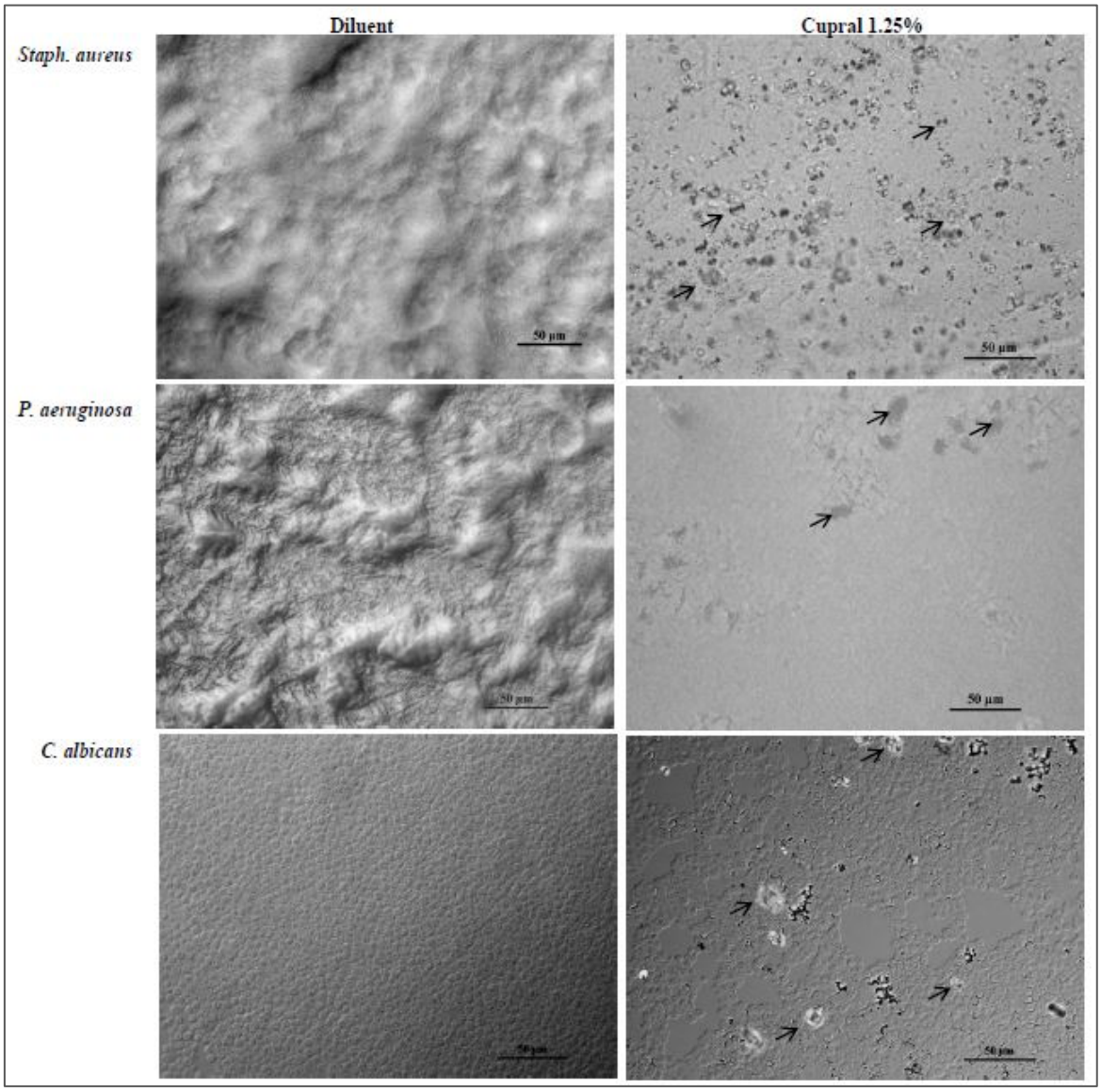


Fig. 7 Morphology of preformed biofilm treated or not with Cupral. *Staph.aureus*, *P. aeruginosa* and *C. albicans* were allowed to produce biofilm by incubation 24 h on coverslips in the presence of CO_2; then biofilms were exposed or not to Cupral 1.25% for additional 24 h. Morphology was evaluated by optical microscope. The left panels show representative images of 48 h microbial biofilms in medium. The right panels illustrate biofilms exposed to Cupral 1.25%. The bars in Fig. indicate 50 µm. The arrows indicate aggregates/debris.

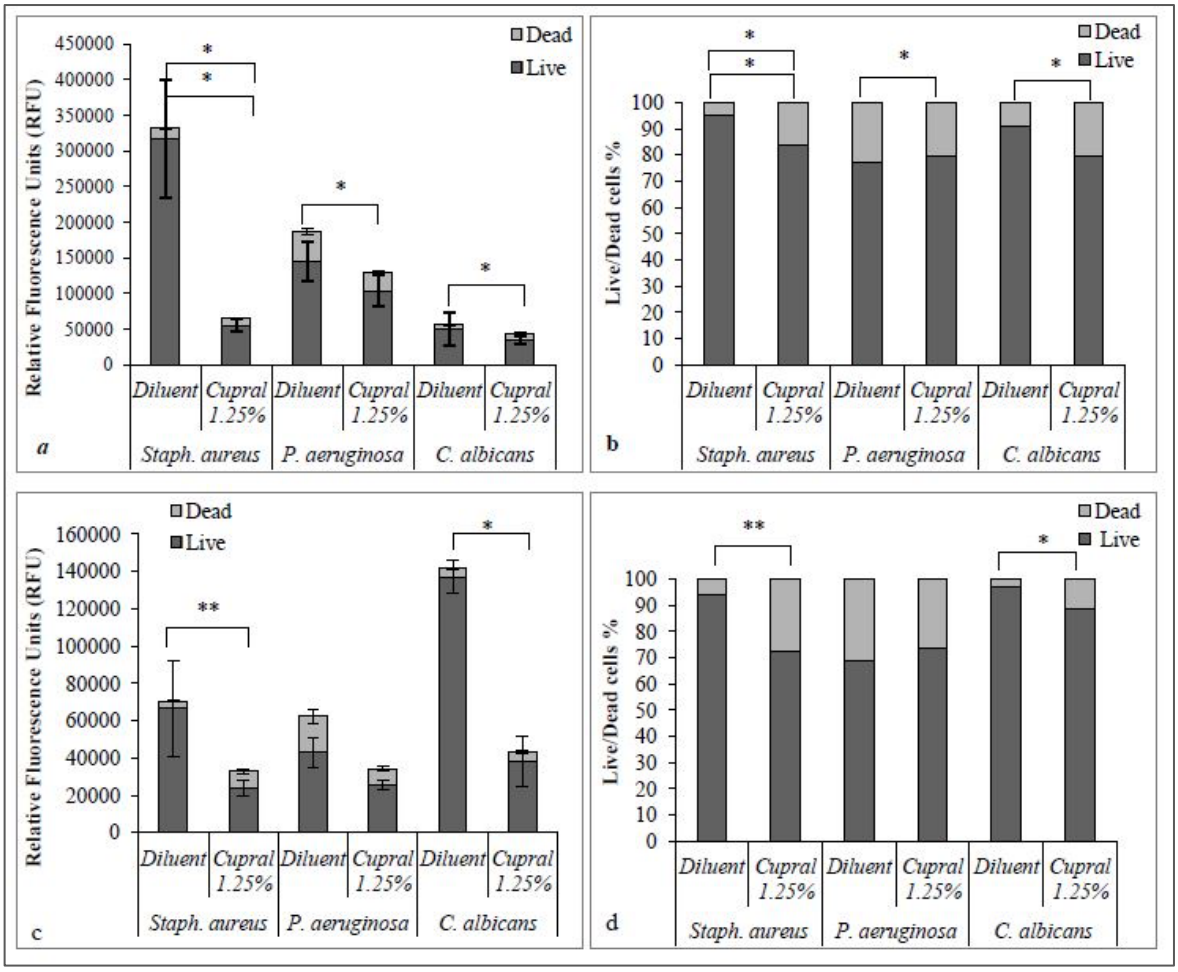


Fig. 8 Cupral effects on biofilm vitality. Left panels: Relative Fluorescence Units (RFU) mean of live (dark grey) and dead (light grey) cells was determined in *Staph. aureus*, *P. aeruginosa* and *C. albicans* biofilms (24 h), treated or not with Cupral 1.25% for additional 6 h (a) and 24 h (c). Right panels: percent of live and dead cells in each group at 6 h (b) and 24 h (d). The data shown are representative of two experiments with three replicates. The dotted lines refer to the dead cells, while the continuous lines refer to the live cells. **p* ≤0.05, ***p* ≤0.01.

DISCUSSION

In the present study, the antimicrobial capacity of Cupral has been assessed using microorganisms, such as *Staph. aureus* (Gram+), *P. aeruginosa* (Gram−), and *C. albicans* (fungus), known to commonly colonize human oral cavity, where they produce biofilm onto biotic and abiotic surfaces. Moreover, such microorganisms are considered among the most resistant species detectable in infected root canals, thus being often associated with endodontic treatment failures[4].

The formation and development of biofilm is a process that begins with adhesion of microorganisms as planktonic forms, followed by growth, extracellular polymeric matrix production, detachment and readhesion, in a dynamic and continuous reorganization that leads to the formation of a sessile structure[32]. Of clinical importance is the fact that, once produced, biofilm is extremely resistant to common disinfectants, antimicrobial drugs and host immune defenses. In particular, endodontic failures often occur when permanent sterility cannot be achieved inside treated root canals[33] especially if biofilm has been produced. Accordingly, a crucial aim of root irrigation and disinfection with antimicrobial products is the elimination of microbial cells, even if organized as biofilm, particularly in intra-canals surfaces not easily reached by endodontic instruments[34]. Sodium hypoclorite (NaOCl) is a frequently used as irrigating solution in endodontics because of its ability to dissolve necrotic tissues as well as its potent antimicrobial action[35]; yet, caustic and toxic effects to vital tissues are often noted. Other compounds, such as chlorhexidine (CHX)[7] and cetrimide, are less effective than NaOCl in eradicating for instance *Enterococcus faecalis* biofilm[35]; nevertheless, CHX[7,9], has the ability to inhibit bacterial adhesion to dentin. Furthermore, the use of calcium hydroxide as an intracanal medication has been associated with periradicular healing and high antibacterial efficacy[36], this basic formulation in clinical practice can be associated with other antimicrobial molecules such as iodoform[37], propolis[38,39], royal jelly[40] or copper hydroxide[27,28], as in Cupral composition. Cupral is a suspension based on copper-calcium-hydroxide, with bactericidal effects due to alkaline proteolysis, as reported by Knappwost[27]; on these bases, it is expected that, *in vivo*, when entering into contact with irrorated tissues, it reacts with buffer solution of carbonic acid/hydrogen carbonate of the blood and forms a calcium carbonate mineral membrane that increases overtime in thickness to the borderline of blood-supplied tissues. In turn, this membrane prevents the entry of OH^- ions into the tissues, thus neutralizing the acid environment of the inflamed site providing analgesic effects[27]. Furthermore, it is likely that calcium hydroxide, present in Cupral paste, reacts with copper sulphate in alkaline environment to form copper hydroxide and possibly depending on pH variation, the reaction and dissociation between calcium

hydroxycuprate and copper hydroxide may occur[27]. Our present study provides the first evidence on the *in vitro* efficacy of Cupral on growth of planktonic microorganisms and on their ability to produce and persist as biofilm. In particular, by spectrophotometric and CFU assay, we demonstrate the antimicrobial effects of Cupral against bacterial and fungal planktonic cells. Interestingly, the efficacy of Cupral has been demonstrated in the presence and absence of CO_2. The rational for including CO_2 in our study derives from the fact that such a weak dioxide acid is expected to reduce the pH, by reacting with copper and calcium hydroxide present in Cupral. Thus, we assume that, because of such acidic property, CO_2 tends to mimic the *in vivo* environment where microorganisms grow releasing acid products; under such conditions, Cupral has been found to exert its antimicrobial activity.

We demonstrate that Cupral drastically affects microbial cells interfering with their cell cycle in a dose-dependent manner; indeed, the time of microbial cell replication drastically augments, irrespective of the microorganisms investigated; particularly, *Staph. aureus* and *C. albicans* exposed to Cupral 12.5 and 1.25% become unable to complete their cell-cycle, while *P. aeruginosa* appears somehow less affected. These results are in line with what observed in terms of microbial cell growth and biofilm formation, namely both CFU and OD values are significantly reduced in microorganisms treated with Cupral with respect to their relative controls, again, the observed trends are dose-dependent and have been detected in the presence or absence of CO_2, thought to a different extent. CO_2 partially reduces Cupral efficacy; indeed, when used at 1.25%; the CFU and OD levels of Cupral-treated *C. albicans* are higher in the presence than in the absence of CO_2. Thus, under acidic conditions closely mimicking the *in vivo* situation (addition of CO_2), Cupral happens to be not as effectiveness; yet, growth of microbial cells exposed to 10 times diluted Cupral (1.25%) still remains much below the control levels. The antimicrobial activity of Cupral has also been demonstrated by the disk diffusion test, where the inhibition halo of *Staph. aureus* exposed to Cupral 12.5% is very close to that obtained with gentamicin (13 *vs* 16 mm), used as a positive control. We are aware that the disk diffusion may not be the most appropriate assay to assess Cupral that, as a gelatinous formulation, diffuses with difficulty and in an uneven manner within

constituted MH agar meshes (culture medium used in the assay); yet, the row data produced strengthen the efficacy of Cupral as antimicrobial compound.

By a previously established model[41], here we provide initial evidence on the efficacy of Cupral against preformed microbial biofilms. A marked inhibitory effect has been observed with respect to each of the three microorganisms tested, as shown by the significant and dose-dependent reduction of microbial biomass. It should be noted that *Staph. aureus* biofilm is still affected at the lowest concentration of Cupral (0.125%), when pH is close to neutrality. On this basis, we can speculate that Cupral acts not only via its high alkalinity, but also because of the presence of elements such copper, known to exert antimicrobial activity[42]. In fact, copper is an essential nutrient for both human and microbial cells, while, at high doses, it can exert toxic effects. The exact mechanism(s) of copper action is not clear, yet; many hypotheses have been formulated, including a) loss of potassium or glutamate through the outer cell membrane, b) interference in the osmotic equilibrium, c) binding to proteins that do not require copper ions, d) oxidative stress induction with hydrogen peroxide generation[42,43]. It is worth noting that the Cupral effective dose of 1.25% contains approximately 350 μg/mL of copper ions, a value very close to the MIC previously established for copper against *Staph. aureus*[44]; to our opinion, this strengthens the hypothesized antimicrobial role of such ion in our model.

Old data[27] ascribe to Cupral some cytotoxicity *in vitro* (undiluted paste onto murine 3T3 fibroblasts, for 24 h); yet, no clinical evidence exists on its *in vivo* toxic or side effects. An obvious advantage of using our *in vitro* system is the possibility of easily testing serial dilutions. The demonstration that 10- and 100-fold diluted Cupral still retains relevant antimicrobial effects opens to novel formulations, that optimizing the dosage of the active principles, may implement clinical treatment.

Confocal microscopy of a 24 h-old *P. aeruginosa* biofilm shows that Cupral 1.25% prevents biofilm formation, as demonstrated by the absence of a threedimensional structure, while cellular aggregates and water channels are expected and easily detectable in control samples. Also, optical microscopy of preformed biofilm of *Staph. aureus*, *P. aeruginosa* and *C. albicans*, treated with Cupral 1.25% for additional 24 h, points out major differences in morphology between treated and

untreated biofilm, with lack of a three-dimensional architecture and little residual cells in Cupral-treated samples. In line with these results, further data, obtained by a "live/dead cell" staining, reveal that Cupral 1.25% reduces the number of alive cells embedded in biofilms, both at 6 and 24 h of treatment, no matter whether considering bacterial or fungal biofilms. In parallel, the number of dead cells is higher in Cupral-treated samples as compared to their respective control groups, both in *Staph. aureus* and *C. albicans*; on the contrary, *P. aeruginosa* biofilm treated with Cupral has fewer dead cells as compared to its control. This last result can be interpreted considering the contribution of extracellular DNA (eDNA). Notoriously, eDNA is abundantly accumulated during *P. aeruginosa* biofilm formation by lysis of a subpopulation of bacteria[23,24], by mechanisms dependent on acyl homoserine lactone (autoinducer 1) and quinolone signalling during quorum sensing attainment[45]. Thus, eDNA may initially facilitate bacterial adhesion and cell-to-cell aggregation; then, as an important component of biofilm matrix, eDNA enhances stability of the sessile community and strengthens its resistance to antibiotics or detergents[46]. We can envisage that the increased PI RFU observed in *P. aeruginosa* biofilm may be due to eDNA accumulation, in turn possibly protecting cells from Cupral's action. Another quorum sensing-mediated phenomenon, in *P. aeruginosa* biofilm, is pyoverdine production. Our study shows that pyoverdine levels in untreated planktonic cells result very low in comparison with those observed in biofilm cells; interestingly, the latter are inhibited in a dose-dependent fashion by Cupral. Taken together, these findings add further evidence on the ability of Cupral to prevent *Pseudomonas* production and persistence biofilm.

CONCLUSION

Currently, Cupral is an endodontic paste used in root treatment and, more recently, also in gingivitis and indirect covering of dental pulp, with the aim of obtaining a clinical improvement. Here, we provide *in vitro* novel insights on the antimicrobial efficacy of Cupral, demonstrating its ability to affect *Staph. aureus*, *P. aeruginosa* and *C. albicans* as well; growth of planktonic cells is impaired, biofilm formation is prevented and preformed biofilm is damaged. Thus, our results suggest

that Cupral, a promising clinically used compound, may be a good candidate for treatment of oral infections, including biofilm-associated ones, irrespective of the fact that a Gram+, Gram− or fungal cell maybe the causative agent.

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CHAPTER 2

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CASE REPORT

Efficacy of a Copper–Calcium–Hydroxide Solution in Reducing Microbial Plaque on Orthodontic Clear Aligners: A Case Report

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Keywords: clear aligners, copper–calcium–hydroxide, orthodontic therapy, microbial plaque

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Abstract

The aim of this study was to investigate the ability of a copper–calcium–hydroxide-based compound to remove microbial plaque naturally produced onto orthodontic clear aligners. A commercially available dental paste, named Cupral, based on copper–calcium–hydroxide, was used. A healthy volunteer (female, 32 years old), undergoing orthodontic treatment with thermoplastic clear aligners was enrolled. By conventional/confocal microscopy and colony-forming unit (CFU) assay, 2-week used aligners were examined for microbial plaque, prior and following exposure to Cupral. Confocal microscopy revealed abundant plaque irregularly distributed onto the aligner surface. Following Cupral treatment, a drastic decrease occurred in plaque thickness and matrix presence. As assessed by the CFU assay, total microbial load approached 10^9 CFUs/aligner, with slight differences in aerobiosis and anaerobiosis culture conditions; six macroscopically different types of colonies were detected and identified by matrix-assisted laser desorption ionization-time of flight mass spectrometry. Following Cupral treatment, microbial load dropped to undetectable levels, irrespectively of the conditions considered. Exposure of clear aligners to Cupral results in the elimination of contaminating microorganisms; the antimicrobial activity is retained up to 1.25% concentration. Overall, our data describe a novel use of Cupral, a copper–calcium–hydroxide-based compound, in daily hygiene practices with promising results.

EUR J DENT 2019 Jul;13(3):478-484. doi.org/ 10.1055/s-0039-1695653

Introduction

Notoriously, the conventional orthodontic treatment with stainless appliances[1] is related to reduce patient's ability to maintain oral hygiene increasing the risk of periodontal disease via accumulation of microbial plaque.[2-4] Plaque-embedded microorganisms activate several genes involved in virulence factors[5,6] reducing susceptibility to detergents or antibiotics as well as to host-mediated immune defenses.[7-10]

Recently, clear aligners use in dentistry allows the gradual move of teeth into an ideal position (computerized technology), minimizing microbial risk,[11,12] dental trauma, and apical resorptions.[4] The orthodontic protocol includes 20/24 hours aligners use, removal during meals or sugar containing drinking beverages, followed by tooth and device brushing before re-wearing.[11,12] A helpful method to visualize microbial and device-associated plaque is through the use of disclosing agents, which stain microbial pellicle.[13,14]

Cupral (a copper–calcium–hydroxide-based compound) is a dental paste clinically used in periodontal infections, being endowed with antimicrobial activity.[15,16] Moreover, recent clinical experience demonstrates that Cupral efficiently counteracts endodontic infections.[17,18] Yet, its mechanisms of action have not been investigated; neither data exist on Cupral use in other dentistry fields.

The purpose of this pilot study was to investigate the ability of Cupral to remove microbial plaque, naturally produced onto orthodontic aligners. Briefly, clear aligners, regularly used by a healthy volunteer undergoing standard orthodontic therapy, were exposed or not to Cupral and assessed for total and residual microbial population, by quali-quantitative analysis.

Case Report

A healthy volunteer (female, 32-year old) undergoing orthodontic treatment for 12 months with thermoplastic clear aligners was enrolled. She started treatment on December 2017 using the Nuvola (Rome, Italy) system, that included 8 months of active treatment (replacing the aligners every 2 weeks) and 4 months of stabilization period (replacing the aligners every 2 months), the latter known as limited retention before the fixed retention applied from the clinical orthodontist. The volunteer was

informed about the nature of the study, performed on individual basis that did not directly involve her as a patient, but only the used aligners at the time of their disposal. She was asked to provide the upper and lower aligners pieces, on winter and summer months of 2018. In particular, at the times when the aligners had to be replaced with new pairs, the used ones were carefully removed from the mouth using sterile gloves and inserted in a bottle with sterile physiological saline solution (200 mL); then, the bottle was delivered to the Microbiology Laboratory for microbiological analysis. Totally, six couples (upper and lower) of 2-weeks-used aligners were provided, focusing on winter and summer months (January–March and June–August).

Cupral

We used a commercially available compound (HUMANCHEMIE GmbH; Alfeld, Germany), containing highly dispersed calcium and copper hydroxide. A starting solution of Cupral was prepared by diluting 1 g of compound in 4 mL of distilled water (weight/volume 25%) sterilized by autoclave, and stored at 4 °C prior to be used in the study. Operationally, Cupral's solution was diluted in culture medium or microbial cultures up to the final working conditions (1.25%).

Confocal Microscopy on Cupral-Treated and Untreated Aligners

The aligners were washed with phosphate-buffered saline (PBS) at room temperature and then treated for 30 to 60 seconds with plaque disclosing tablets (MGS GAP Research Co, Ltd, Northfleet, Kent, United Kingdom) containing erythrosine red, to stain microbial plaque. Then, the aligners were further washed and brushed using running water, to mimic the daily hygiene practice. After this step, the upper and lower aligner were split in two equal pieces (right and left), transferred in two different bottles and processed in parallel; the right half upper and lower aligners were maintained in saline buffer (control group), while the left half upper and lower aligners were treated with Cupral 1.25% (treated group), the procedure was done for 1 hour at room temperature. Finally, all the pieces were brushed again, processed using standard procedures and analyzed by transmitted illumination confocal microscopy (Nikon LV 150 Confovis Microscope, Japan).

Microbiological Analysis on Clear Aligners Exposed or Not to Cupral

The procedure is detailed in ►Fig. 1. The clear aligners were split in two equal parts and inserted in two sterile bottles, one containing saline buffer and the other Cupral 1.25%. After an hour at room temperature, both bottles were sonicated at 3500HZ for 15 minutes to disaggregate microbial plaque. Then, the suspension was seeded uniformly onto two series of tryptic soy agar (TSA; Oxoid, Milan, Italy) and Sabouraud (SAB) plates (100 µL/plate in triplicates), to assess total bacterial and fungal load, by colony-forming unit (CFU) assay. Two sets of TSA and SAB plates were prepared to assess microbial growth in aerobiosis and anaerobiosis conditions. Following 24 hours of incubation at 37 °C, the number of colonies/plate were counted and the results were expressed as mean of CFUx10^7/aligner.

According to macroscopic aspect, namely color, morphology and size, the colonies were clustered and counted. Then, to identify the isolates, representative colonies from each typology were subcultured in Columbia agar plates (OXOID; Milan, Italy); the growing colonies were then Gram stained, observed by optical microscope and identified by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS, Biomerieux, France).

Optical Microscopy of the Isolated Colonies

Representative colonies, isolated after 24 hours incubation in TSA or SAB, were observed and photographed by inverted light microscope (Nikon Eclipse TS 100, Japan).

Results

Cupral Effects on Microbial Plaque Produced onto Clear Aligners: Confocal Analysis

To investigate the structure of biofilm produced on the aligners, we performed a topographic analysis (three-dimensional [3D] mapping) by transmitted-illumination confocal microscopy, before and after Cupral treatment. ►Figure 2 (upper panel) shows the overall architecture of biofilm on untreated aligner; microbial plaque and polysaccharide matrix were easily detectable (A). To evaluate biofilm thickness, a 3D

EUR J DENT 2019 Jul;13(3):478-484. doi.org/ 10.1055/s-0039-1695653

reconstruction of biofilm structure was obtained from the same capture, by means of a mathematical linearization of the sample (B); according to the chromatic scale (C), the plaque thickness in the untreated device varied from 4.5 to 8 μm, as indicated by the yellow, red, and white colors.

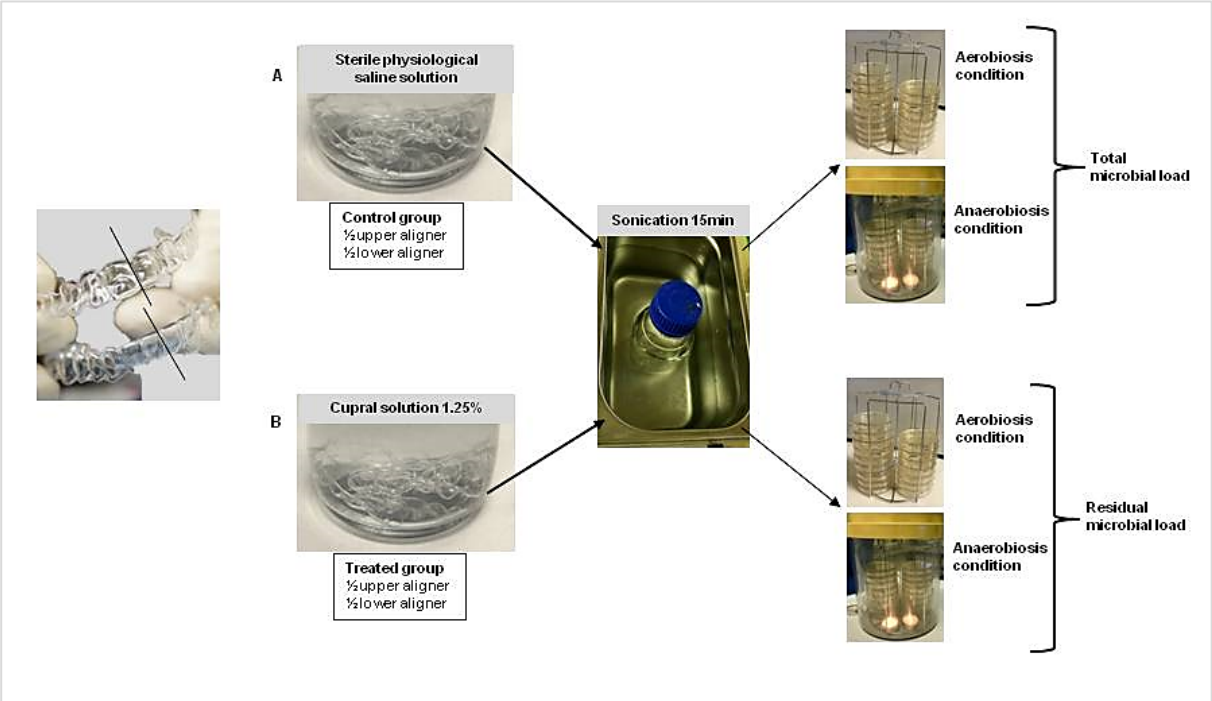

Fig. 1. Flowchart of the microbiological analysis performed on clear aligners. Two-weeks-used clear aligners (upper and lower) were split in two equal parts. A part was maintained in saline buffer (A), while the other was exposed to Cupral 1.25% (B); both groups were incubated for 1 hour and sonicated for 15 minutes. The supernatants were harvested, diluted, and plated on TSA and SAB plates; each set of plates was incubated, under aerobiosis or anaerobiosis conditions, for 24 hours. Total and residual microbial loads were evaluated by colony-forming unit analysis; each group was tested in triplicate plates.

EUR J DENT 2019 Jul;13(3):478-484. doi.org/ 10.1055/s-0039-1695653

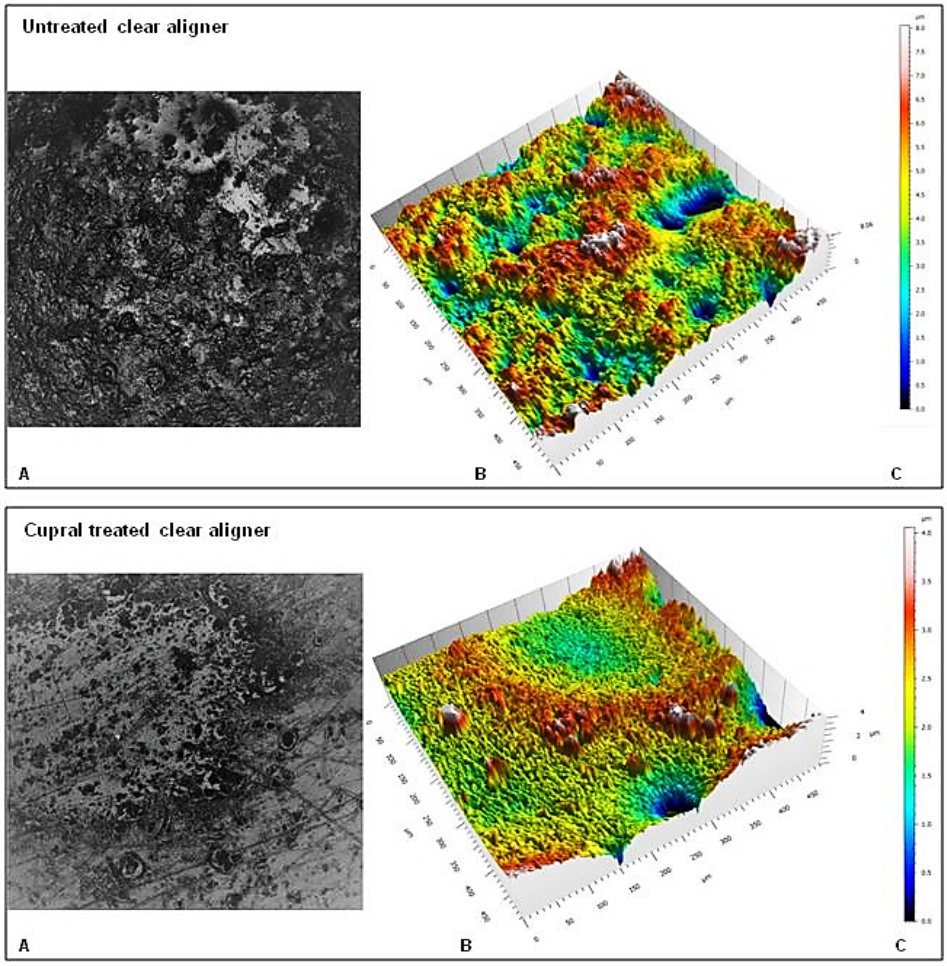

Fig. 2 Confocal microscopy of microbial plaque on orthodontic clear aligner. The upper panel shows a representative image of the untreated clear aligner; an abundant microbial biofilm is observed (A), as confirmed by the three-dimensional (3D) reconstruction of the same capture (B), according to the thickness scale (C). The lower panel shows a representative image of the Cupral-treated aligner; the device surface with minimal residual cells is observed (A), as confirmed by the 3D reconstruction of the same capture (B), according to the thickness scale (C).

The lower panel shows an image from Cupral-treated aligner (1 hour with 1.25%); only a scant 3D structure with minimal polysaccharide matrix was detected on the surface of the device (A). Furthermore, the 3D reconstruction (B) indicated the predominance of peaks chromatically quite uniform (mostly green and yellow colors), indicating a reduced thickness that, based on the reference scale (C), was around 1 to 1.5 µm, in several areas.

Cupral Effects on Clear Aligners-Associated Microbial Load: CFU Evaluation and MALDI-TOF Identification

Microbial load was quantified in clear aligners, used for 2 weeks, during winter (►Fig. 3) and summer months (►Fig. 4). In particular, ►**Figure 3A** indicates the total (untreated aligners) and residual (Cupral treated aligners) microbial load, using TSA and SAB medium in aerobiosis and anaerobiosis conditions. The colonies obtained in TSA ranged around 200 to 400 CFUx10^7/aligner in both conditions. In contrast, no microbial growth was detected in SAB plates in aerobiosis condition, while only few colonies were counted in anaerobiosis condition. Following Cupral treatment, no residual microbial load was ever observed, at any of the conditions tested. ►**Figure 3B** depicts the isolated species, expressed in percent with respect to the total number of colonies. While no growth occurred in SAB under aerobiosis conditions, few colonies were detected in anaerobiosis and were identified as *Rothia aeria* and *Streptococcus sanguinis*.

EUR J DENT 2019 Jul;13(3):478-484. doi.org/ 10.1055/s-0039-1695653

A

	Aerobiosis condition		Anaerobiosis condition	
Medium	TSA	SAB	TSA	SAB
Total microbial load (Untreated aligners)	397.0	n.d.	241.0	8.5
Residual microbial load (Cupral treated aligners)	n.d.	n.d.	n.d.	n.d.

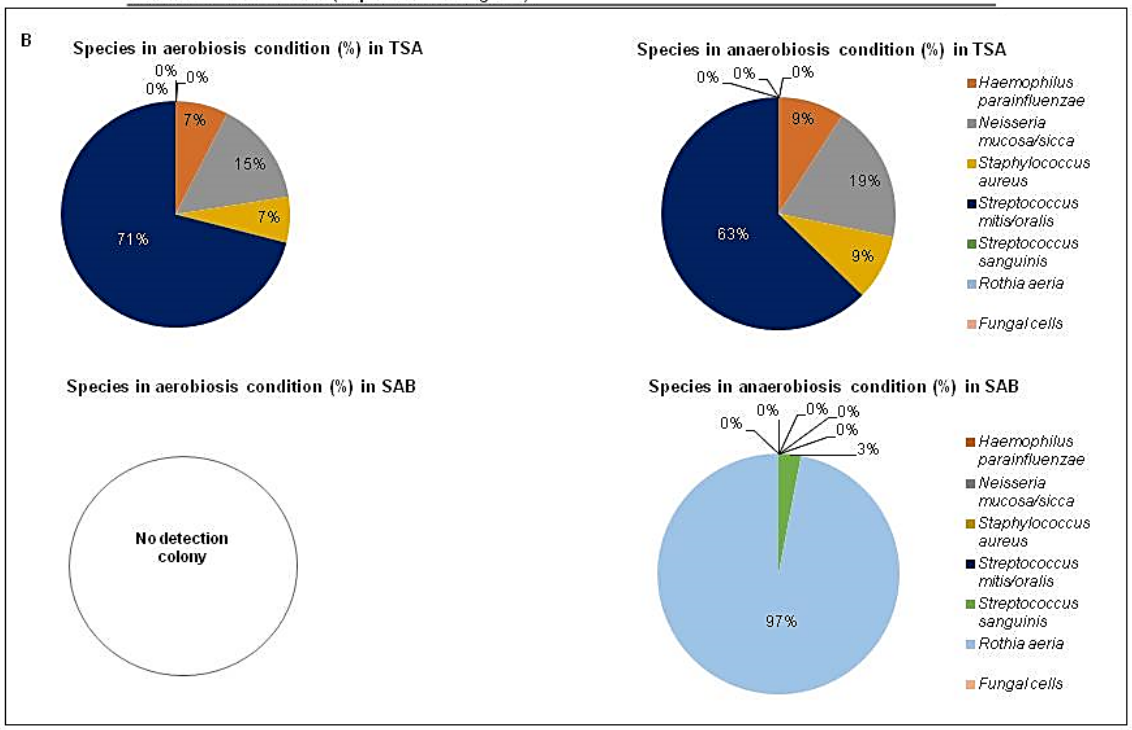

Fig. 3 TSA Total and residual microbial load detected on winter months. The clear aligners were exposed or not to Cupral 1.25%. The colonies isolated after incubation in TSA or SAB medium were counted and clustered. The upper table shows the total and residual microbial load expressed as colony-forming units/aligners. The values represent the mean of three independent analyses, where each sample had been assessed in triplicate. The standard errors, less than 10%, are omitted. The lower pie graphs represent the percent of species detected in aerobiosis and anaerobiosis conditions. **n.d.**: not detected.

As detailed in ►**Fig. 3B**, **S***treptococcus mitis/oralis* was the most common isolated species in TSA, both under aerobiosis and anaerobiosis conditions (71% and 63%, respectively).

►**Figure 4A** shows the data obtained analyzing the aligners during summer times. The CFU/aligner in TSA ranged around 160 to 210 CFUx10^7/aligner, while in SAB, the CFU values varied from 1.5 to 23 CFUx10^7/aligner, in both conditions. Also, in this case, following Cupral treatment, no residual microbial load was ever observed, at any of the conditions tested. ►**Figure 4B** displays the isolated species, expressed

in percent, with respect to the total number of colonies. Here, once again *Streptococcus mitis/oralis* was found as the most abundant isolated species (96% of colonies in TSA aerobiosis and 84% in TSA anaerobiosis, 99% in SAB aerobiosis and 100% in SAB anaerobiosis).

According to morphology, color and size, the isolated species were clustered in six different types of colonies, as depicted in ►**Fig. 5**. In particular, the colonies grown on aligners used during winter months included Gram-negative coccobacilli and diplococci, Gram-positive cocci and streptococci. On aligners used during summer months, we observed a similar trend with the most representative isolates being Gram-positive streptococci. By MALDI-TOF analysis, they were identified as *H. parainfluenzae*, *N. mucosa/sicca*, *S. aureus*, *S. mitis/oralis*, *S. sanguinis*, and *R. aeria*.

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A

	Aerobiosis condition		Anaerobiosis condition	
Medium	TSA	SAB	TSA	SAB
Total microbial load (Untreated aligners)	165.5	1.5	210.4	22.6
Residual microbial load (Cupral treated aligners)	n.d.	n.d.	n.d.	n.d.

B

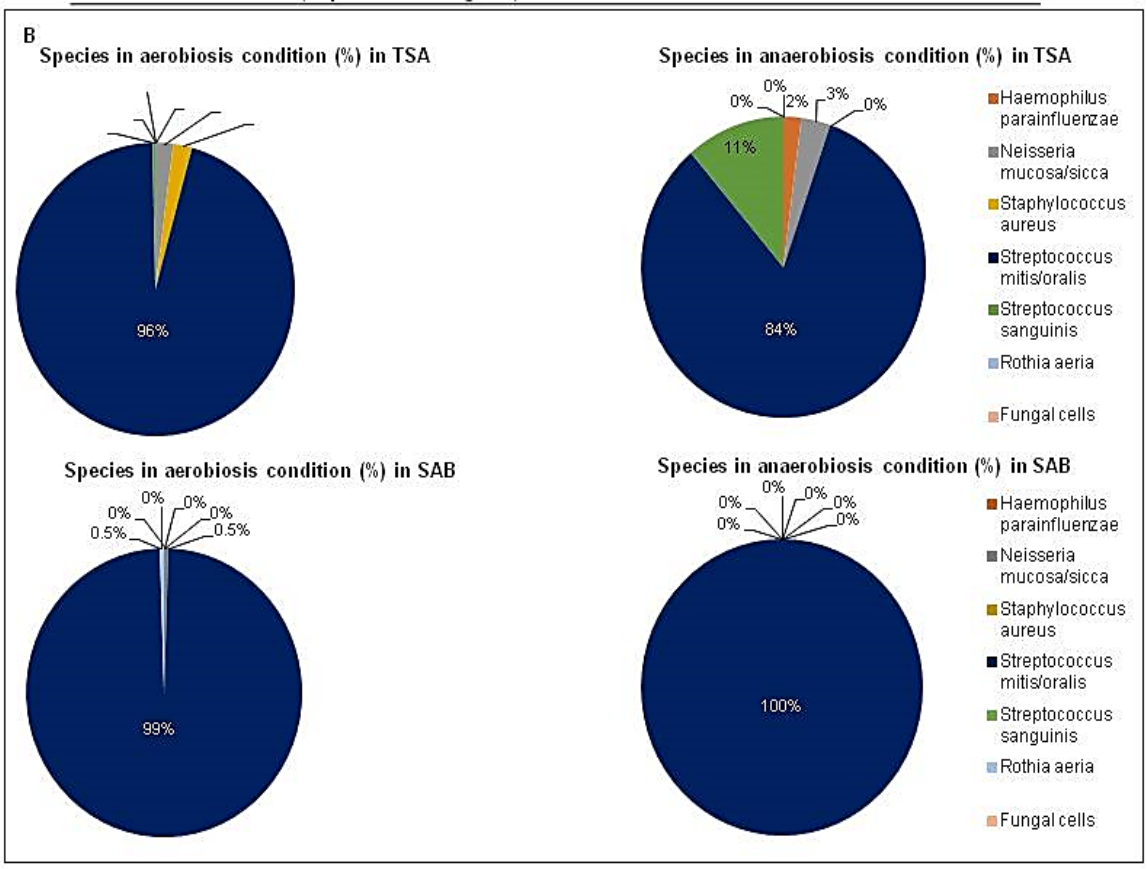

Fig. 4 Total and residual microbial load detected on summer months. The clear aligners were exposed or not to Cupral 1.25%. The colonies isolated after incubation in SAB medium were counted and clustered. The upper table shows the total and residual microbial load expressed as colony-forming units/aligners. The values represent the mean of three independent analyses, where each sample had been assessed in triplicate. The standard errors, less than 10%, are omitted. The lower pie graphs represent the percent of species detected in aerobiosis and anaerobiosis conditions. **n.d.**: not detected.

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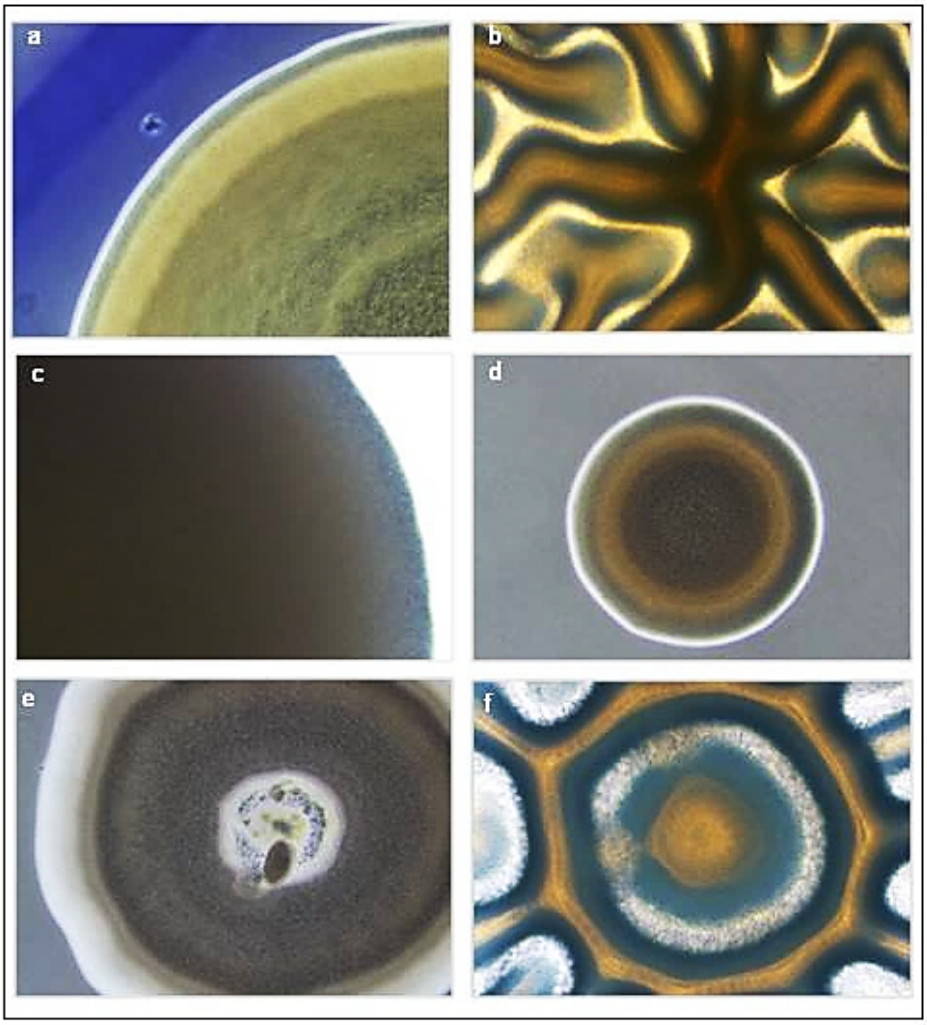

Fig. 5 Morphological peculiarities of the isolated colonies observed by inverted light microscope. Images (10x magnification) of the most representative colonies in TSA or SAB medium: (**A**) translucent white, coccobacilli Gram- (*Haemophilus parainfluenzae*); (**B**) wrinkled large yellow, diplococci Gram- (*Neisseria mucosa/sicca*); (**C**) large white, cocci Gram+ (*Staphylococcus aureus*); (**D**) small white, streptococci Gram+ (*Streptococcus mitis/oralis*); (**E**) irregular margins, streptococci Gram+ (*Streptococcus sanguinis*); (**F**) large beige, cocci Gram+ (*Rothia aeria*).

Discussion

Here, we show that clear aligners, regularly worn for ~2 weeks by a healthy subject, are abundantly contaminated by different bacterial species, with total microbial load ranging around 10^9 per device. As predictable, microbial biofilm is naturally produced onto clear aligners; despite daily washing and brushing, the presence of microbial plaque is easily detectable by staining with plaque disclosing

tablets (data not shown). According to culture-dependent analysis, Gram staining/morphology, and MALDI-TOF identification of the isolates, several bacterial species have been obtained, including *Haemophilus parainfluenzae*, *N. mucosa/sicca*, *S. aureus*, *S. mitis/oralis*, *S. sanguinis*, and *R. aeria*. In contrast, no cultivable fungal isolates have been detected, although SAB has been used as a fungi-preferential medium. Unexpectedly, under anaerobic conditions, SAB returns colonies belonging to the *Streptococcus* genus; likely, as facultative anaerobic species, streptococci easily grow in such conditions, because of the large amount of dextrose present in such medium, as an important element for their cellular metabolism.

It is well known that in orthodontic treatment, not only the active period but also the retention time is important because the extended periodontium must be stabilized.[19] In our study, irrespective of the season time, *Streptococcus mitis/oralis* happens to be the most representative species on aligners. These findings are in line with an extensive literature[20,21] describing such species as commonly found in oral cavity, among the early colonizers of mucosa and dental surfaces.

In our hands, slight winter–summer variations occur. In particular, about one log reduction is observed in terms of total microbial load/aligner, when using TSA in aerobiosis conditions during summer months in comparison with winter months. Nevertheless, it is worth noting that *S. aureus*, *H. parainfluenzae*, and *N. mucosa/sicca* are vice-versa more abundant on January–March sampling (10^8 CFU and 10^7 CFU/aligners on winter and summer season, respectively). This result may be explained considering that such microorganisms are, indeed, easily found in the upper respiratory tract (including oral cavity) during winter season. When considering summer season, *S. mitis/oralis* represents the most detectable microbial species within the device-associated bacterial plaque. Overall, the relative homogeneity in terms of microbial population observed during the 1-year analysis argues on a good and regular hygiene of the oral cavity and excludes a major impact of seasonal climate on aligners' colonization.

Recently, we provided *in vitro* evidence on the efficacy of Cupral as antimicrobial compound, being effective both against planktonic and biofilm communities.[16]

Moreover, clinical evidence exists on the successful treatment of periodontal disease patients by Cupral.[17,18] Here, we show that Cupral can be successfully employed to perform aligners hygiene; microbial contamination is totally removed, even when Cupral is diluted up to 1.25%. As shown by CFU assay, no residual viable cells have ever been detectable upon Cupral treatment of the aligners. Moreover, confocal data document the abundant presence of a biofilm on aligner's surface, where microbial cells aggregate in a tridimensional architecture of irregular peaks. Interestingly, Cupral profoundly affects such biofilm; the 3D construction indicates residual material, related to debris and dead cells according to the fact that no growth has ever been obtained by the CFU assay. It is well known that not only oral hygiene but also the chemical composition of the aligners plays an important role in conditioning microbial adhesion on the devices and, in turn, persistence in the oral cavity; indeed, microcracks, abraded areas, and localized hard deposits are often visible, likely facilitating microbial establishment and long-term maintenance.[22] To our knowledge, this is the first evidence describing a novel usage of Cupral, as a promising tool capable of eliminating aligner-associated microbial contamination. Such present data encourage clinical studies to establish whether the regular use of Cupral for clear aligners' disinfection may allow amelioration of gingival health and integrity during orthodontic treatment.

Conclusion

Cupral, a copper–calcium–hydroxide-based compound, exerts potent effects against microbial plaque, naturally occurring on clear aligners; tridimensional biofilm structure appears profoundly affected as well as no bacterial growth ever occurs following exposure to Cupral. While expanding the knowledge on Cupral antimicrobial activity, our data open to its use as a novel tool for clear aligners' daily hygiene.

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CHAPTER 3

Int. J. Mol. Sci. 2019, 20, 1868; doi:10.3390/ijms20081868

ARTICLE

Evaluation of Biological Response of STRO-1/c-Kit Enriched Human Dental Pulp Stem Cells to Titanium Surfaces Treated with Two Different Cleaning Systems

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Keywords: human dental pulp stem cells; stemness properties; titanium surface properties

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Abstract

Peri-implantitis—an infection caused by bacterial deposition of biofilm—is a common complication in dentistry, which may lead to implant loss. Several decontamination procedures have been investigated to identify the optimal approach being capable to remove the bacterial biofilm without modifying the implant surface properties. Our study evaluated whether two different systems—Ni-Ti Brushes (Brush) and Air-Polishing with 40 µm bicarbonate powder (Bic40)—might alter the physical/chemical features of two different titanium surfaces—machined (MCH) and Ca^{++} nanostructured (NCA)—and whether these decontamination systems may affect the biological properties of human STRO-1^{+}/c-Kit^{+} dental pulp stem cells (hDPSCs) as well as the bacterial ability to produce biofilm. Cell morphology, proliferation and stemness markers were analysed in hDPSCs grown on both surfaces, before and after the decontamination treatments. Our findings highlighted that Bic40 treatment either maintained the surface characteristics of both implants and allowed hDPSCs to proliferate and preserve their stemness properties. Moreover, Bic40 treatment proved effective in removing bacterial biofilm from both titanium surfaces and consistently limited the biofilm re-growth. In conclusion, our data suggest that Bic40 treatment may operatively clean smooth and rough surfaces without altering their properties and, consequently, offer favourable conditions for reparative cells to hold their biological properties.

1. Introduction

The use of dental implants in daily clinical practice is currently widespread and in huge growth: the modern implant therapy allows in fact not only to offer a biological and functional advantage for many patients, compared with fixed or removable prosthetic solutions but also to obtain excellent long-term results, as confirmed by previous studies reporting survival rates of 95.7% and 92.8% after 5 and 10 years, respectively [1]. However, despite these high survival rates, implant rehabilitation can fail. The most relevant troubles concerning osseointegrated implants are the peri-implant diseases, such as mucositis (reversible) and peri-implantitis (not reversible). Peri-implantitis has been defined as a disease with infectious pathogenesis characterized by a mucosal lesion often associated with bleeding, suppuration, increased probing depth always accompanied by marginal bone loss [2]. In this context, several factors affecting the systemic health status of the patients, that is, chronic diseases, autoimmune/inflammatory diseases, in combination with poor oral hygiene and smoking might influence the host-microbial interface very early in the healing phase following dental implant thus increasing the risk of peri-implant diseases [3].

The formation and organization of a bacterial biofilm on the implant portions exposed to the oral cavity is the initial cause of the peri-implant diseases and its removal is the goal to prevent or treat these diseases. Microbial biofilm commonly occurs in the oral cavity; it consists of a multispecies community, including Gram+ and Gra$^-$ bacteria as well as fungal cells, organized as sessile cells, tightly embedded in a matrix of polysaccharide origin. Biofilm formation and development is a process that begins with adhesion of initially planktonic microorganisms, followed by growth, extracellular polymeric matrix production, detachment and delocalization. Microbial biofilm occurs both in biotic and abiotic surfaces; once structured, biofilm-associated cells acquire enhanced resistance to cleaning treatment, antimicrobial drugs and host immune defences with respect to their planktonic counterparts [4]. To date, several methods have been developed with the aim of decontaminating implants: mechanical systems (Titanium brushes, Air-polishing systems, Ultra-Subsonic systems, Laser, Curettes) and chemical systems or antimicrobial solutions

(Chlorhexidine in solution or in gel, Stannous fluoride, Tetracycline, Minocycline, Citric acid, Hydrogen peroxide and Gel etching with 35% phosphoric acid) [5–7]. Most of these decontaminating treatments might alter the chemical-physical properties of the implant surface [8–11]. Therefore, the ideal decontamination system is expected to be capable of breaking and removing the bacterial biofilm, without modifying the surface properties and thus maintaining the implant surface biologically favourable to adhesion and differentiation of the reparative cells grown onto the implant. It is well known indeed that the micro/nanotopography and the chemical composition of implant surfaces might influence the osseointegration process, by affecting and modifying the biological properties of the cells that interact with the surface [12–15]. Based on these considerations, among several decontamination techniques investigated, Nickel Titanium (Ni-Ti) brushes and Air Polishing systems have aroused interest. Ni-Ti is a resistant material with high flexibility when subjected to heating/cooling alternations. Moreover, the centrifugal force of the motor movement, the pressure exerted by the operator's hand and the heating/cooling alternations allow the brush to effectively reach even the most diffcult spaces to clean. In fact, Ni-Ti brushes proved more effective in removing plaque than steel or plastic curettes, being, at the same time, less aggressive towards the implant surfaces [16–19]. Likewise, air-polishing devices based on the use of bicarbonate powder, which historically represents the first type of substance used in this approach, were shown to be highly effective in the mechanical removal of biofilm from different surface types, such as machined, SLA, sandblasted and TPS, with previous data showing that this approach provided better outcomes when compared to other instruments, such as plastic inserts of sonic devices [9,20,21].

Human DPSCs can be easily obtained from routine tooth extraction procedures and own self-renewal properties and a high regenerative potential [22]. Although dental pulp stem cells are a heterogeneous cell population, the immune selection against the stemness markers c-Kit and STRO-1 allows to isolate an adequate source of pre-osteoblast/odontoblast cells [23,24] which represent the ideal cell candidate in order to mimic in vitro the physiological processes of osseointegration

and to investigate how different cleansing approaches might modify the cells-implants interactions.

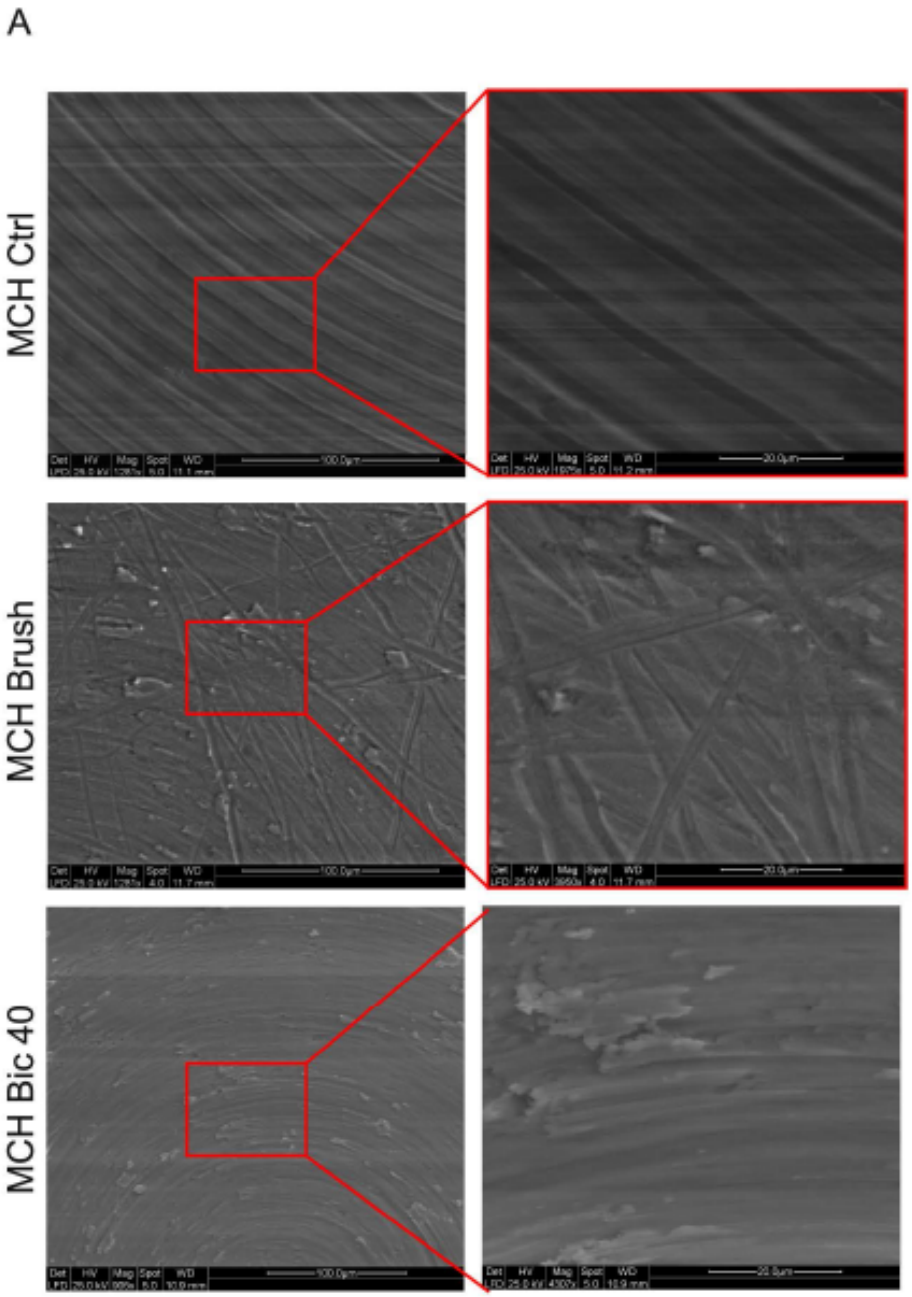
The aim of this study was to investigate whether the use of two different mechanical decontamination systems, namely Ni-Ti Brushes and Air-Polishing System with 40 μm bicarbonate powder, might alter the roughness and chemical composition of two different implant surfaces (Machined and Ca^{++} Nano-incorporated) and whether the two decontamination systems may impact the biological and stemness features of DPSCs as well as the bacterial ability to produce biofilm.

2. Results

2.1. Titanium Surface Characterization

Scanning electron microscopy (SEM) analysis carried out onMCHsurfaces from each experimental group is shown in Figure 1A. At lower magnifications, MCH control surface displayed concentric irregularities according to our previous findings [15]. The polishing treatment with Ni-Ti brush was characterized by grooves oriented in all the directions through the entire surface of the disks, as reported in either lower and higher magnifications. On the MCH titanium surfaces treated with Bic40, the presence of slight alterations of the whole surface was observed. Particularly, newly formed irregularities were detected on the entire treated disk (Figure 1A).

Int. J. Mol. Sci. 2019, 20, 1868; doi:10.3390/ijms20081868



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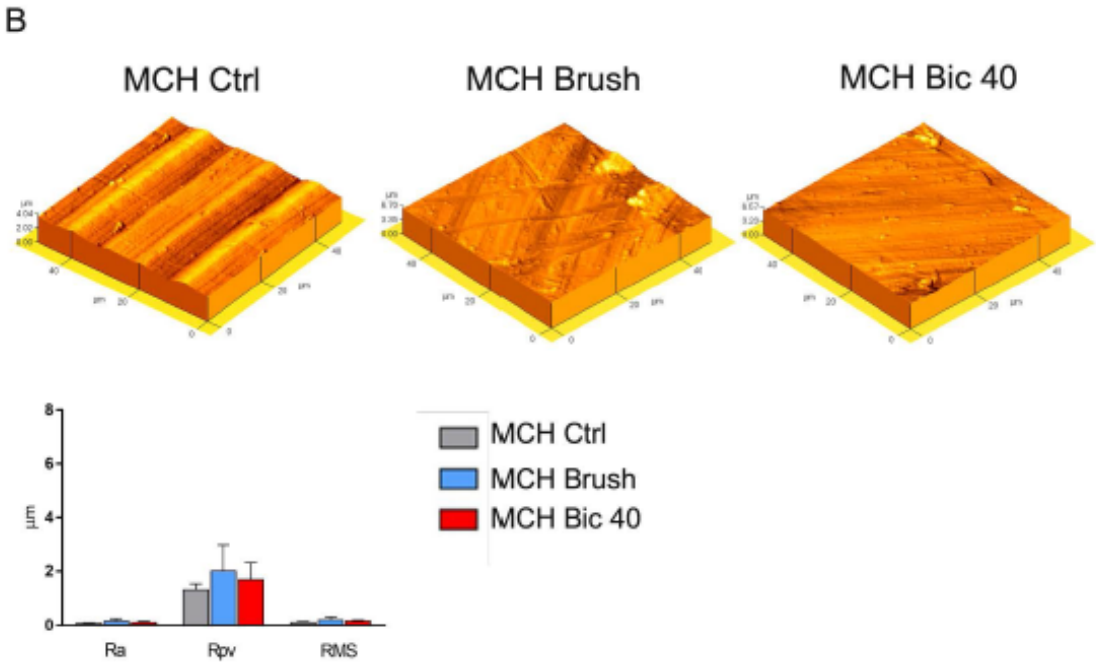

Figure 1. Machined (MCH) surfaces characterization after cleaning treatments. (**A**) Scanning electron microscopy (SEM) analysis at different magnifications was carried out on MCH titanium surfaces from the three experimental groups (Ctrl, Brush and Bic40, as indicated) in order to evaluate the surface topography. Scale bars: 100 µm (left) and 20 µm (magnifications on the right). (**B**) Atomic force microscopy (AFM) analysis of MCH surfaces. Histograms report the surface roughness expressed as Ra, Rpv and Rsm values. Values represent mean ± SD of three independent experiments; one-way ANOVA followed by Newman-Keuls post-hoc test.

Furthermore, atomic force microscopy (AFM) analysis was performed to evaluate the roughness of MCH disks following the different polishing treatments. In particular, as shown in Figure 1B, Ra, Rpv and Rsm were determined for each experimental group. With regard to Rpv parameter, higher values were recorded in MCH Brush group, in comparison to control MCH and MCH Bic40, although these differences were not statistically significant (Figure 1B). At the same time, SEM analysis was carried out on NCA surfaces from the three experimental groups. Data are reported in Figure 2A. According to previous data from our group [15], control NCA were characterized by homogeneous irregularities spread through the whole analysed area. The polishing treatment with Brush induced a notable modification of the surface: particularly, a flattening of the peaks typical of NCA surface was observed at lower and higher magnifications. Conversely, the air-

polishing treatment with Bic40 did not induce any relevant alteration of the nanotopography of the surface. These observational data were not confirmed by AFM analyses, actually, Ra, Rpv and Rms parameters did not differ among the three experimental groups. Likely, this evidence might be due to the AFM instrumental sensitivity (Figure 2B). Taken together, data on surface roughness of MCH and NCA surfaces treated with Bic40 did not show any significant difference, in terms of nanotopography, from both MCH and NCA controls.

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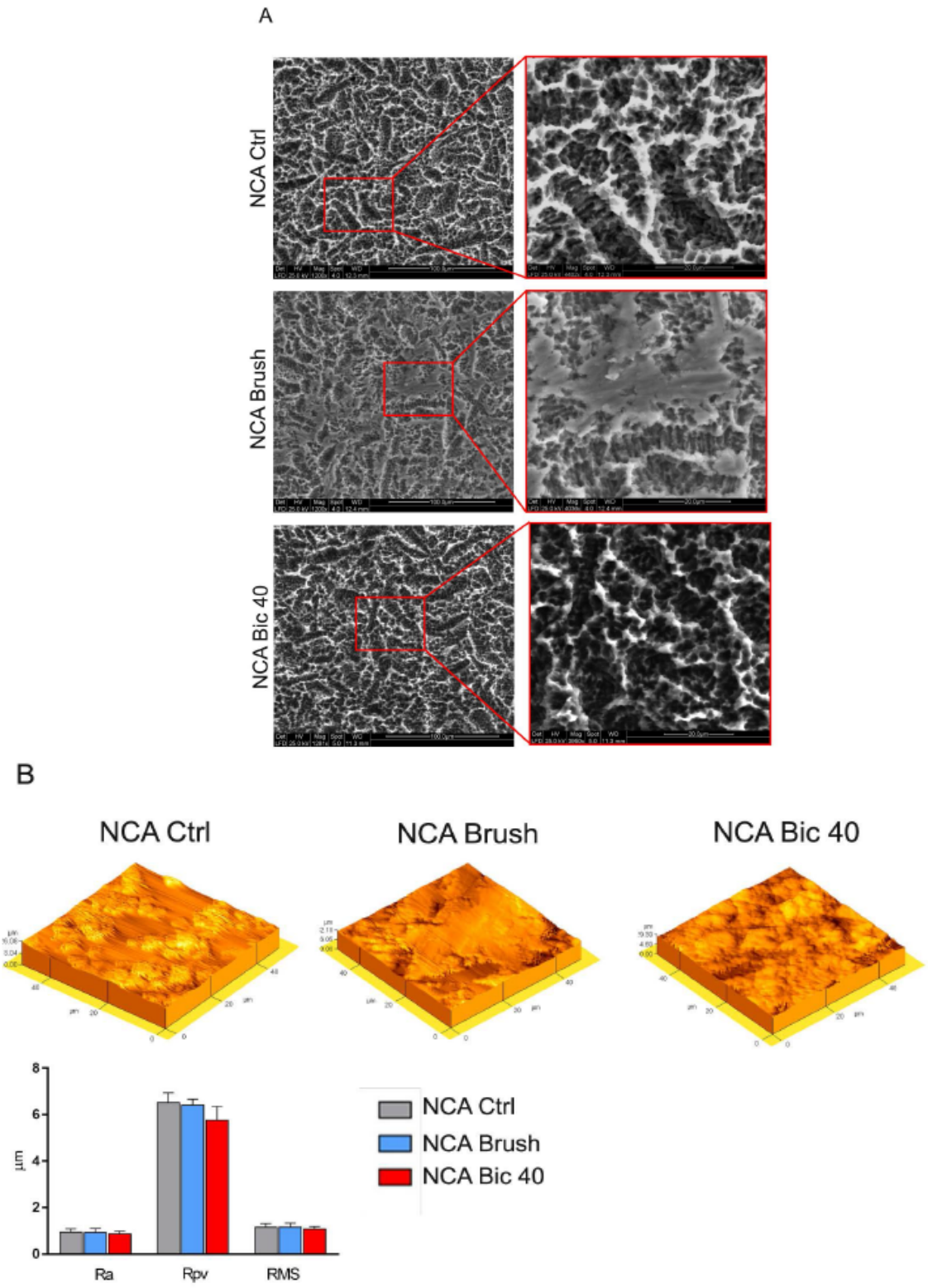

Figure 2. NCA surfaces characterization after cleansing treatments. (**A**) SEM analysis at different magnifications was carried out on NCA titanium surfaces fromthe three experimental groups (Ctrl, Brush and Bic40, as indicated) in order to evaluate the surface topography. Scale bars: 100 µm (left)

and 20 μm (magnifications on the right). (**B**) AFM analysis of NCA surfaces. Histograms report the surface roughness expressed as Ra, Rpv and Rsm values. Values represent mean ± SD of three independent experiments. No statistically significant difference was detected among the groups; one-way ANOVA followed by the Newman-Keuls post-hoc test.

2.2. Stem Cells Morphology and Proliferation on Titanium Surfaces after Polishing Treatments

The hDPSCs morphology was evaluated by confocal microscopy, as shown in Figures 3 and 4. Cells were stained with phalloidin and DAPI. After 7 days of culture under standard conditions on MCH surfaces, hDPSCs displayed a fibroblast-like morphology with cells being arranged parallel to the surface grooves without showing significant differences following the Brush and Bic40 polishing treatments. With regard to the distribution of cells through the surface area, we noticed that hDPSCs cultured on MCH Brush oriented not only along the grooves due to the industrial fabrication but also along the scratches created after the Brush cleaning. No differences were observed in hDPSCs seeded on MCH Bic40 when compared to the control group (Figure 3A). Also, no differences in terms of proliferation rate were observed among the three experimental groups as indicated by histograms (Figure 3B).

Int. J. Mol. Sci. 2019, 20, 1868; doi:10.3390/ijms20081868

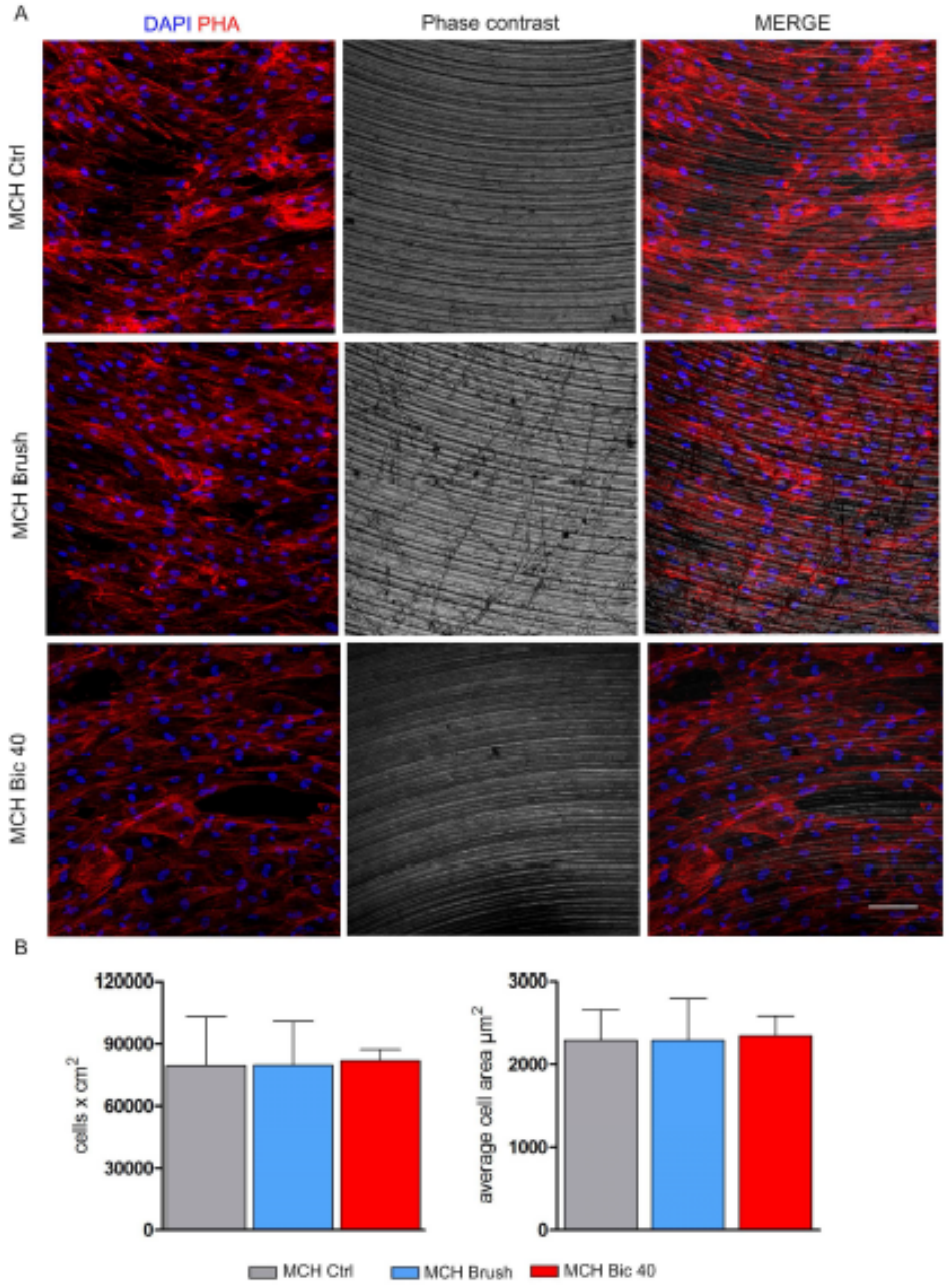

Figure 3. Evaluation of hDPSCs morphology and proliferation on MCH titanium surfaces. (**A**) hDPSCs morphology was assessed by confocal microscopy with phalloidin staining after 7 days of culture on MCH Ctrl, MCH Brush and MCH Bic40 surfaces. (**B**) Cell proliferation on titanium disks was measured by counting cell nuclei after DAPI staining. Histograms show cell numbers after 7 days of culture on titanium surfaces from the three experimental groups. Values represent mean ± SD. No statistically significant difference was detected among the groups; one-way ANOVA followed by the Newman-Keuls post-hoc test. Scale bar: 100 µm.

Figure 4 shows the morphology, distribution and proliferation of hDPSCs after 7 days of culture on NCA surfaces following Brush and Bic40 treatments. As formerly described, the culture on NCA disks induced a morphology alteration in hDPSCs. In particular, cells were homogeneously spread through the whole area and showed an irregular shape with reduction of the average cell area. The same features were observed in hDPSCs cultured on NCA Brush disks. Interestingly, when NCA disks were cleaned with Bic40 hDPSCs grew still homogeneously although recovering their typical fibroblast-like morphology, as reported when cultured on MCH disks. This shift in morphology was reflected also by an increased proliferation rate and by values of average cell area of hDPSCs cultured on NCA Bic40, with respect to NCA Ctrl and NCA Brush (* p <0.05, Figure 4B).

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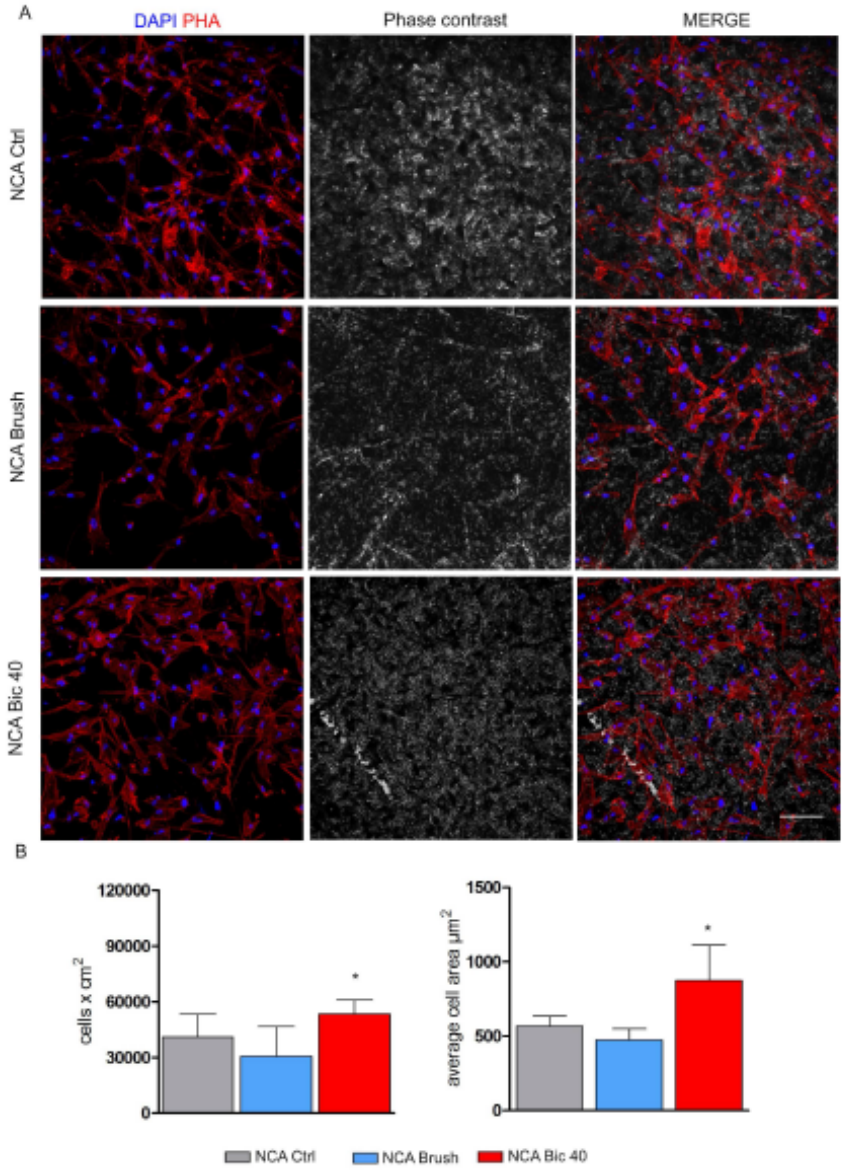

Figure 4. Evaluation of hDPSCs morphology and proliferation on NCA titanium surfaces. (**A**) hDPSCs morphology was assessed by confocal microscopy with phalloidin staining after 7 days of culture on NCA Ctrl, NCA Brush and NCA Bic40 surfaces. (**B**) Cell proliferation on titanium disks was measured by counting cell nuclei after DAPI staining. Histograms show cell numbers and average cell area after 7 days of culture on titanium surfaces from the three experimental groups. Values represent mean ± SD. * p <0.05 NCA Bic 40 vs. NCA Brush, NCA Bic40 vs. NCA Ctrl; one-way ANOVA followed by the Newman-Keuls post-hoc test. Scale bar: 100 µm.

2.3. Expression of Stemness Markers

Human DPSCs were immune-selected against STRO-1 and c-Kit. After 7 days of culture on MCH and NCA surfaces, immunofluorescence analysis was performed in

Int. J. Mol. Sci. 2019, 20, 1868; doi:10.3390/ijms20081868

order to evaluate the maintenance of their biological properties. The expression of STRO-1 and c-Kit, two typical mesenchymal stem cells markers, were investigated in MCH and NCA after cleaning treatments.

As reported in Figure 5, hDPSCs cultured on MCHCtrl showed the expression of both mesenchymal markers. These markers were still observed in hDPSCs cultured either on MCH Brush and MCH Bic40.

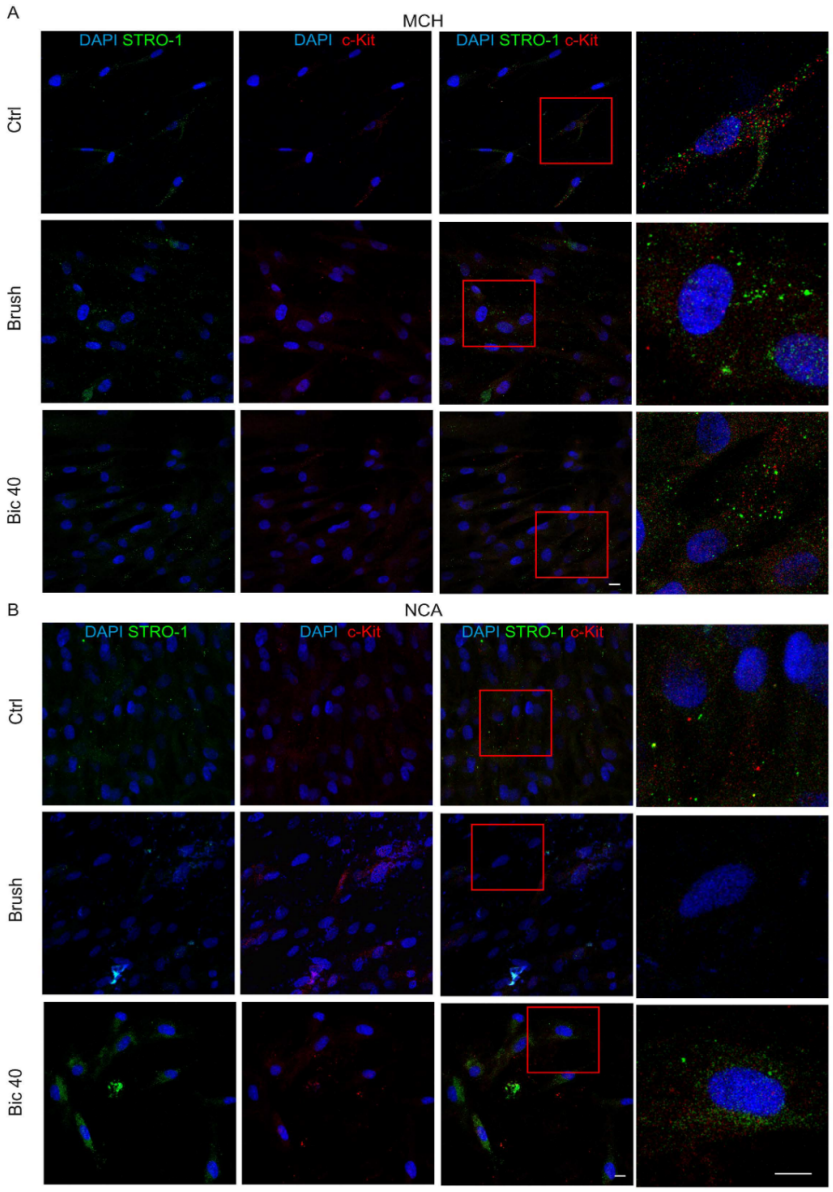

Figure 5. Evaluation of stemness markers of hDPSCs on titanium surfaces after cleaning treatments. Immunofluorescence analysis of STRO-1 and c-Kit in hDPSCs cultured for 7 days on MCH (**A**) and NCA (**B**) surfaces from the three experimental groups. Red squares indicate details reported at higher magnifications on the right. Nuclei were stained with DAPI. Scale bar: 10 μm.

On the contrary, we noticed a reduction in STRO-1 and c-Kit expression in hDPSCs grown on NCA Ctrl and NCA Brush after 7 days of culture. Conversely, the expression of these markers was evident in hDPSCs cultured on NCA Bic40. The morphology data in association with stemness evaluation indicate that after cleansing with Bic40, the NCA surface appeared more favourable/suitable to the growth of hDPSCs in their physiological microenvironment.

2.4. Microbial Biofilm Formation onto Titanium Disks

Based on the biological results concerning morphology, proliferation and stemness markers, it was then evaluated whether the polishing treatment with Bic40 on NCA surface may affect the microbial growth. Thus, *Pseudomonas aeruginosa* (10^6/mL) was seeded at time 0, in two sets of wells, containing or not the titanium disks; then, the plate was incubated at 35 °C for 24 h and microbial growth was assessed by bioluminescent analysis. As shown in Figure 6A, a superimposable trend was observed in the two groups of wells (control: no disks; sample: with disks); moreover, at 24 h, comparably high levels of totalmicrobial load were achieved, being $2.6x10^8$/mL and $3.4x10^8$/mL, the CFU/mL detected in control and sample groups, respectively. These data indicated that the presence of titanium disks did not affect microbial growth. To assess the occurrence of biofilm onto such disks, the latter were exposed to bacteria for 24 h, washed twice with PBS to eliminate the non-adherent microbial cells and then the residual bioluminescent signal was evaluated, as measure of formed biofilm. The obtained bioluminescent signal was converted in CFU and indicated that biofilm was produced at amounts as high as $1.95x10^8$ CFU/mL (data not shown).

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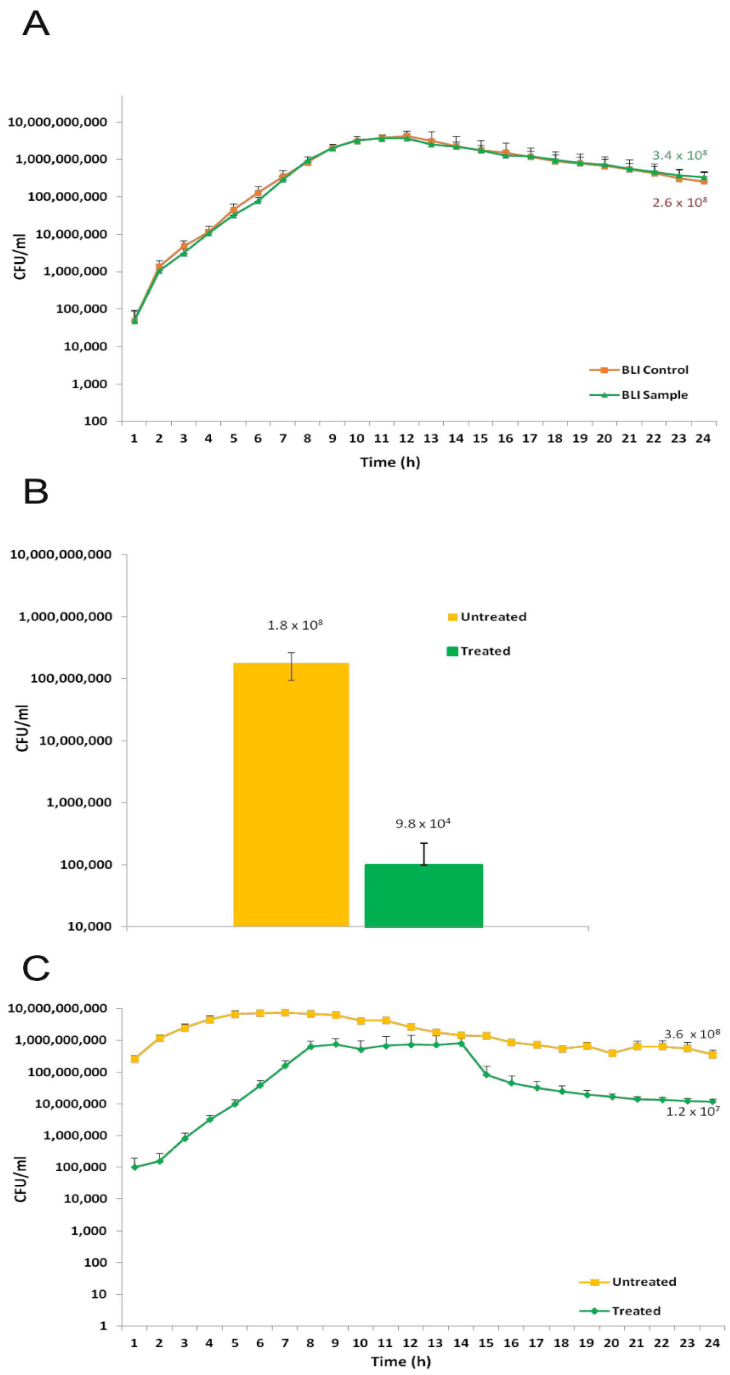

Figure 6. *P. aeruginosa* growth and biofilm formation on titanium disks, treated or not with Bic40 procedure, as assessed by a bioluminescent (BLI) bacterial strain. (**A**) *P. aeruginosa* growth was not affected by the presence of titanium disks. BLI-*Pseudomonas* (10^6/mL) in TSB plus 2% sucrose was seeded in 96 black well-plate and incubated at 35 °C in the presence (orange) or absence (green) of titanium disks (1/well). The plates were then incubated by Fluoroskan and the bioluminescent signal was recorded in real time (h), up to 24 h. By the calibration curve, the RLUs were converted in CFU/mL + SEM (Standard Error Mean), as indicated in the Y axis. (**B**) Effects of the cleaning

procedure on titanium disks-associated biofilm. Microbial biofilm produced onto titanium disks (by 24 h incubation, as above) was exposed or not to the Bic40 cleaning procedure (treated *vs* untreated group); the residual bioluminescent signal was measured and converted, by the calibration curve, in CFU/mL ± SEM, as indicated in the Y axis. (**C**) Real time monitoring of *P. aeruginosa* re-growth on treated and untreated titanium disks. The microbial re-growth, occurring in Bic40-treated and untreated disks, was evaluated in real time, for additional 24 h. Then, the bioluminescent signal was measured and converted in CFU, returning values of $3.6x10^8$/mL and $1.2x10^7$/mL, in untreated and treated groups, respectively.

2.5. Microbial Biofilm Removal from Titanium Disks

To assess the efficacy of the Bic40 cleaning procedure against a preformed biofilm, 4 disks containing a 24 h-old biofilm were treated with Bic40 procedure, as detailed above (treated group), while 3 disks, used as controls were not treated (untreated group); then, each disk was transferred in a new well with fresh medium and incubated for additional 24 h. As shown in Figure 6B, when compared to the untreated groups, the Bic40-treated disks displayed an about 3 logs drop in terms of CFU/mL.

2.6. Microbial Re-Growth on Treated and Untreated Titanium Disks

As detailed in Figure 6C, the microbial load in the untreated group remained almost constant during the subsequent 24 h range. In contrast, treated disks showed a delayed and time-related increase in CFU. In particular, the major differences between treated and untreated disks occurred within the first 5–6 h; then, microbial load on treated disks reached a plateau level, which consistently remained about 1 log below the values observed in the untreated disks until at 24 h.

3. Discussion

The peri-implant disease is due to the colonization of the implant surfaces by pathogens that constitute a biofilm [25]. Bacterial adhesion and biofilm formation play a fundamental role not only into the pathogenesis of peri-implantitis but also into the implant survival [12,13,26]. In a previous study [27], we observed that the biofilm occurred regardless of the degree of surface roughness. Therefore, the removal of the biofilm from the implant surface, indistinctly smooth or rough, is the primary objective. It is well known that the response of cells and tissues to a biomaterial

depends on the properties of the material itself such as surface topography, chemical composition and capability to interact with body fluids [15,28]. Following bacterial contamination, the procedures used to decontaminate the implant surface can cause alterations of its topography and its chemical composition. To this regard and to the best of our knowledge the optimal cleansing approach is expected to effectively remove the bacteria biofilm without altering the chemical and physical properties of the implant and consequently the biological properties of cells involved in the osseointegration process.

In this study we analysed two different titanium surfaces, before and after the treatment with two cleansing approaches, mimicking what physiologically occurs in terms of cells/implant interactions after decontamination procedures.

Qualitative analysis of surface morphology revealed how in both MCH and NCA groups the surfaces treated with Ni-Ti brushes are morphologically different from the untreated surfaces, in agreement with Park et al. [18]. In fact, SEM analysis showed the presence of deep grooves heterogeneously distributed over the MCH surface and flattened area over the NCA surface. Slight although not statistically significant differences were revealed by Ra, Rpv and Rsm physical parameters. Conversely, the treatment with Air-polishing system with Bic40 produced not significant alterations of both the MCH and NCA surfaces in terms of physical parameters. Subsequently, the aim of the study was to evaluate the biological features of stem cells that embryologically participate to the osseointegration process. The use of dental pulp stem cells represents a suitable choice in terms of stemness properties and ease of isolation. Although hDPSCs are a heterogeneous population, we used a stem cell population enriched for the expression of the stemness markers STRO-1 and c-Kit, which represent a strictly mesenchymal origin able to differentiate in bone, adipose and myogeniclineages. To the best of our knowledge, we noticed that on MCH titanium surface before and after both the cleansing treatments, hDPSCs maintained their fibroblast-like morphology without any alteration of cell proliferation. The only difference observed concerned the hDPSCs distribution pattern through the MCH Brush surface, in fact cells were spread along the grooves produced by Ni-Ti brush.

On the contrary, hDPSCs grown on MCH Bic40 and MCH ctrl surfaces did not show any difference.

Regarding NCA surface, a change in cell morphology was observed in NCA ctrl and NCA brush, in accordance with our previous findings [15]. After the treatment with Bic40 hDPSCs showed their typical cell morphology and also demonstrated a statistically significant higher proliferation rate, when compared to NCA ctrl and NCA Brush surfaces: this phenomenon might be attributed to the interaction of calcium ions incorporated on the NCA surface with bicarbonate ions from the cleansing treatment with Bic40. These observations were confirmed by the evaluation of stemness markers on MCH and NCA surfaces before and after the cleansing treatments. In particular, whereas no differences were detected in any MCH group, we noticed that STRO-1 and c-Kit expression were maintained in NCA Bic40 group. As a matter of fact, the maintenance of stemness is a primary requirement to preserve the biological properties including self-renewal and differentiation capabilities and immunomodulatory properties and consequently to avoid cell senescence. Based on these results, Bic40 might represent the most suitable cleansing treatment. To further confirm the efficacy of Bic40, we also performed microbiology assays. Using a recently established model precious in assessing microbial biofilm formation onto medical devices [29], here we showed that BLI-*Pseudomonas* has the ability to adhere to the titanium disks and to form a consistent biofilm on their surface. Moreover, the cleaning treatment with Bic40 μm is capable of reducing the biofilm of about 99% with respect to untreated control group (100% *vs* 0.05%, respectively). In particular, the microbial load, evaluated as RLU and converted in CFU/mL, has been reduced of more than 3 logs in the treated groups as compared to their controls. Furthermore, microbial re-growth in treated disks remains consistently below the control values (difference of about 1 log). We may hypothesize that the combination of the physical treatment (dry spray) and the hypertonic condition (sodium bicarbonate accumulation onto titanium disks) negatively impact on microbial cell-viability. Furthermore, the enrichment of air-polishing powders with antimicrobial fillers such as Ciprofloxacin and/or mucosal

defensive agents such as Zinc L-carnosine might improve the antibacterial action of the cleaning tool and its biocompatibility towards soft tissues [30].

In conclusion, we demonstrated that Bic40 provides a suitable cleansing approach either on smooth and rough surfaces.

4. Materials and Methods

4.1. Human DPSCs Isolation and Immune Selection

This study was carried out in compliance with the recommendations of Comitato Etico Provinciale—Azienda Ospedaliero-Universitaria di Modena (Modena, Italy), which provided the approval of the protocol (Ref. number 3299/CE; 5 September 2017). Human DPSCs were isolated from third molars of adult subjects (n = 3; 30–35 years) undergoing routine dental extraction. All subjects gave written informed consent in accordance with the Declaration of Helsinki. Cells were isolated from dental pulp as previously described [23]. Briefly, dental pulp was harvested from the teeth and underwent enzymatic digestion by using a digestive solution, (3 mg/mL type I collagenase plus 4 mg/mL dispase in α-MEM). Pulp was then filtered onto 100 µm Falcon Cell Strainers, in order to obtain a cell suspension. Cell suspension was then plated in 25 cm^2 culture flasks and expanded in standard culture medium [α-MEM supplemented with 10% heat inactivated foetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin] at 37 °C and 5% CO_2. Following cell expansion, human DPSCs were immune-selected by using MACS® separation kit according to manufacturers' instructions. The immune-selections were performed by using primary antibodies: mouse IgM anti-STRO-1 and rabbit IgG anti-c-Kit (Santa Cruz, Dallas, TX, USA). The following magnetically labelled secondary antibodies were used: anti-mouse IgM and anti-rabbit IgG (Miltenyi Biotec, Bergisch Gladbach, Germany). The immune-selection resulted in the isolation of a homogeneous hDPSCs population expressing STRO-1 and c-Kit. All the experiments were performed using STRO-1^+/c-Kit^+ hDPSCs.

4.2. Titanium Surfaces Characterization

A total of 50 titanium disks (MegaGen Co. Ltd., Daegu, South Korea) measuring 13 mm in diameter and 3 mm in thickness were used in this study. Particularly, two different titanium surfaces were used: machined (MCH) and Ca^{2+} incorporated (NCA). The treatment processes are hold by the manufacturer. For this study, titanium surfaces were divided into 3 different experimental groups: (1) control surfaces (Ctrl), (2) surfaces cleaned with Ni-Ti brushes (Brush) and (3) surfaces cleaned by air-polishing with $NaHCO_3$ 40 μm (Bic40).

The cleansing of the disks was carried out using two mechanical methods: (1) Nickel-Titanium Brushes (I.C.T. Brush micro, Hans Korea Co. Ltd., Gyeonggi-do, Korea/ De Ore, Verona, Italy) and (2) Air-Polishing System (Combi-Touch, Mectron spa, Carasco, Genova, Italy) with 40 μm bicarbonate powder.

Briefly, the “I.C.T.” (Implant Cleaning Technique) nickel-titanium brushes, made up of about 40 super elastic filaments with a diameter of 0.07–0.13 mm, were used at 400 rpm and 600 rpm, respectively, for two sequential rounds of 45 s each. The total duration of each surface treatment was 90 s and a 25 g pressure calibrated on an electronic scale was used, with 100 N of torque. All the treatments were performed by the same operator under irrigation with buffer saline solution (0.9% NaCl).

The “Combi-Touch” air polishing system with sodium bicarbonate particles (ø 40 μm) was used for 30 s at a distance of 5 mm. In particular, the operating principle of “Combi Touch” air-polishing system consists in the mechanical action of compressed air spreading an accelerated flow of particles onto the titanium surface. When the particles hit the surface, their kinetic energy is dissipated almost completely, thus producing a gentle although effectively cleansing action. The cleaning treatment is completed by a water jet that is arranged in the form of a bell around the main flow and that uses the pressure drop originated around the nozzle to prevent the powder cloud from bouncing and being dispelled and, at the same time, to dissolve the powder by washing the surface.

After the cleansing treatments, surface morphology for each experimental group was qualitatively evaluated through Scanning Electron Microscopy (EVO MA 10-Carl Zeiss, Oberkochen, Germany) working at 25 keV. Moreover, surface roughness was

determined by Atomic Force Microscopy (Nanoscope IIIa, Veeco, Santa Barbara, CA, USA) and Ra, Rpv and Rms parameters were obtained. Ra (Roughness average) measures the average surface roughness considering the peaks and the valley means. The Rpv (peak to valley distance) describes the maximum observed range in a sample area and it is given by the distance between the highest peak and the lowest valley on a measured surface. Rms parameter describes the density of micropores on the surface.

4.3. Cell Morphology and Proliferation

Undifferentiated STRO-1$^+$/c-Kit$^+$ hDPSCs at passage 1 were seeded at a density of 2.5x10^3 cell/cm^2 on titanium disks in 12-multiwell units and expanded under standard culture conditions. After 7 days of culture, cells were fixed in ice-cold paraformaldehyde 4% for 15 min without dissociating them from the titanium disks. The cells were subsequently permeabilized with 0.1% Triton X-100 in PBS for 5 min, stained with AlexaFluor 546 Phalloidin (Thermo Fisher Scientific) and rinsed with PBS 1%. Nuclei were stained with 1 µg/mL 4',6-diamidino-2-phenylindole (DAPI) in PBS 1%. Titanium disks were mounted with DABCO anti-fading medium on cover glasses. Cell proliferation and morphology were assessed using confocal microscopy (Nikon A1), as formerly described by Bianchi et al. [24].

Cell proliferation was measured by counting the DAPI-positive nuclei on 10 randomly selected fields measuring 2.85x10^5 cm^2 for each disk by a blind operator. At the same time, average cell area was measured on hDPSCs from 10 randomly selected fields, measuring 2.85x10^5 cm^2, on 3 disks for each experimental group.

4.4. Evaluation of Stemness Markers in hDPSCs Cultured on Titanium Disks

After 1 week of culture on each disk, cells were fixed in 4% ice-cold paraformaldehyde in PBS for 15 min and then processed as previously described [31]. The following primary Abs diluted 1:100 were used: mouse IgM anti-STRO-1 and rabbit IgG anti-c-Kit (Santa Cruz, Dallas, TX, USA). Secondary Abs (goat anti-mouse IgM Alexa488, goat anti-rabbit Alexa546) were diluted 1:200 (Thermo Fisher Scientific, Waltham, MA, USA). Nuclei were stained with 1 µg/mL 4',6-diamidino-2-phenylindole (DAPI) in PBS 1%. The multi-labelling immunofluorescence

experiments were carried out avoiding cross-reactions between primary and secondary Abs.

Confocal imaging was performed using a Nikon A1 confocal laser scanning microscope, as previously described [32]. The confocal serial sections were processed with ImageJ software to obtain 3-dimensional projections and image rendering was performed by Adobe Photoshop Software.

4.5. Microbial Strain

We used the bioluminescent *Pseudomonas aeruginosa* strain (P1242) (BLI-*Pseudomonas*) previously engineered in order to express the luciferase gene and substrate under the control of a constitutive P1 integron promoter 2 [33]; thus, live cells constitutively produce a detectable bioluminescent signal. To quantify the bioluminescence emission by BLI-*Pseudomonas* in the experimental groups, a calibration curve was created allowing to express such values in terms of colony forming units (CFU)/mL; in particular, serial dilutions (starting from $1x10^8$/mL) of a bacterial suspension in Tryptic Soy Broth (TSB) (OXOID, Milan, Italy) with 2% sucrose were prepared and a volume of 100 µL of each dilution was seeded in a black-well microtiter plate. The plate was immediately read by using a Fluoroskan Luminescence reader (Thermo Fisher Scientific, Waltham, MA, USA).

4.6. Biofilm Formation onto Titanium Disks

In order to allow biofilm formation onto Ca-structured titanium disks, 180 µL of overnight cultures of BLI-*Pseudomonas* (10^6/mL) in TSB plus 2% sucrose were seeded in 96 black well-plate, containing 1 disc/well. In parallel, BLI-*Pseudomonas* was seeded in wells without the titanium disks. The plates were then incubated at 35 °C for 24 h, into the Fluoroskan reader and the bioluminescence was detected at every hour to evaluate in real time, the total microbial load. After incubation, the disks were washed twice with phosphate buffered saline (PBS) (EuroClone, Wetherby, UK) at room temperature (RT), transferred into new wells and the bioluminescence signal was again measured; the obtained values were referred to the amounts of biofilm formed onto disk surfaces. Through the calibration curve, the relative luminescent units (RLU) obtained in each experiment were converted in CFU/mL.

4.7. Biofilm Re-Growth onto Treated and Untreated Titanium Disks

Following biofilm formation, the disks were split in two groups and the cleaning treatment was performed, as detailed above; then, controls (untreated) and cleaned (exposed to Bic40 µm for 30 s/surface) were transferred into new wells containing fresh medium and further assessed for microbial residual load and time-related re-growth. Briefly, treated and untreated disks were analysed by Fluoroskan reader, immediately (residual biofilm) and at any hour during a further 24 h incubation at 35 °C. Through the calibration curve, the RLU were converted in CFU/mL.

4.8. Statistical Analysis

All experiments were performed in triplicate. Data were expressed as mean ± standard deviation (SD) + SEM. Differences between two experimental conditions were analysed by paired, Student's *t*-test. Differences among three or more experimental samples were analysed by ANOVA followed by Newman–Keuls post hoc test (GraphPad Prism Software version 5 Inc., San Diego, CA, USA). In any case, p value < 0.05 was considered statistically significant.

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CHAPTER 4

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COMMUNICATION

Differential Efficacy of Two Dental Implant Decontamination Techniques in Reducing Microbial Biofilm and Re-Growth onto Titanium Disks *In Vitro*

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Keywords: Brush; BIC-40; BLI-*P. aeruginosa*; dental implants; decontamination systems; titanium disks

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Abstract

Dental implants are crucial therapeutic devices for successful substitution of missing teeth. Failure cases are mainly pathogen-associated events, allowing clinical progression toward peri-mucositis or peri-implantitis. The aim of this study was to compare the performance of two mechanical decontamination systems, Nickel-Titanium brush (Brush) and Air-Polishing system with 40 µm bicarbonate powder (BIC-40), by means of a novel bioluminescence-based model that measures microbial load in real time. Briefly, 30 disks were contaminated using the bioluminescent *Pseudomonas aeruginosa* strain (BLI-*P. aeruginosa*), treated with Brush (30 s rounds, for 90 s) or BIC-40 (30 s, at 5 mm distance) procedure, and then assessed for microbial load, particularly, biofilm removal and re-growth. Our results showed that Brush and BIC-40 treatment reduced microbial load of about 1 and more than 3 logs, respectively. Furthermore, microbial re-growth onto Brush-treated disks rapidly occurred, while BIC-40-treated disks were slowly recolonized, reaching levels of microbial load consistently below those observed with the controls. In conclusion, we provide evidence on the good performance of BIC-40 as titanium device-decontamination system; the clinical implication for such findings will be discussed.

1. Introduction

To achieve a successful oral rehabilitation, aimed at substituting one or more missing teeth via dental implants, such as titanium (Ti) element or titanium alloys [1], it is important not only to obtain implant osseointegration but also to limit, as much as possible, the risk of peri-implant disease [2]. As recently underlined [3], much has to be done in such topic. Peri-implantitis is a chronic, irreversible, multifactorial condition, triggered by microbial biofilm formation, and persistent inflammation around dental implants [3–5], especially in patients with insuciently poor oral hygiene, smoking, diabetes, history of periodontitis, trauma, or fracture due to uncontrolled implant-overload [6,7]. Massive and long-lasting inflammation of the surrounding area will bring to local tissue de-structuration, bone-degradation and eventually loss of the involved implant(s) [8]. It has also been hypothesized that some subjects are genetically predisposed to the onset and progression of peri-implantitis. This would be due to an incorrect modulation of the RANK/RANKL systems, regulators of immune responses, and bone physiology [9–13]. In particular, three molecules, the receptor activator of NF-kappa B (RANK), its ligand RANKL, and the decoy receptor of RANKL, osteoprotegerin (OPG), attracted the attention of scientists and pharmaceutical companies alike. Genetic experiments evolving around these molecules established their pivotal role as central regulators of osteoclast function [14].

Notoriously, microbial contamination and biofilm formation may take place onto biotic and abiotic surfaces, including dental implants. Once established as a sessile structure, microbial community acquires enhanced resistance to cleansing techniques, antimicrobial drugs, and host immune defenses with respect to their planktonic counterpart [15,16]. Several studies [17,18] focused on implant and abutment colonization, stating that the characteristics of their surface(s) significantly influence clinical outcome, affecting microbial localization on the device(s) and around the peri-implant area. Moreover, by an experimental study in dogs, Albouy et al. [19] demonstrated that peri-implantitis progression is more rapid when using rough surface implants rather than machined ones.

Over the years, different methods, such as mechanical and chemical systems or antimicrobial solutions, have been used for implant decontamination, thus achieving good results [20,21]. Nevertheless, undesired alteration(s) of implant surfaces can happen to a different extent [22]. Current efforts are aimed to implement microbial biofilm removal, minimizing changes/damages on implant surface-structure(s) and, at meantime, maintaining the implant biologically adequate and favorable for adhesion and persistence of reparative host cells [23,24].

Among several decontamination systems, Nickel Titanium brushes (Brush) and Air Polishing system with 40 μm bicarbonate powder (BIC-40) are receiving interesting attention. The former is known to be resistant and high flexible material when undergoing heating/cooling variations [25]. The latter, employing bicarbonate powder is often used by clinicians for its efficacy in mechanical removal of biofilm, providing satisfactory clinical outcomes [26].

The set-up of new experimental models, such as the recently described use of an engineered laboratory strain of *Pseudomonas aeruginosa* (*P. aeruginosa*) to assess, in real time, biofilm formation onto medical devices [27], opens new pathways for a better comprehension of the events involved in microbial biofilm formation onto abiotic surfaces and its abatement by chemical and/or mechanical procedures.

The aim of the present study was to compare two decontamination systems, Brush and BIC-40, in their ability to impair *P. aeruginosa* contamination onto titanium disks by a bioluminescence-based assay. The null hypothesis was that biofilm persistence and microbial re-growth on titanium disks were comparable, irrespectively of the decontamination system employed.

2. Materials and Methods

2.1. Titanium Disks

We tested 30 disks (MegaGen Co. Ltd., Daegu, South Korea), with Calcium-incorporated titanium surface, having dimensions of 13 mm in diameter, 3 mm in thickness, and an arithmetic average (Ra) value of 0.93 μ. The titanium disks were

firstly sterilized by autoclave. Then, microbial contamination and cleaning/decontamination were performed as below.

2.2. *Pseudomonas aeruginosa*

We used the bioluminescent *P. aeruginosa* strain P1242 (BLI-*Pseudomonas*). As previously described [28], such cells were engineered in order to express the luciferase gene and its substrate under the control of a constitutive P1 integron promoter 2, thus constitutively producing a bioluminescent signal. Bacteria from -80 °C glycerol stocks were initially seeded onto Tryptic Soy Agar (TSA) (OXOID, Milan, Italy) plates and incubated overnight at 37 °C. Then, isolated colonies were collected, suspended with 10 mL of Tryptic Soy Broth (TSB) (OXOID, Milan, Italy), and allowed to grow overnight at 37 °C with gentle shaking. Bacterial concentrations were then assessed by the McFarland standard curve and working dilutions ($1x10^6$/mL, in TSB) were prepared and used according to the protocols detailed below.

2.3. Microbial Growth and Biofilm Formation onto Titanium Disks

Overnight cultures of BLI-*Pseudomonas* (10^6/mL) were seeded in 96 black well-plates, in the presence or not of titanium disks (1 disk/well), to allow microbial growth and biofilm formation. The plates were then incubated at 35 °C for 24 h, into the Fluoroskan Luminescence reader (Thermo Fisher Scientific, Waltham, MA, USA), and the bioluminescence was detected hourly. The BLI signal was automatically recorded (total microbial load). At time 24 h, the disks were washed twice with phosphate buffered saline (PBS) (EuroClone, Wetherby, UK) at room temperature (RT) to eliminate the planktonic cells, transferred into new wells and the bioluminescence signal was again measured (biofilm produced onto disk surfaces). The recorded bioluminescence values were expressed as Relative Luminescence Units/sec (RLU/sec) and converted in colony forming units (CFU/disk), based on an internal reference curve.

2.4. Titanium Disk Decontamination Systems

BLI-*Pseudomonas* contaminated disks were treated with Nickel-Titanium Brushes, named Brush (Implant Cleaning Technique, Hans Korea Co. Ltd., Gyeonggi-do,

Korea/De Ore, Verona, Italy) and Air-Polishing system (Combi-Touch, Mectron spa, Carasco, Genova, Italy) with 40 μm bicarbonate powder (BIC-40). The Brush, composed of about 40 super elastic filaments with a diameter of 0.07–0.13 mm, was used at 400 and 600 rpm, for three sequential rounds of 30 s each with 25 g pressure calibrated on an electronic scale of 100 N torque. The BIC-40 system (30 s at a distance of 5 mm) consisted in the mechanical action of compressed air spreading a flow of particles onto the titanium surface, as previously demonstrated by the authors [25]. The BIC-40 system was executed for 30 s at a distance of 5 mm, consisting in the mechanical action of compressed air spreading the flow of particles onto the titanium surface. When the particles hit the surface, their kinetic energy is dissipated almost completely, thus producing a gentle and effective cleansing action. The cleaning treatment was completed by a water jet that was arranged in the form of a bell around the main flow. This flow used the pressure drop originated around the nozzle to prevent the powder cloud from bouncing and being dispelled and, at the same time, to dissolve the powder by washing the surface.

All the decontamination treatments were performed by the same operator.

2.5. Residual Biofilm and Microbial Re-Growth after Decontamination

The BLI-*Pseudomonas*-contaminated titanium disks were divided into three groups and processed as: untreated controls, Brush-treated, and BIC-40-treated groups. Upon treatment, the disks were transferred in new wells containing fresh medium and immediately analyzed by the Fluoroskan reader (residual biofilm). Subsequently, the disks were incubated at 35 °C into the Fluoroskan reader and hourly evaluated for BLI signal for further 24 h (microbial regrowth). The values obtained were expressed as RLU/sec and CFU/disk.

2.6. Statistical Analysis

Each experiment was performed twice; each protocol included 15 disks. Each condition (i.e., control, Brush and BIC-40) was tested using five replicates. Data that had a normal distribution were expressed as RLU/sec or CFU/disk. The results are shown as mean ± standard error (SEM). Statistical analysis was performed using the Student's *t*-test that allowed comparing treated *vs* untreated groups.

3. Results

3.1. Total Microbial Growth and Biofilm Formation onto Titanium Disks

Initially, to exclude any effect of titanium on total microbial growth, BLI-*P. aeruginosa* (10^6/mL) was seeded at time 0 in two sets of wells, containing or not titanium disks (1 disk/well). Then, the plate was incubated at 35 °C for 24 h, and microbial growth was kinetically assessed, by bioluminescence analysis. Superimposable and time-related trends were observed in the two groups (both above 10^8 CFU/mL, at time 24 h), indicating that the presence of titanium disks did not affect *P. aeruginosa* growth.

Next, the formation of *P. aeruginosa* biofilm onto titanium disks was assessed. In particular, at 24 h, the disks were washed twice with PBS to eliminate the non-adherent microbial cells, transferred into new wells, and then tested for residual bioluminescent signal. As shown in Figure 1 (upper panel, control group), BLI-RLU remained at levels as high as 0.8 ± 0.12 RLU/s, indicating that a strong biofilm had been formed onto the disks. In order to express the BLI results as microbial load/disk, an internal calibration curve was used and the CFU/disk were calculated, as shown in Figure 1 (lower panel, control group).

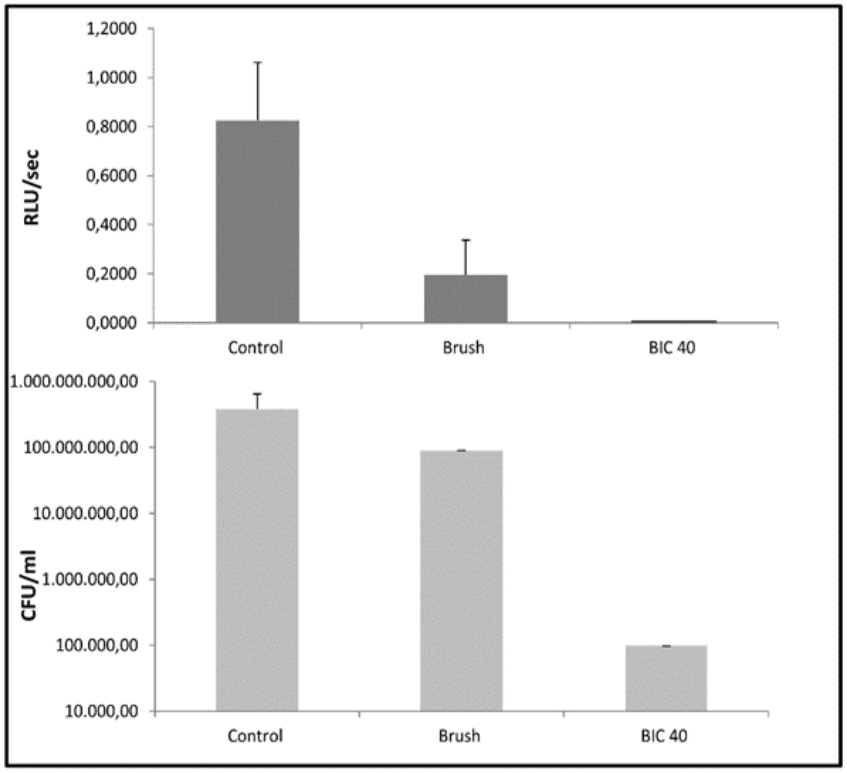

Figure 1. *P. aeruginosa* biofilm formation onto Titanium disks and residual microbial load, following Ni-Ti brush and AirPol BIC-40 decontamination treatments. Microbial load was assessed on treated and untreated disks, by bioluminescence assay; the results are given as RLU/s and number of viable cells (CFU/disk).

3.2. Microbial Biofilm Removal from Titanium Disks by the Two Decontamination Systems

To assess the efficacy of the two cleaning systems, two sets of disks, housing a 24 h-old *P. aeruginosa* biofilm, were treated with Brush or BIC-40 procedures, as detailed in Section 2. In parallel, a third set of disks remained untreated (control group). Then, each disk was transferred into a new well, with fresh medium, and the RLU/s were immediately detected. As shown in Figure 1, when compared to the untreated control group, both decontamination systems were capable of impairing biofilm level, although to a different extent. In particular, when expressing such decreases as percent, the Brush procedure caused 76% biofilm reduction, while the BIC-40 treatment allowed 99.9% of biofilm reduction.

3.3. Microbial Re-Growth onto Treated Titanium Disks

The microbial re-growth onto decontaminated titanium disks was measured hourly, for additional 24 h, incubating again the plate at 35 °C in the Fluoroskan reader. The results are given as microbial load ratio between treated and untreated disks, as shown in Figure 2. We found that, following the Brush procedure, the microbial load ratios rapidly increased, up to about 1.5 logs within the initial 5 h. Then, between treated and untreated groups, values remained constantly close to 1 up to 24 h, indicating that microbial load in treated and untreated disks was comparable.

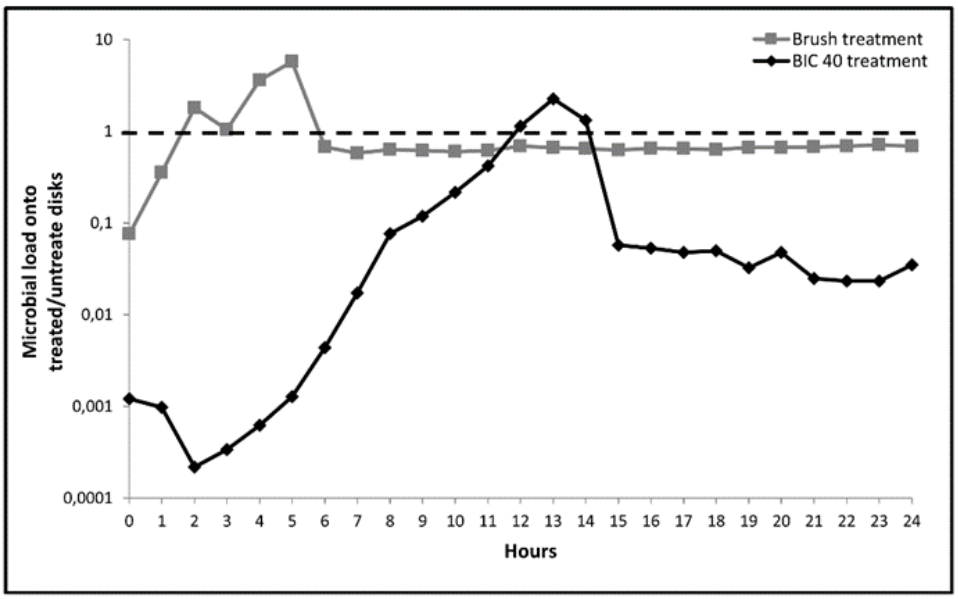

Figure 2. Kinetic evaluation of *P. aeruginosa* biofilm re-growth onto titanium disks, following decontamination by Ni-Ti Brush and AirPol BIC-40 systems. Microbial load was kinetically assessed on treated and untreated disks, by bioluminescence assay. The results are expressed as microbial load ratio between treated and untreated disks. The ratio = 1 (dotted line) indicates superimposable microbial load in treated and untreated groups.

Differently, when using the BIC-40 procedure, the microbial load ratios further decreased at time 2–4 hours of incubation. Then, a gradual but slow enhancement was observed up to 12–13 hours. After that, the values decreased again and remained stable by the time, the ratio between treated and untreated disks ranging between 0.1 and 0.01 log. These data indicated that BIC-40–treated disks

maintained, by the time, a significantly lower microbial load with respect to both the Brush-treated as well as the untreated control counterpart.

4. Discussion

Here, we show that BIC-40 system performs better than Brush in reducing *P. aeruginosa* biofilm and microbial re-growth onto titanium disks.

Dental implants are widely used in different clinical situations; osseointegration ability and long-lasting integrity bring about successful results [29]. Vice versa, microbial localization on the implant affects the surrounding area, eventually leading to osseointegration loss [3,4]. As described in [30], microbial biofilm may happen, despite titanium disks' surface sharpness. This outline needs a targeted clinical action for patient benefit. Besides antibiotics or other therapies [31], decontamination techniques of dental implant surfaces deserve special attention as local cleaning systems. Such treatments seem to be less effective when applied to rough surfaces rather than to smooth surfaces. Furthermore, depending upon cleaning procedure used, undesirable effects may occur on implant surface morphology [32–34].

By a recently established model assessing in real time microbial biofilm formation onto medical devices [27], here we show that BLI-*Pseudomonas* has the ability to adhere onto the titanium disks, thus rapidly forming a consistent biofilm on their surfaces within 24 h. Moreover, we provide the first evidence on the different effcacy of the two decontamination treatments, since Brush and BIC-40 are capable of impairing biofilm up to 76% and 99.9%, respectively. In particular, as evaluated by RLU analysis and subsequent conversion in CFU/disk, we show that microbial load is reduced of about 1 log following Brush treatment, while more than 3 logs decrease occurs upon BIC-40 treatment. Furthermore, kinetic assessment of BLI signal demonstrates that microbial re-growth onto BIC-40-treated disks is delayed and remains mostly below the control values (about 1 log). In the Brush-treated disks, microbial re-growth occurs, rapidly reaching levels similar to those observed in the untreated control disks. It should be noted that *Pseudomonas* produces one of the most complex and diffcult to treat biofilms [35] even on dental implants [36]. Thus, we assume that the successful decontamination obtained by BIC-40, in our model, would

likely be as effective when applied against other clinically relevant biofilms, including those produced during peri-mucositis/peri-implantitis.

As recently published [25], a mechanical treatment for decontamination of titanium disks, such as the use of Brush, deeply affects the device surface producing deep grooves on it. In contrast, lack of damage occurs in BIC-40 treated titanium surface(s). Taken together, our previous [25] and present data favor the conclusion that the use of BIC-40, avoiding formation of niches onto device surface, would in turn prevent/minimize microbial adhesion, survival, and persistence. Moreover, we favor the hypothesis that the good performance of BIC-40 can also be related to the fact that sodium bicarbonate particles, remaining onto BIC-40-treated titanium disks [25], likely render the device surface hostile to subsequent microbial re-growth. In any case, using a BLI-based system that allows kinetic monitoring of microbial load onto medical devices [27], we have been able to show a different efficacy of the two decontamination procedures not only in impairing preformed biofilm but also in limiting subsequent re-growth onto titanium disks.

Finally, it is worth noting that the present data have been obtained using titanium disks with rough surfaces, as representative of the worst condition clinically possible. Indeed, previous studies investigating the correlation between implant surface morphologies and microbial localization around the peri-implant areas demonstrate a more pronounced progression of the pathology around rough surface rather than machined ones [17–19]. From here, we assume that BIC-40 performance would further improve when used to decontaminate machinated implants.

5. Conclusions

This *in vitro* study provides evidence on the relevance of BIC-40 as dental implant decontamination system. Besides avoiding titanium surface damage [25], such treatment consistently impairs biofilm and microbial re-growth. Given the relevance of microbial contamination in implant failure, these findings open to a clinical use of BIC-40 in the treatment of pathogen-related peri-mucositis and peri-implantitis.

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CHAPTER 5

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ARTICLE

Propolis Affects *Pseudomonas aeruginosa* Growth, Biofilm Formation, eDNA Release and Phenazine Production: Potential Involvement of Polyphenols

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Keywords: *P. aeruginosa*; virulence factors; phenazines; eDNA; MIC; antimicrobial; antibiofilm; propolis; polyphenols.

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Abstract

Pseudomonas aeruginosa (*P. aeruginosa*) is an opportunistic pathogen responsible for a wide range of clinical conditions, from mild infections to life-threatening nosocomial biofilm-associated diseases, which are particularly severe in susceptible individuals. The aim of this *in vitro* study was to assess the effects of an Albanian propolis on several virulence-related factors of *P. aeruginosa*, such as growth ability, biofilm formation, extracellular DNA (eDNA) release and phenazine production. To this end, propolis was processed using three different solvents and the extracted polyphenolic compounds were identified by means of high-performance liquid chromatography coupled to electrospray ionization mass spectrometry (HPLC-ESI-MS) analysis. As assessed by a bioluminescence-based assay, among the three propolis extracts, the ethanol (EtOH) extract was the most effective in inhibiting both microbial growth and biofilm formation, followed by propylene glycol (PG) and polyethylene glycol 400 (PEG 400) propolis extracts. Furthermore, *Pseudomonas* exposure to propolis EtOH extract caused a decrease in eDNA release and phenazine production. Finally, caffeic acid phenethyl ester (CAPE) and quercetin decreased upon propolis EtOH extract exposure to bacteria. Overall, our data add new insights on the anti-microbial properties of a natural compound, such as propolis against *P. aeruginosa*. The potential implications of these findings will be discussed.

1. Introduction

In nature, many microbial species use a cell-to-cell signaling system, named quorum sensing (QS), to form biofilms on both biotic and abiotic surfaces. Microorganisms embedded in a biofilm acquire resistance to drugs and detergents and make host defenses less efficient [1–3]. Microbial biofilms consist of sessile cells, embedded in a self-produced matrix of polysaccharides, proteins, lipids and extracellular DNA (eDNA) [4,5]. *Pseudomonas aeruginosa* (*P. aeruginosa*), a Gram-negative, aerobic (and at times facultative anaerobic), encapsulated, rod-shaped bacterium, is an opportunistic pathogen responsible for nosocomial infections, which can be particularly severe and life threatening in susceptible individuals [6]. *P. aeruginosa* is responsible also for oral infections in patients with clinical conditions, such as apical periodontitis, pulp necrosis, pulpitis or mandibular/maxillary alveolitis [7]. Indeed, the onset and development of infections are mostly related to the wellestablished ability of *P. aeruginosa* to produce biofilm, either onto biotic or abiotic surfaces.

Several virulence factors of *P. aeruginosa*, including biofilm formation, pyoverdine and pyocyanin (PYO) production as well as eDNA release, are regulated by QS. In particular, eDNA release occurs through mechanisms involving the autoinducer N-acyl-L-homoserine lactones (AHL) and the *Pseudomonas* quinolone signaling (PQS) molecules. QS-independent mechanisms, via flagella and type IV pili, have also been documented as contributing to eDNA release [8]. PYO derives by the precursor phenazine-1-carboxylic acid (PCA), which is then converted in phenazine-1-carboxamide (PCN) and 1-hydroxy-phenazine (1-OH-PHZ) [9]; it is a virulence factor known to contribute to eDNA release by its interaction with molecular oxygen to form reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), which cause cell lysis. In turn, the released eDNA promotes *Pseudomonas* adhesion and increases biofilm thickness and biomass content [10]. Recently, a rapid and easy-to-perform *in vitro* model has been described for real-time monitoring of *P. aeruginosa* growth and biofilm formation onto endotracheal tubes using a bioluminescent strain [11].

Traditional treatments of infectious diseases have broad-range efficacy. However, the frequency of microbial genetic mutations is drastically enhanced, resulting in an increased incidence of antibiotic resistance. Therefore, there is an increasing need to find other molecules, such as natural compounds, capable of killing or inhibiting microbial growth likely without promoting resistance mechanisms. In this context, propolis (bee-glue) is a well-known mixture of resinous and balsamic substances that, collected by honey-bees from tree and herb buds, are transformed with the help of salivary secretions into a peculiar product. This natural-resinous hive substance has been considered as an official drug by London's pharmacopoeia since the 17th century. The name propolis, derived from the ancient Greek, "pro" and "polis", meaning "before or in defense of the city". Indeed, propolis is used for beehive defense, as well as to close hive holes and cracks, in order to avoid contact with harmful agents, such as other insects, fungi, bacteria, etc. In Europe, propolis became very popular between the 17th and 20th centuries [12,13]. Its chemical composition and biological properties vary according to the geographical area and climate characteristics [14].

The main components of propolis are flavonoids, phenolic acids, terpenes, aromatic acids and other molecules, most of which are lipophilic and, therefore, easily dissolved in organic solvents, such as ethanol [15,16]. The biological activity of propolis is mostly linked to flavonoids and phenolic acids [17]. To date, propolis is considered as a potent antiseptic substance, successfully used in several clinical settings, without concomitant toxic/deleterious effects [12]. In particular, propolis has gained attention in different dentistry fields [17]; some clinical studies have demonstrated its preventive effect against dental caries [18] as well as its therapeutic benefits on oral surgery and periodontology [19], oral mucositis [20], plaque inhibition [21], pulp capping [22,23] or its efficacy as an endodontic irrigant [24]. Ethanol extract of propolis [25] has been demonstrated to possess also anti-inflammatory and regenerative effects, and this observation is confirmed by experiments in vivo of teeth pulpotomy in piglets [22]. Other important biological properties of propolis, ranging from antimicrobial [16], antibiofilm [26], anticancer [27], antioxidant [16,28],

anti-inflammatory activities to wound-healing promotion [29] have been described; all these properties are attributed to its high polyphenols content.

The aim of this study was to assess *in vitro* the anti-*P. aeruginosa* activity of an Albanian propolis with respect to some virulence factors, such as biofilm formation, eDNA release and phenazine production. In addition, by high-performance liquid chromatography (HPLC) coupled with mass spectrometry (MS), the profile of propolis extracts was characterized, focusing on some specific polyphenols before and after exposure to *P. aeruginosa* cells.

2. Materials and Methods

2.1. Microbial Strain

The bioluminescent *P. aeruginosa* strain (P1242) (BLI-*Pseudomonas*) was maintained and handled as previously described [11]. This strain had been engineered to express the luciferase gene and luciferase substrate under the control of a constitutive P1 integron promoter. Thus, live cells constitutively produced a detectable bioluminescent signal [30]. Bacteria from −80 °C glycerol stocks were initially seeded onto Tryptic Soy Agar (TSA) (OXOID, Milan, Italy) plates and incubated overnight at 37 °C; then, isolated colonies were collected, added to 10 mL of Tryptic Soy Broth (TSB) (OXOID, Milan, Italy) and allowed to grow overnight at 37 °C with gentle shaking. Bacterial concentrations were then measured by the McFarland standard curve and appropriate dilutions prepared, according to the protocol.

2.2. Propolis

Crude propolis, produced by *Apis mellifera* L., was collected on June 2018, in north Albania (Puka, Thethi and Velë forest areas), far from asphalted urban or interurban roads. The woodland vegetation in this area, mainly consists of *Picea abies* L., *Populus alba* L., *Aesculus hippocastanum* L., *Castanea sativa* Miller, *Arbutus unedo* L., *Quercus pubescens* Wild.,*Prunus dulcis* (Mill.) D.A. Webb, *Prunus avium* L., *Helianthus annuus* L., *Cucumis sativus* L., *Ulmus* L., *Thymus vulgaris* L., *Cornus mas* L., *Salvia officinalis* L., *Acacia penninervis* DC, *Salix* L.,

Cytisus scoparius L. & Link, *Trifolium* L. and *Carpinus* L. The crude propolis appeared as a mild amorphous mass, with an aromatic balsamic smell, in dark yellow to light brown color, with a bitter and burning taste numbing the oral mucosa. It was delivered at room temperature (RT) in the dark and kept at −80 °C until the extraction procedure.

2.3. Chemicals and Solvents

HPLC-grade acetonitrile (ACN), formic acid (HCOOH), analytical grade absolute ethanol (EtOH), propylene glycol (PG), polyethylene glycol 400 (PEG 400) and methanol (MeOH) were purchased from Sigma (Milan, Italy). Water (H_2O) was purified using a Milli-Q Plus 185 system from Millipore (Milford, MA, USA).

2.4. Extraction of Phenolic Compounds from Crude Propolis

One gram of an Albanian frozen propolis (kept at −80 °C) was grinded in a mortar and reduced to uniform particle size powder. The extraction was carried out by dynamic maceration with 10 mL of solvent (i.e., EtOH, PG and PEG 400) under the dark for 24 h, at RT. The extracts were centrifuged for 5 min at 4000 rpm. The supernatant solutions were filtered in a vacuum into a 10 mL volumetric flask and the solvents were added to the final volume. The extraction procedure was repeated twice for each solvent tested.

2.5. Spectrophotometric Analysis of Total Phenolics

The total phenolic content was determined by using the Folin-Ciocâlteu colorimetric assay with some modifications [31]. A solution of gallic acid in water, at different concentration (2–20 µg/mL), was used as the reference. The total phenolic content was assessed by using 50 µL of each extract, previously diluted 1:1 with H_2O. Fifty µL of pure solvent (EtOH, PG and PEG 400) were used as the blank, respectively. Then, 500 µL of the Folin-Ciocâlteu reagent and 1 mL of sodium carbonate (Na_2CO_3) saturated solution were added. The solution was then adjusted to the final volume of 10 mL with H_2O. Afterwards, the solutions were incubated at RT in the dark and, after 2 h, the absorbance was evaluated at 760 nm wavelength. The phenolic content was determined from the equation of the regression curve and expressed as mg of gallic acid equivalents for mL of propolis extract (mg GAE/mL).

2.6. Sample Preparation for HPLC Analysis

An aliquot of 200 µL of each propolis extract was properly diluted with 1 mL of EtOH in a volumetric flask, filtered through a 0.45 µm PTFE filter into a HPLC vial and injected in the HPLC system. All sample preparations were carried out in duplicate.

2.7. HPLC Analysis of Phenolics in Propolis Extracts

Chromatography was performed using an Agilent Technologies (Waldbronn, Germany) modular model 1100 system, consisting of a vacuum degasser, a quaternary pump, an autosampler, a thermostated column compartment and a diode array detector (DAD). The chromatograms were recorded using an Agilent ChemStation for LC and LC-MS systems (Rev. B.01.03).

The analysis was carried out on an Ascentis C_{18} column (250×4.6 mm I.D., 5 µm, Supelco, Bellefonte, PA, USA). The mobile phase was composed by (A) 0.1% HCOOH in H_2O and (B) ACN. The gradient elution was modified as follows: 0–3 min 25% B, 3–10 min linear gradient from 25% to 30% B, 10–40 min from 30% to 40% B, 40–60 min from 40% to 60% B, 60–80 min from 60% to 90% B, 80–92 min 90% B. The post-running time was 5 min. The flow rate was 1.2 mL/min. The column temperature was set at 30 °C. The sample injection volume was 5 µL. The UV/DAD acquisitions were at 265 nm (for chrysin and galangin), 290 nm (for cinnamic acid, pinocembrin and pinobanksin), 320 nm (for caffeic acid, p-coumaric acid and ferulic acid), 338 nm (for apigenin and luteolin) and 370 nm (for quercetin, isorhamnetin and kaempferol).

HPLC coupled with electrospray ionization mass spectrometry (ESI-MS) analyses were performed using an Agilent Technologies modular 1200 system, equipped with a vacuum degasser, a binary pump, an autosampler, a thermostatted column compartment and a 6310 A ion trap mass analyzer with an ESI ion source. The HPLC column and the applied chromatographic conditions were the same as reported for the HPLC-DAD system. The flow-rate was split 6:1 before the ESI source. For ESI-MS2, the parameters were set as follows: the capillary voltage was 3.5 kV, the nebulizer (N_2) pressure was 20 psi, the drying gas (N_2) temperature was 350 °C, the drying gas flow was 9 L/min and the skimmer voltage was 40 V.

Data were acquired by Agilent 6300 Series Ion Trap LC/MS system software (version 6.2). The mass analyzer was used in the full-scan positive and negative ion modes in the *m*/*z* range 100–1000. MS^2 spectra were automatically performed with helium as the collision gas by using the SmartFrag function.

2.8. Minimal Inhibitory Concentration (MIC) Assay

The MIC assay was performed by the microbroth dilution method according to the Clinical and Laboratory Standards Institute/National Committee for Clinical Laboratory Standard (CLSI/NCCLS M7-A6) [32]. According to the experimental protocol, each propolis extract was tested at final dilutions, ranging from 500 µg GAE/mL to 1.9 µg GAE/mL. In parallel, each solvent (at the corresponding dilutions) and gentamicin (2 mg/mL) were included as negative and positive controls, respectively. A bacterial cell suspension (5×10^5 cells/mL in Mueller Hinton plus 2% sucrose, obtained from overnight cultures) was seeded (100 µL/well) in a 96 U-bottom microtiter-plate; then, the bacterial cells were added with medium (100 µL/well) or treated with scalar doses of propolis extracts or their respective solvent (100 µL/well). Therefore, the plate was incubated at 37 °C for 24 h. The MIC of each extract was defined as the lowest concentration that inhibited visible *Pseudomonas* growth and in which the relative concentration of the solvent showed the minimal toxicity.

2.9. Assessment of Propolis Effects on Microbial Growth and Early Biofilm Formation

BLI-*Pseudomonas* is known to produce biofilm, as detailed elsewhere [11]. In order to monitor the total microbial growth under different experimental conditions, 100 µL of overnight cultures of BLI-*Pseudomonas* (5×10^5/mL) in TSB plus 2% sucrose were seeded in a 96 black well-plate, containing 100 µL of TSB 2% sucrose (untreated) or propolis extract (treated) or solvent (control). The plates were then incubated at 35 °C for 16 h, into the Fluoroskan reader and the bioluminescence was detected at every hour. The values collected in real time as a bioluminescence signal and expressed as relative luminescence units (RLU)/s, indicated the total microbial growth; based on an internal reference curve, such values could be converted in colony forming units (CFU)/mL.

In order to measure biofilm production by BLI-*Pseudomonas*, the samples were incubated for 16 h, washed twice with phosphate buffered saline (PBS) at RT to remove the planktonic cells and then the bioluminescence signal was measured. Such values were referred to the amounts of early biofilm formed onto plate surfaces, in treated or untreated samples.

2.10. Assessment of Phenazines and Propolis Polyphenols in Cell-Free Supernatants

In order to evaluate the amount of phenazines and to determine the levels of propolis polyphenols in supernatants from *Pseudomonas* exposed or not to propolis, a suitable HPLC-ESI-MS analysis was performed. To do this, all supernatants were filtered by Amicon Ultra-0.5 10 K centrifugal filter devices and diluted 1:5 (*v/v*) with 5% MeOH—0.2% HCOOH in H_2O. The HPLCESI-MS instrument used was an UltiMate 3000 system, consisting of an online degasser, a binary pump HPG 3400RS, a well plate autosampler WPS 3000RS and a thermostatted column compartment TCC 3000RS coupled to a Q Exactive hybrid quadrupole–orbitrap mass analyzer via a HESI-II heated electrospray ion source (Thermo Scientific). Chromatographic separation of a 5 µL sample injection was performed on a Zorbax SB-C_{18} RRHT (50×2.1 mm I.D., 1.8 µm) column (Agilent) at 25 °C and a 0.3 mL/min flow rate. A linear gradient elution scheme was used with mobile phase components, being 0.1% HCOOH in H_2O (A) and MeOH (B). The gradient started at 2% B, which was maintained for 0.5 min, then raised up to 42% B for 30 min and up again to 95% B for 4 min. The column was kept at 95% B for 4.4 min; then, the starting conditions were restored in 0.1 min and maintained for 11 min pending a successive injection. Electrospray ionization was operated in positive ion mode, using N_2 as the sheath gas (40 arbitrary units), auxiliary gas (290 °C, 30 arbitrary units) and sweep gas (two arbitrary units). The sprayer voltage was kept at 3.5 kV and the transfer capillary temperature was set at 320 °C. The Q-Exactive was operated in Full MS/dd-MS^2 mode. The full MS scan range was set from *m*/*z* 150 to 1000 at 70,000 full widths at half maximum FWHM resolution (*m*/*z* 200). The automaticgain control (AGC) target was set at 1.0×10^6 with a maximum injection time (IT) of 200 ms. Datadependent MS^2 (dd-MS^2) acquisitions at 17,500 FWHM resolution (*m*/*z* 200) were triggered for

the Top 3 precursor ions following each full MS scan. The intensity threshold for precursor ion selection was set to 1.0×10^5 then dynamic exclusion was active for 20.0 s. AGC target and maximum IT for the MS^2 experiments were set to 5.0×10^5 and 80 ms.

2.11. Assessment of Propolis Effects on eDNA Release

For the analysis of eDNA, 100 µL of overnight cultures of BLI-*Pseudomonas* (5×10^5/mL) in TSB plus 2% sucrose were seeded in 96 well-plates, containing 100 µL of medium (untreated) or propolis extract (treated) or solvent (control). The plates were then incubated at 35 °C for 16 h. The supernatants were collected and centrifuged twice at 14,000 rpm for 15 min and filtered by Amicon Ultra-0.5 10 K centrifugal filter devices in order to remove any remaining bacteria. To exclude residual viable bacteria, 50 µL of the supernatants were seeded onto TSA plates and incubated for 48 h at 37 °C under aerobic conditions; no bacterial CFU on TSA plates were ever observed. To quantify eDNA concentration in the cell-free supernatants, 100 µL of each sample were incubated with Propidium Iodide (PI) (1 µg/mL) for 15 min at 37 °C; then, the fluorescence emission was quantified by Fluoroskan reader (excitation/emission: 584/612).

2.12. Statistical Analysis

Quantitative variables were tested for normal distribution. Statistical differences between propolis treated and untreated (solvent) samples were analyzed according to Kruskal-Wallis test by using GraphPad prism 8. Values of p <0.05 were considered significant.

3. Results

3.1. Total Phenolic Compounds in Propolis Extracts

Initially, the total polyphenol content of the three different propolis extracts (obtained by EtOH, PG and PEG 400) was determined by means of the Folin-Ciocâlteu colorimetric method. In particular, the PEG 400 extract showed the highest polyphenol content (5.8 ± 0.2 mg GAE/mL), while PG and EtOH extracts showed values of 4.8 ± 0.5 and 4.1 ± 0.4 mg GAE/mL, respectively.

3.2. HPLC Analysis of Polyphenols in Propolis Extracts

In order to investigate the presence of polyphenols on the three propolis extracts, a HPLCUV/DAD analysis was performed and representative chromatograms are shown in Figure 1.

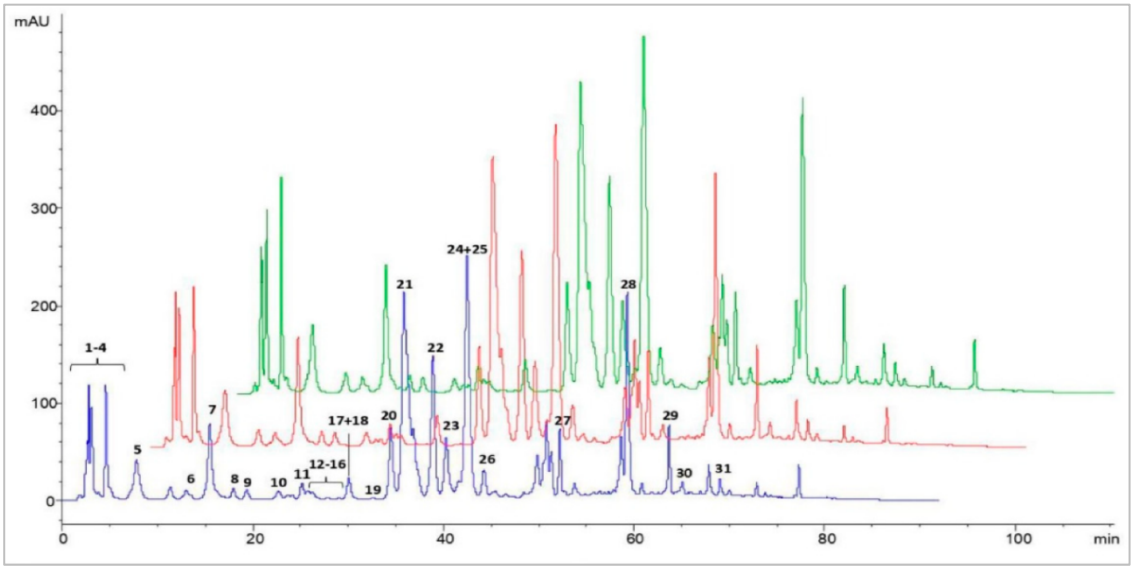

Figure 1. HPLC-UV/DAD chromatograms of EtOH (blue line), PG (red line) and PEG 400 (green line) propolis extracts. Data are from a representative experiment, out of two performed. For peak identification, see Table 1.

The compounds present in propolis extracts were identified by comparing the retention times of each peak with those of the standards, and by UV/Vis, MS and MS^2 data [33]. The list of the polyphenols detected in propolis is shown in Table 1. The composition of the Albanian propolis extracts appeared qualitatively similar,

irrespectively of the solvent used, likely because of the similar extraction properties of the solvents applied in this work.

Table 1. HPLC-UV/DAD, HPLC-ESI-MS and MS^2 data obtained for the analysis of propolis constituents.

Peak Number	Compounds	UV λ_{max}(nm)	$[M + H]^+$	$[M - H]^-$	MS^2 Fragments (*m/z*)
1	Caffeic acid	298,324		179	135
2	*p*-Coumaric acid	298,310		163	119
3	Ferulic acid	298,324		193	149, 134
4	Isoferulic acid	296,321		193	149, 134
5	3,4-Dimethyl-caffeic acid (DMCA)	296,322		207	163, 133
6	Quercetin	256,372	303		285, 257, 229, 165, 153, 149
7	Pinobanksin-5-methyl-ether	288,318 sh	287		269, 241, 152, 91
8	Quercetin-3-methyl-ether	256,358	317		302, 165, 153, 137
9	Chrysin-5-methyl-ether	264,314	269		254, 167
10	Apigenin	267,338	271		253, 153, 119
11	Pinobanksin	291,330 sh	273		255, 227, 153
12	Isorhamnetin	255,372	317		302, 285, 177, 153
13	Luteolin-methyl-ether	266,350	301		286, 217
14	Quercetin-dimethyl-ether	254,356	331		316, 301, 299
15	Galangin-5-methyl-ether	260,302 sh, 352	285		270, 239, 167
16	Pinobanksin-5-methyl-ether-3-*O*-acetate	288,326	329		287, 241
17	Cinnamilidenacetic acid	312	175		157, 129
18	Quercetin-7-methyl-ether	256,372	317		302, 271, 243, 179, 167
19	Quercetin-dimethyl-ether	256,357	331		316, 299
20	Caffeic acid prenyl ester	298,326		247	179, 135
21	Chrysin	268,314 sh	255		209, 153, 129
22	Pinocembrin	290,330 sh	257		215, 153, 131, 103
23	Galangin	260,308 sh, 360	271		165, 153, 105
24	Caffeic acid phenylethyl ester (CAPE)	298,328		283	179, 135
25	Pinobanksin-3-*O*-acetate	294,332 sh	315		273, 255, 227, 153
26	Methoxy-chrysin	266,310 sh, 340 sh	285		270, 257, 242
27	Pinobanksin-3-*O*-propionate	292,330 sh	329		273, 255, 227, 153
28	Pinobanksin-3-*O*-butyrate *	268,310 sh	343		273, 255, 227, 153
29	Pinobanksin-3-*O*-pentanoate *	292,332 sh	357		273, 255, 227, 153
30	Pinobanksin-3-*O*-hexanoate *	282	371		273, 255, 227, 153
31	*p*-Methoxy cinnamic acid cinnamyl ester	278	295		149

Experimental conditions as in Section 2.7. * Or positional isomers. Data are from a representative experiment, out of two performed.

3.3. Antibacterial Activity of Propolis

The antimicrobial activity of the three propolis extracts was evaluated *in vitro*, according to the standardized CLSI/NCCLS method [32]. In detail, nine different dilutions of each extract and their corresponding solvent dilutions were assessed by microbroth dilution. The MIC values were obtained for both propolis EtOH and PG extracts 15.6 µg/mL. Differently, the MIC obtained for propolis PEG 400 extract was as high as 62.5 µg/mL. Based on these results, a kinetic analysis of *Pseudomonas* growth upon exposure to each of the three propolis extracts was carried out, by means of a BLI-based assay known to provide direct and real time assessment of viable cells [11]. For each propolis extract, the MIC values and their corresponding diluted solvent or the medium alone were used (Figure 2). As depicted in Figure 2A, the RLU/s observed in the two controls (medium and EtOH) were similar, although an appreciable anticipation of the curve was observed with EtOH; in contrast, minimal or no RLU/s were ever detected in propolis extract treated samples. These differences were statistically significant, when comparing propolis treated- to solvent treated-*P. aeruginosa*, within the 12 to 16 h time frame. As shown in Figure 2B, in the medium and PG solvent controls, the RLU/sec had comparable time-related trends, with a slight anticipation by the latter. Propolis PG extract also significantly affected bacterial growth; in particular, a detectable signal occurred at 9 h reaching the highest value (30.47 RLU/s) at 15 h. Statistical significance (p <0.05) was achieved at 12–14 h when comparing propolis extract with its solvent. Then, a slight decrease occurred in the luminescence signal down to 26.5 RLU/s at 16 h. When *P. aeruginosa* was grown in the presence or absence of PEG 400 propolis extract, a major toxicity of the solvent per se was evident (Figure 2C). In fact, all the RLU/s were consistently lower than the corresponding medium values. As for PG, the PEG 400 propolis extract did not completely affect bacterial growth. Statistical significance (p <0.05) was reached within the 13–15 h time frame, when comparing propolis extract with solvent. When in parallel groups, gentamicin was used as the positive control; a complete inhibition of BLI-*Pseudomonas* growth was observed, as shown by the little or no luminescence signal detected (Panels A, B and C). Moreover, as depicted in Figure 2 (right panels), the conversion of the RLU in

CFU/mL at 16 h allowed to underline that the most consistent inhibitory effects were indeed observed upon exposure to propolis EtOH extract (approximately 2.5 log decrease).

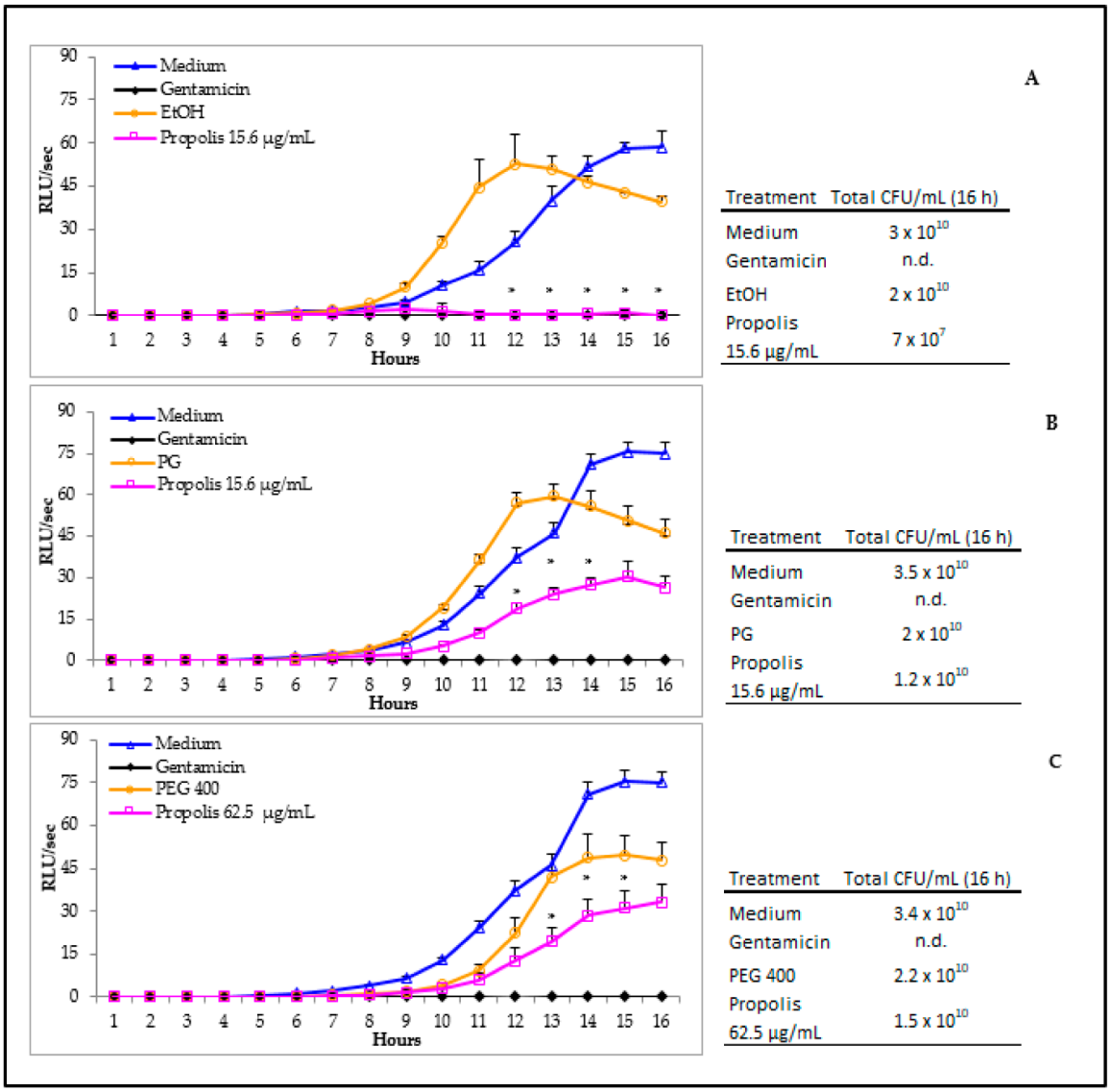

Figure 2. *P. aeruginosa* growth in the presence or absence of the three propolis extracts. The left panels show the total growth of *Pseudomonas* exposed to EtOH (**A**), PG (**B**) and PEG 400 (**C**) propolis extracts at their MICs, as measured kinetically by the BLI-based assay; in the right panels, the data are shown as total CFU/mL at 16 h. The respective solvents at the same dilutions were tested as negative controls; gentamicin (2 mg/mL) was used as the positive control. The results were expressed as mean ± SEM of the RLU/sec of 6–8 replicate samples obtained in two independent experiments. An internal calibration curve was used to convert to the RLU/s in total CFU/mL detectable at time 16 h. **n.d.:** not detectable. Statistical significance was set at * p <0.05 propolis treated *vs* solvent.

3.4. Propolis effects on P. aeruginosa Early Biofilm Formation

Bacterial cells, exposed or not to each propolis extract (used at its own MIC, i.e., 15.6 µg GAE/mL for propolis EtOH and PG extracts and 62.5 µg GAE/mL for propolis PEG 400 extract), were allowed to form a 16-h-old biofilm. Then, the wells were washed to remove the planktonic cells and the residual bioluminescent signal was evaluated to measure the early biofilm produced under the different experimental conditions (Figure 3). Propolis EtOH extract greatly prevented biofilm formation, as indicated by the low bioluminescent signal (panel A, pink column: 3.4 RLU/s). As shown in the right insert of Figure 3A, this drop corresponded to a significant inhibition (81%) in biofilm formation, when propolis extract was compared with the solvent. Differently, *Pseudomonas* cells exposed to EtOH alone produced a biofilm comparable to that developed in the presence of the medium alone (6% inhibition). When BLI-*Pseudomonas* was exposed to propolis PG extract, a still significant biofilm reduction (39%) was observed when propolis extract was compared with the solvent (right insert of Figure 3B), while the solvent per se had irrelevant effect (5% inhibition only). When PEG 400 was employed, an inhibitory effect by the solvent per se was observed (31.7%, with respect to medium); a further decrease occurred upon propolis PEG 400 extract treatment (38%, propolis *vs* solvent) as shown in the right insert of Figure 3C.

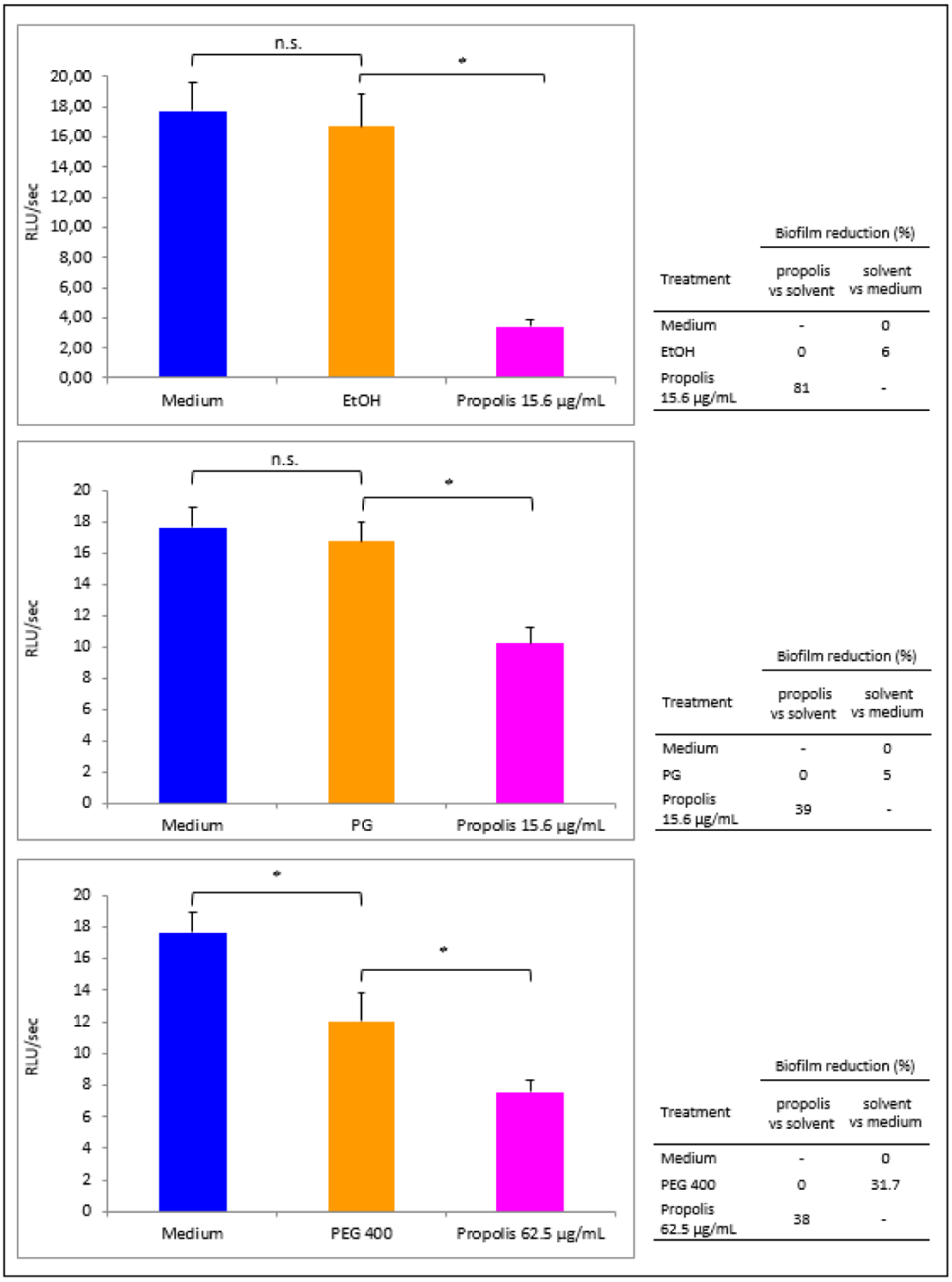

Figure 3. Propolis effects on early biofilm formation by *P. aeruginosa*. BLI-*Pseudomonas* cells were cultured for 16 h in medium or in the presence of EtOH (**A**), PG (**B**) and PEG 400 (**C**) propolis extracts, used at their MICs. Then, the wells were washed and the biofilm formation was quantified by a BLI assay. The luminescence values were plotted as mean ± SEM of 6–8 replicate samples of three independent experiments. Statistical significance was set at * p <0.05. **n.s.:** not significant. The biofilm reduction (%) related to each condition is shown in the right inserts.

3.5. Propolis Effects on Phenazines Release by P. aeruginosa

Phenazines are relevant virulence factors of *P. aeruginosa*. They are essential for adhesion and biofilm formation; they are also involved in oxidative stress, causing cell injury and death [9,10]. For this reason, the levels of three phenazines (PCA, PYO and 1-OH-PHZ) were assessed by HPLC-ESIMS analysis, using *P. aeruginosa* cell-free supernatants from bacteria exposed to propolis EtOH extract, solvent or medium for 16 h. The results showed that propolis extract influenced the release of phenazines to a different extent, depending on the dose used. In particular, as shown in Table 2, the peak areas of PCA, PYO and 1-OH-PHZ decreased and this decrease (expressed as reduction percentage) ranged between 55% and 92.2%, depending on the propolis concentration. In all the cases, a relevant reduction was due to the solvent *per se*, when used at the condition corresponding to the highest propolis concentration.

Table 2. Propolis effects on phenazines release by *P. aeruginosa*.

	Phenazines								
	PCA			PYO			1-OH-PHZ		
		Reduction (%)			Reduction (%)			Reduction (%)	
Treatment	Peak Area	Propolis vs. Medium	Propolis vs. Solvent	Peak Area	Propolis vs. Medium	Propolis vs. Solvent	Peak Area	Propolis vs. Medium	Propolis vs. Solvent
Medium	2.7×10^9	0	-	5.1×10^9	0	-	4.1×10^7	0	-
EtOH	3×10^9	-	0	5.3×10^9	-	0	4.4×10^7	-	0
Propolis 15.6 µg/mL	9.7×10^8	64	68	2.3×10^9	55	56.6	1.7×10^7	58.5	61.4
EtOH	1.7×10^9	-	0	3×10^9	-	0	2.4×10^7	-	0
Propolis 31.2 µg/mL	2.2×10^8	92	87.1	5×10^8	90.2	83.4	3.2×10^6	92.2	86.7

The supernatants of BLI-*Pseudomonas* exposed to the medium, propolis EtOH extract or solvent for 16 h were collected and tested for phenazines levels by HPLC-ESI-MS analysis. The peak area values of the three phenazines (PCA, PYO and 1-OH-PHZ) in their specific chromatographic runs were used for semiquantitative evaluation. The percentage reduction was expressed with respect to the medium alone or to the solvent. The results shown are from a representative experiment out of two performed.

3.6. Propolis effects on eDNA release by P. aeruginosa

It is known that eDNA is a relevant component of *P. aeruginosa* biofilm, essential for the initial adhesion and stability of the sessile community [2,8]. In order to assess

the capacity of *P. aeruginosa* to release eDNA, the bacteria were allowed to produce biofilm in the presence or absence of propolis extract for 16 h; then, cell-free supernatants were tested for the presence of eDNA, as detailed in the Materials and Methods. As shown in Table 3, eDNA release by *P. aeruginosa* was affected by propolis; in particular, the eDNA release decreased in a dose-dependent fashion, to 24.8% and 43.8% when using 15.6 and 31.2 µg/mL propolis, respectively. A partial reduction was also observed by the solvent *per se* (0.081 *vs* 0.121 RFU; 33.1% decrease), when used at the condition corresponding to the propolis dose of 31.2 µg/mL. Moreover, a reduction of eDNA release was observed when comparing propolis *vs* solvent; in this case, the RFU% reduction was 27.8 and 16.1 at 15.6 and 31.2 µg/mL.

Table 3. Propolis effects on eDNA release by *P. aeruginosa*.

Treatment	RFU	eDNA Reduction (%)	
		Propolis vs. Medium	Propolis vs. Solvent
Medium	0.121	0	-
EtOH	0.126	-	0
Propolis 15.6 µg/mL	0.091	24.8	27.8
EtOH	0.081	-	0
Propolis 31.2 µg/mL	0.068	43.8	16.1

The eDNA content was determined in 16 h cell-free supernatants from *P. aeruginosa,* exposed or not to propolis. PI was added before fluorescence reading, as detailed in the Materials and Methods. The results were expressed as mean fluorescence values (RFU) of triplicate samples. Standard deviations values < 5% were omitted. The reduction (%) was calculated with respect to the medium and the solvent. These values are from a representative experiment out of two performed.

3.7. Polyphenol Content in Propolis Exposed or Not to P. aeruginosa

Propolis is a complex mixture of components with a broad spectrum of activities, including antimicrobial, antioxidant, anti-inflammatory, anti-proliferative and anti-angiogenic effects [14–16]. A selected group of compounds, occurring in propolis and known for their biological activities, were analyzed by means of HPLC-ESI-MS. In particular, the peak areas observed in the propolis extract (15.6 µg/mL) exposed or not to *Pseudomonas* for 16 h were compared. The overlapped chromatographic

peaks of eight polyphenols are shown in Figure 4. A reduction in the peak areas of quercetin and CAPE was observed upon propolis exposure to *P. aeruginosa* (black lines) with respect to the controls (propolis extract alone; red lines). A slight decrease was also observed for the pinobanksin-3-*O*-acetate and pinobanksin-3-*O*-butyrate peak areas, while that of chrysin moderately increased.

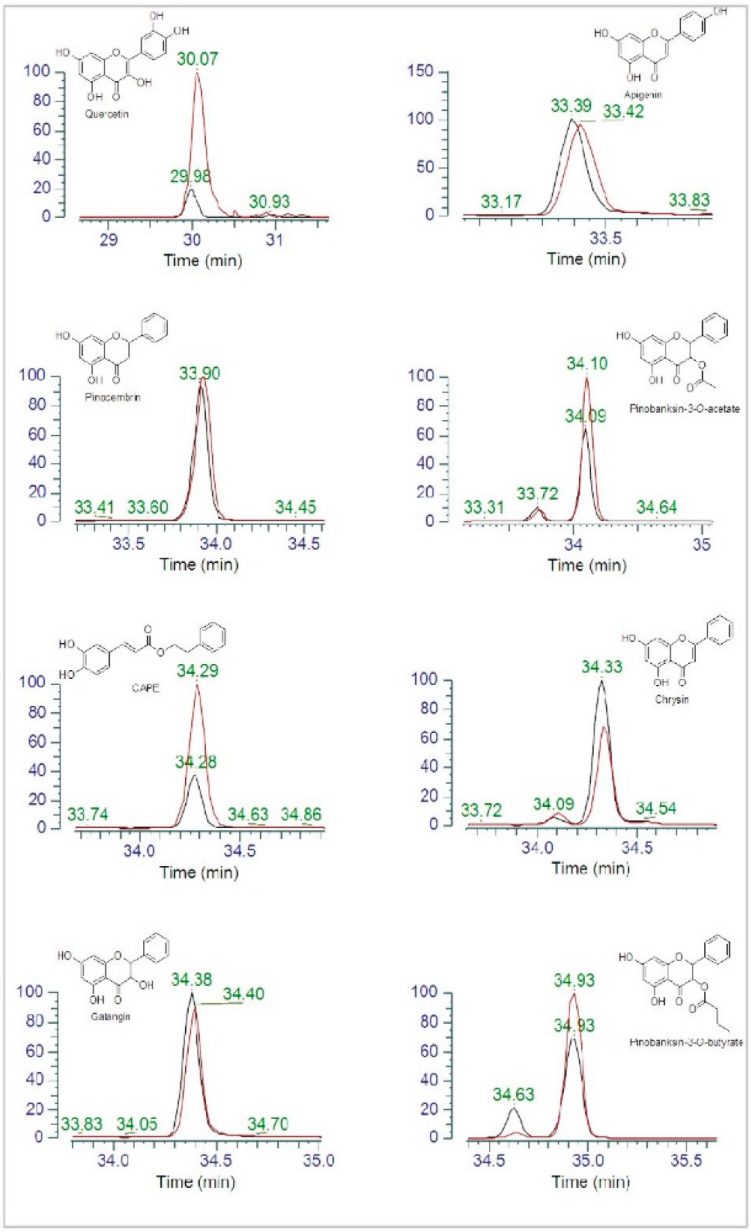

Figure 4. Overlapped peaks of eight polyphenols in propolis extract alone (red line) and upon exposure to *Pseudomonas* (black line). The chemical structure of each compound is also shown. Chromatograms were acquired in the negative ion mode. Data are shown according to the retention times (min).

4. Discussion

Propolis is known to exert antimicrobial activity more efficaciously against Gram-positive than Gram-negative bacteria [34,35]; this is likely due to the peculiar structure of the latter as well as to their ability of producing a wide range of hydrolytic enzymes, which in turn likely break down the active compounds of propolis [34,35]. On these bases, our study has focused on *Pseudomonas*, as a prototype of a hardly attackable pathogen, capable to express numerous virulence factors; its susceptibly to propolis has been evaluated in terms of variation in growth ability, biofilm formation, production of phenazines and eDNA release. In particular, an Albanian propolis, previously described for its therapeutic efficacy in dentistry settings [22], has been extracted using three different solvents, EtOH, PG and PEG 400, and then analyzed by HPLC-UV/DAD and HPLC-ESI-MS. The three profiles obtained, which happen to be comparable irrespectively of the solvent used, show that the most abundant flavonoids are chrysin, galangin, pinocembrin and pinobanksin (and its esters). Moreover, among phenolic acids, caffeic acid derivatives, such as CAPE, also occur in high amounts. Overall, the main components identified in the Albanian propolis closely recall those previously described for propolis samples of Italian origin and, more generically, of European origin [33]. Hereafter, the rough extracts have directly been tested (i.e., without solvent removal) against *Pseudomonas* to avoid loss of any volatile compounds, likely mediating antimicrobial activities [33].

When assessed by MIC assay, the anti-*Pseudomonas* activity of each propolis extract varies from 15.6 µg/mL for EtOH and PG extracts to a four-fold higher value (62.5 µg/mL) for the PEG 400 extract. To better investigate the phenomenon, a highly sensitive bioluminescence-based model has been used [11], in order to real-time monitor both total microbial growth and viable cells organized as biofilm. Preliminary results, aimed at testing serial dilutions in the sub-MIC range, provide evidence for inconsistent inhibitory effects (data not shown), thus orienting the focus of our efforts on the MIC condition. As detailed in the Results section, relevant differences among the three extracts have been observed. In particular, the propolis EtOH extract happens to be the most efficient in decreasing the total microbial growth (the solvent *per se* showing no toxic effects). In contrast, the efficacy of the

other two extracts is partially hampered by the toxicity of both PG and PEG 400 solvents. The anti-*Pseudomonas* effect of propolis EtOH extract, demonstrated by the persistently low BLI signal, is further highlighted when converting the bioluminescence values in CFU/mL at 16 h. Thus, propolis EtOH extract appears as the most interesting anti-*Pseudomonas* among the three extracts tested. Further analysis has revealed that EtOH propolis significantly reduces (81% decrease) the amounts of viable-metabolically active cells, capable of producing biofilm; in contrast, only a partial reduction (26%) of the total biofilm mass occurs under that same condition, as assessed by crystal-violet staining (data not shown), thus suggesting that the amounts of viable cells and, to a lesser extent, the polymeric extracellular matrix accumulation are indeed affected by propolis.

The BLI-based assay allowed us to demonstrate a significant reduction (39%), also when using propolis PG extract. Differently, the propolis PEG 400 extract inhibitory effects are somehow hampered by the action due to the solvent per se (31.7% inhibitory effect). Thus, by a highly sensitive BLI-based system, these findings provide novel evidence on the anti-*Pseudomonas* activity of propolis extracts, underlying that its efficacy also depends on the solvent used. Whether the anti-*Pseudomonas* effects of propolis are mainly due to a direct antibacterial activity or rather to a specific antibiofilm effect remains to be elucidated. Further experiments aimed at evaluating propolis effects on preformed *Pseudomonas* biofilm have revealed a consistent lack of activity independently on the propolis extract considered (data not shown), thus implying that propolis acts as an anti-*Pseudomonas* agent when the pathogen is in a planktonic stage, while an already structured sessile microbial community appears to be insensitive.

P. aeruginosa produces a large amount of water-soluble blue-green phenazine pigments, known to exert antimicrobial [36] and antifungal [37] activities. Phenazines control colony size, favor bacterial adhesion and increase thickness and biomass of *P. aeruginosa* biofilm [38,39]. Moreover, PYO, one of the most studied phenazines, interacts with molecular oxygen to form reactive oxygen species, like H_2O_2, which modify the redox balance. By our study, we expanded the knowledge on *Pseudomonas* as a phenazine producer, demonstrating not only PYO but also PCA

and 1-OH-PHZ release. Moreover, a semiquantitative analysis, performed by comparing the peak areas of each compound under different conditions, indicated that phenazine production occurs to a similar extent in untreated cells (i.e., grown in medium) and in cells exposed to EtOH solvent. Differently, when comparing controls vs. propolis treated groups, a dose-dependent reduction in the peak areas is observed, implying that the overall release of such virulence factors is affected (always >50% decrease) in the presence of propolis. A further analysis, carried out by normalizing phenazine production with respect to the area described under the cell growth curve within the period of 16 h (i.e., total viable cells), indicated an increase of several fold in phenazine production upon propolis treatment (see Table S1). This apparent discrepancy suggests that the single cell secretory activity of the surviving/persisting cells is indeed increased, opening to the hypothesis of a microbial reaction to propolis-mediated insult. Further in-depth studies are needed to elucidate this point.

eDNA, which has been demonstrated to support biofilm stability, is abundantly released by *Pseudomonas* [5,8]. In our model, eDNA levels detected following propolis treatment are impaired in a dose-dependent manner. This decrease is probably related to the reduced number of cells detected at the end of the treatment. In any case, in line with other studies [10], the reduction of eDNA is shown to parallel with the decreased production of PYO.

It is well-known that propolis composition may vary as a function of the geographical area of production, botanical sources, season of collection, etc. [15]. Its therapeutic efficacy is mainly related to its antimicrobial, antioxidant, and antiproliferative effects. The capacity of propolis to modulate the immune system has also been described [40–42]. As main propolis components, polyphenols have been investigated to understand the mechanisms involved in propolis-mediated effects [43]. It has been reported that the antibacterial mechanism of quercetin probably depends on the disruption of target membrane and inactivation of extracellular proteins by forming irreversible complexes [44]. In addition, quercetin and its derivatives may reduce the expression of some inflammatory genes. The effects of this compound have been described in the murine RAW264.7 macrophage cell line,

in terms of oxigenase-1 protein production, transduction of nuclear factor NFkB, decrease in Nrfk2 gene expression and inactivation of miR-155 [45]. Caffeic acid and CAPE have a significant role in cellular cycle and cancer cell apoptosis; bacterial replication also seems to be affected [46,47]. Chrysin and its phosphate ester exert a strong inhibitory effect on Enterovirus EV71 [48]. Galangin significantly suppresses growth of vancomycin-intermediate *Staphylococcus aureus* (VISA) strain Mu50 [49]. Veloz et al. [50] have shown that pinocembrin and apigenin are able to modify the architecture of *S. mutans* biofilm, reducing its thickness; antimicrobial activity against *S. mutans* has also been documented [50]. In our *in vitro* model, BLI-*Pseudomonas* exposure to propolis has been shown to reduce the levels of several phenolic compounds, as shown by a decrease in their corresponding peak areas; in particular, quercetin and CAPE are consistently affected, while pinobanksin-3-O-acetate and pinobanksin-3-O-butyrate appear to slightly decrease when compared to the control (propolis alone). On the contrary, chrysin slightly increases in the supernatants of bacteria treated with propolis. Given the antimicrobial properties of quercetin and CAPE, we favor the idea that these compounds can directly interact with bacteria, thus explaining the decrease observed upon propolis exposure to *Pseudomonas*. A similar phenomenon also occurs when considering pinobanksin esters, known for their antioxidant effects [51]. Whether these compounds may be involved in the inhibition or modulation of oxidation reactions remains to be investigated. Taken together, these data provide initial evidence that *Pseudomonas* affects several polyphenols present in propolis; further in-depth studies are warranted to better address this issue.

5. Conclusions

Our results strengthen the relevance of propolis as a natural antimicrobial product against *P. aeruginosa*, a Gram-negative opportunistic pathogen, known to be highly refractory to disinfectants, antibiotics and host defense mechanisms due to its multiple virulence-factors. By a highly sensitive luminescence-based model, here, we provide the first evidence that *Pseudomonas* exposure to propolis impairs its growth ability, production of biofilm and capacity to release molecules, such as phenazines

and eDNA. Being these peculiarities closely related to *Pseudomonas* pathogenic potential, we might envisage propolis as a precious source of natural compounds for the development of new therapeutic strategies, particularly against biofilm-related infections. In this perspective, it will be interesting to evaluate the antibacterial activity of specific propolis components, widening the panel of both polyphenolics and microbial agents as well.

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CHAPTER 6

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ARTICLE

Antibacterial Effects of MicroRepair®BIOMA-Based Toothpaste and Chewing Gum on Orthodontic Elastics Contaminated *In Vitro* with Saliva from Healthy Donors: A Pilot Study

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Abstract

Several new products with innovative formulations are being proposed to facilitate oral care. Here, we evaluated the effects of a commercially available product, a toothpaste and chewing gum named Biorepair Peribioma, on oral microorganisms of healthy subjects. Saliva from six volunteers was collected during 20 min of mastication of a traditional gum (gum A) and the Biorepair Peribioma gum (gum P). Orthodontic elastics (OE) were *in vitro* contaminated with salivary samples, both A and P, and subsequently exposed or not to a Biorepair Peribioma toothpaste-conditioned supernatant (Tp-SUP). The salivary samples were tested for initial microbial load; hence, the contaminated OE were assessed for microbial growth, adhesion, biofilm formation and persistence; moreover, species identification was assessed. We found that the salivary samples A and P had similar microbial load; upon contamination, microbial adhesion onto the OE was detected to a lower extent when using Saliva P with respect to Saliva A. Microbial growth and biofilm formation, assessed at 24 h, remained at lower levels in OE exposed to Saliva P, compared to Saliva A. This difference between salivary samples A and P was confirmed when measuring biofilm persistence (48 h), while it was lost in terms of microbial re-growth (48 h). The Tp-SUP treatment drastically affected microbial load at 24 h and strongly impaired biofilm formation/persistence, in OE exposed to both salivary samples A and P. Finally, such treatment resulted in consistent overgrowth of Lactobacilli, bacterial species originally present both in the Biorepair Peribioma toothpaste and gum. In conclusion, by an *in vitro* pilot study, we show that the Biorepair Peribioma toothpaste and gum deeply affect oral microorganisms' behavior, drastically impairing their ability to contaminate and produce plaque onto orthodontic devices.

1. Introduction

The oral cavity is a highly complex ecological niche, comprising resident microbial communities that crucially participate at the maintenance of local homeostasis. The latter can be affected by several factors, such as poor oral hygiene, poor diet, and use of drugs, as well as occurrence of dental appliances. In addition, immunodeficiency or a variety of systemic diseases greatly impact on oral health [1,2]. When the homeostasis of the oral microbiota is perturbed by external factors, high numbers of opportunistic pathogens can prevail locally and organize themselves in microbial biofilms. The latter predispose to the onset of oral diseases, by promoting a persistent and deleterious inflammatory process [3].

Saliva is a fundamental element in oral health. It is responsible for continuous clearing of substances and acids neutralization as well; in addition, because of its high content in inorganic ions, it also helps the remineralization process, constantly occurring on teeth surfaces [4]. The protection of oral tissues provided by saliva occurs also by moisturizing and buffering them with high calcium and phosphate concentrations [5]. Saliva contains antimicrobial compounds, such as lysozyme, lactoferrin and lactoperoxidase, which control both resident microbiota and potential oral pathogens, by interfering with their multiplication rate or by a direct killing activity. Lysozyme acts on peptidoglycan, the fundamental component of bacterial cell wall. Lactoferrin acts as an iron-sequestering agent, therefore depriving bacteria of an important element for their metabolic processes. Peroxidase intervenes in the redox balance of the oral environment, by neutralizing the hydrogen peroxide produced by oral bacteria [6]. Saliva also contains essential biological components, such as salivary mucins and glycoproteins, which promote epithelial cell health as well as early dental plaque development [7]. Mucins are very resistant to a variety of proteolytic enzymes occurring in the bacterial plaque around the teeth and in the crevicular area, especially in patients with periodontitis. In addition, mucin is protective and prevents ulceration in soft tissues membranes. In inflammatory diseases, proteases are also generated by polymorphonuclear (PMN) leukocytes. Bacterial and PMN proteases (such as elastase, collagenase and cathepsin) a

ect the integrity of the mucous membranes [8]. Upon secretion, saliva is sterile, but soon it becomes an important vehicle for microorganisms in the oral cavity.

Depending on their ability to closely adhere to biotic and abiotic surfaces and to aggregate with each other, some oral microorganisms can easily produce biofilm both on teeth and on orthodontic appliances and dental prostheses [9]. As a result, they can exert their pathogenic potential locally, negatively a ecting oral homeostasis and/or directly damaging oral tissues, including mucous membrane, enamel and cement [10–13].

The most frequent oral diseases are caries and periodontitis, both associated with the presence of disease-promoting bacterial biofilms on tooth surfaces. Dental caries prevalence is steadily increasing [14] as well as periodontitis, which is commonly observed as a chronic disease, generally developing from gingivitis [15,16]. The microbial species most commonly implicated in caries lesions are *Streptococcus mutans* (*S. mutans*) and *Streptococcus sobrinus* (*S. sobrinus*) [17]. Differently, *Streptococcus salivarius* (*S. salivarius*) is considered for its role in prevention of dental caries [18], periodontal disease [19], and halitosis [20]. In this scenario, the possible synergistic or antagonistic relationships among different microbial species and their impact on tooth structure and/or on periodontium inflammation are being investigated. Interestingly, probiotic bacteria have been proposed as a means to counteract the onset of periodontal disease, thanks to their capacity to inhibit the proliferation of pathogenic germs within the periodontium. In particular, initial studies describe the use of probiotics, such as Lactobacillus and Bifidobacterium spp, to contain the level of *S. mutans*, thus exerting a beneficial role for the oral environment homeostasis [21,22].

Therefore, the “bacteriotherapy” represents a novel approach with enormous potential in the management of oral health and in the containment of local dysmicrobism. In addition, recent studies highlight the effectiveness of various natural substances on supporting oral homeostasis; besides exerting direct antimicrobial activity, such substances have the ability to counteract dental plaque, caries formation, tooth staining, gingival inflammation; also, promotion of enamel recalcification has been documented [23,24].

Several dental malocclusions need to be treated through removable orthodontic clear aligners (Frankel, Bionator, etc.), or fixed orthodontic appliances, such as brackets, tubes or bands, several types of archwires, ligating materials and others. These orthodontic materials, that per se offer a reliable support for microbial adhesion, significantly reduce the efficacy of patient's oral hygiene, making way for debris retention areas; the latter will ultimately facilitate microbial persistence and growth with subsequent development of dental caries, periodontal diseases or other oral infections [25].

Clinical experience reveals that, because of the complexity of brackets design and/or ligation methods, is uncommon to encounter patients with cleaned fixed appliances and microbial plaque carefully removed [26,27]. According to the literature, among different types of orthodontic materials, elastomeric chains or single elastics are highly involved in favoring microbial adhesion/colonization and in turn cross-infections [28,29]. When using these polyurethane devices, it should also be considered that they may undergo alterations to different degree upon contact with physical/chemical agents [30].

The importance of plaque control in maintaining oral health leads to the continuous search for innovative products. In this context, it has recently produced a new fluorine-free toothpaste and a chewing gum, both named Biorepair® PERIBIOMA™ (Coswell S.p.A., Bologna, Italy), have been recently put on the market. These two innovative products contain microRepair®BIOMA (Coswell S.p.A., Bologna, Italy), consisting of biomimetic hydroxyapatite microcrystals combined with selected probiotics aimed at promoting balance of oral microbiota. Such products are expected to repair tooth enamel, protect from microbial plaque formation and help to contain/prevent inflammation and gingival bleeding; in particular, hydroxyapatite crystals are able to bind enamel and dentine, reducing sensitivity and favoring tooth enamel remineralization. The hydroxyapatite crystals also mediate antimicrobial functions by releasing locally calcium, phosphate and zinc ions, especially when the tooth is affected by dental plaque or under acid pH conditions [31,32]. In accordance with the manufacturer's guidelines, they can be used for clinical application at all ages, even in kids, with no risks related to ingestion. Actually, children are possibly

tempted to the idea of chewing a gum and therefore are easily encouraged towards oral hygiene.

Nowadays, there is a lack of microbiological research in this field, which makes the present work a novelty in the field of oral hygiene maintenance, providing an original input for the introduction/implementation of novel easy-to-use tools in clinical practice.

The aim of the present study was to evaluate *in vitro* the effects of the Biorepair Peribioma toothpaste and gum on oral cavity microorganisms. Accordingly, salivary samples, collected from healthy volunteers during gum mastication, were used to contaminate *in vitro* orthodontic elastics (OE); then, microbial growth, biofilm formation and persistence were analyzed at different times in the presence or absence of the toothpaste. Consistent antimicrobial effects were observed. The clinical implications of these findings are discussed.

2. Materials and Methods

2.1. Chewing Gums and Toothpaste-Conditioned Supernatant Preparation

Two different types of chewing gums, a traditional gum (Vigorsol) and the Biorepair® PERIBIOMA™ gum (Coswell S.p.A., Bologna, Italy), both sugar-free, hereafter indicated as gum A and gum P, were provided to the volunteers for saliva collection, as detailed below (see Section 2.2).

The Biorepair® PERIBIOMA™ toothpaste was used to prepare a toothpaste-conditioned supernatant. In particular, 50 gr of toothpaste were suspended in 100 mL of saline solution and incubated for 18 h at 37 °C, under gentle shaking. After incubation, the opalescent solution was centrifuged at 1200 rpm for 10 min and the supernatant collected, aliquoted and frozen at -20 °C (hereafter indicated as Tp-SUP).

2.2. Volunteer Selection and Saliva Collection

Six healthy volunteers were selected according to several inclusions and exclusions criteria, in line with other studies on the efficacy of toothpastes [33,34]. In particular, the inclusion criteria were: both genders, age between 18–64 years, self-

declaration of no basic pathologies and no pregnancy; the exclusion criteria were: non-compliance of the subjects regarding to oral hygiene instructions, use of mouthwashes and antibiotics during the last month. For all the volunteers enrolled, mouthwashes use was prohibited for the entire duration of the study; food and drink were allowed up to 1 h before saliva collection. The enrolled subjects were asked to provide their saliva at least in 3 sessions (every 2 weeks). Here below, are presented the peculiarities of the volunteers: sex: 3 males and 3 females, age range: 25–51; body mass index range: 23, 9–28, 7; no diseases; no pregnancy.

Saliva collection was performed as detailed in the flow chart (Supplementary Material, Figure S1). Firstly, three volunteers (1, 2 and 3) chewed the gum A and the other three volunteers (4, 5 and 6) chewed the gum P, for 20 min, during which saliva samples were collected and named A1, A2, A3 and P4 P5, P6. Secondly, the volunteers were asked to rinse their mouths with fresh water and then the volunteers 1, 2 and 3 chewed the gum P while the volunteers 4, 5 and 6 chewed the gum A, for further 20 min. During that time, a second series of saliva samples (Saliva A4, A5 and A6, and saliva P1, P2 and P3) were collected. Subsequently, all the saliva samples were delivered to the microbiology laboratory, where saliva A1–A6 were pooled as well as the saliva P1–6 (equal volumes from each volunteer were mixed) and immediately used as detailed below.

The present study had been approved by the local Ethics Committee (Prot. AOU: 14075/20; Prot. EC: 0014230/20, dated 21 May 2020).

2.3. Assessment of Microbial Load and Identification of the Main Culturable Species

Initially, the Saliva A and P pools were analyzed to establish the microbial load, by Colony Forming Units (CFU) assay on selective growth media (Tryptic Soy Agar, Sabouraud Dextrose Agar, Mitis Salivarius Agar and De Mann-Rogosa-Sharpe Agar; OXOID; Milan, Italy) under aerobic conditions. The colonies grown after 24 h were phenotypically clustered by color, morphology, size and counted. Subsequently, representative colonies of each type were sub-cultured in Columbia agar plates (OXOID; Milan, Italy) and then identified by MALDI-TOF MS (Bruker Daltonics, Bremen, Germany) and by API® IDENTIFICATION KIT (Biomerieux, Marcy l'Etoile, France).

2.4. Contamination of the Orthodontic Elastics by Saliva

For the present study, the orthodontic elastics (OE) were provided by Leone S.p.A. Florence, Italy. The OE were sterilized by autoclave at 121 °C, for a cycle of 15 min, as indicated elsewhere [35,36]. Then, the OE were exposed to the Saliva A and P pools for *in vitro* contamination, in line with previously described protocols [37]. Briefly, the OE were placed in a 1.5 mL tube containing saliva A or P (100 µL/elastic) and incubated for 1 h at 37 °C with 5% CO_2, under gentle shaking.

2.5. Evaluation of Microbial Adhesion onto OE, Growth and Biofilm Formation

During the 1 h contamination, the samples were concomitantly exposed to the Carboxy-fluorescein Diacetate, Acetoxymethyl Ester (CFDA), that measures both enzymatic activity and cell-membrane integrity (Thermo Fisher Scientific, Waltham, MA, USA), according to manufacturer's instructions. After the 1 h incubation, the elastics were washed with warm saline and transferred to 96 well black microtiter plate, where each well was filled with 100 µL of fresh Tryptic Soy Broth (TSB), supplemented with 2% sucrose (OXOID; Milan, Italy); then, the plate was read at the Fuoroskan microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). The fluorescence signal (excitation/emission: 485/538 nm) was expressed as relative fluorescence units (RFU) and considered as a measure of microbial adhesion to the OE (RFU/OE).

The plate was further incubated for 23 h at 37 °C with 5% CO_2. Then, samples were exposed to saline or Tp-SUP (100 µL/well) and the CFDA reagent was added again for additional 1 h. At the end of such incubation time (24 h), total microbial load/well was assessed by CFU assay and species identification was performed by MALDI-TOF MS and API system, as detailed above. In parallel samples, the OE were washed twice with warm saline, transferred to new wells with fresh TSB plus 2% sucrose and the RFU was measured by Fluoroskan microplate reader. The fluorescence signal (excitation/emission: 485/538 nm) was expressed as RFU and taken as a measure of biofilm formation onto the OE (RFU/OE).

2.6. Evaluation of Microbial Re-Growth and Biofilm Persistence

The OE were further incubated up to 48 h from contamination. Then, microbial re-growth was assessed by CFU analysis and the isolates were identified as detailed above. In parallel groups, the CFDA reagent was added; 1 h later, the OE were washed with warm saline and then residual biofilm was measured by fluorescence analysis, as previously described. The fluorescence emission by live cells (excitation/emission: 485/538 nm) was expressed as RFU and taken as a measure of residual biofilm onto the OE (RFU/OE).

2.7. Statistical Analysis

Data depicted in the graphs are the mean ± SEM from replicate samples of at least 3 independent experiments. Quantitative variables were tested for normal distribution by Shapiro–Wilk test. Statistical differences between groups were analyzed according to Mann–Whitney U test (Figure 1) or Kruskal–Wallis followed by Dunn's multiple comparisons tests (Figures 2 and 3) by using GraphPad Prism 8. Data depicted in the tables are the CFU mean values of 3 independent experiments. Values of **** p <0.0001, *** p <0.001 and * p <0.05 were considered significant.

3. Results

3.1. Initial Microbial Load and Identification of the Main Culturable Species in Saliva A and P

Initially, we evaluated the microbial load and characterized the bacterial species growing in Saliva A and P, under aerobic conditions. As shown in Table 1, the microbial load detected at time 0 in Saliva A was slightly lower as compared to that obtained from saliva P (379.1 *vs* 501 CFU/100 µL×10^4, respectively); such difference did not reach statistical significance. Microbial identification revealed that the most representative species were *Streptococcus mitis/oralis* (*S. mitis/oralis*), reaching 67.01 and 81%, in saliva A and P, respectively. *S. salivarius* was the second most abundant species recovered in saliva A (16.8%) but not in saliva P (2.9%); differently, the latter counted 10.9% of *Neisseria flava/subflava* (*N. flava/subflava*). The third and fourth most abundant species in Saliva A were *S. thermophilus* (8.9%) and *N. flava/subflava* (7.1%), while in Saliva P, the third and fourth most abundant species

were *S. thermophilus* (4%) followed by *S. salivarius* (2.9%). *Rothia aeria* (*R. aeria*) was identified only occasionally in both Saliva A and P, while neither *Candida* nor *Lactobacillus* spp. were ever detected in either salivary sample.

Table 1. Initial microbial load and evaluation of the main microbial species in Saliva A and P.

Species Identified at Time 0 h	**Saliva A**		**Saliva P**	
	CFU	**(%)**	**CFU**	**(%)**
Neisseria flava/subflava	27	7.1	55	10.9
Streptococcus thermophilus	34	8.7	24	4
Streptococcus mitis/oralis	254	67.0	406	81
Streptococcus salivarius	64	16.8	15	2.9
Rothia aeria	<0.1	0.02	1	0.1
Candida Spp.	<0.1	-	<0.1	-
Lactobacillus Spp.	<0.1	-	<0.1	-
Total CFU/100 µL × 10^4	379.1	100	501	100

The two pools of saliva A and P, collected from six healthy volunteers, during mastication of gum A and P, were analyzed to assess the total microbial load, at time 0 h, by CFU assay. The colonies isolated after incubation in selective media were counted and identified by MALDI-TOF MS or API gallery system. The microbial load, expressed as mean of CFU/100 µL×10^4 saliva, and the percentage of bacterial species detected are shown.

3.2. Microbial Adhesion onto OE, Growth and Biofilm Formation

The ability of microorganisms occurring in the saliva A and P to mediate adhesion onto OE was evaluated. Accordingly, two sets of pre-sterilized OE were exposed to each pool of saliva in the presence of CFDA to allow fluorescence staining of cells. After 1 h at 37 °C, a condition commonly used to allow microbial adhesion onto abiotic surfaces [37], the OE were washed with warm saline, transferred to new wells containing fresh medium and analyzed for fluorescence emission. As shown in Figure 1, the fluorescence signal, indicating the amounts of viable and metabolically active cells, was significantly lower in Saliva P, as compared to Saliva A contaminated OE (about 50% difference).

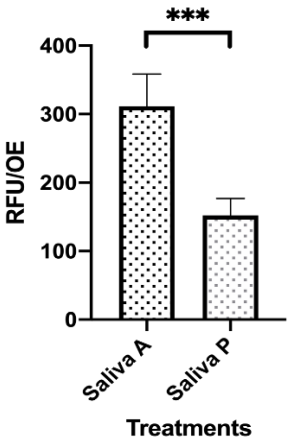

Figure 1. Microbial adhesion onto saliva-contaminated orthodontic elastics (OE). Two sets of OE were exposed to saliva A and P for 1 h at 37 °C. During that time, fluorescence staining of viable cells was allowed by Carboxy-fluorescein Diacetate, Acetoxymethyl Ester (CFDA) addition. Then, the OE were washed with warm saline and transferred to new wells. The plate was read by the Fluoroskan reader and the fluorescence signal was recorded. The depicted values represent the mean (RFU/OE) ± SEM of 48 replicates obtained by 3 independent experiments. *** p <0.001

Subsequently, the contaminated OE were incubated for further 23 h, under standard culture conditions, to allow microbial growth. Then, each set of OE (Saliva A and Saliva P contaminated groups) was split in two sub-groups and exposed to saline or Tp-SUP (in both cases, 100 µL/well were added) for additional 1 h at 37 °C. Subsequently, each sample was assessed for microbial growth by CFU assay, followed by MALDI-TOF MS/API colonies identification. As depicted in Table 2, when comparing the total CFU from Saliva A- and Saliva P-contaminated OE maintained in saline, about 1 log lower values were found in the latter group. Moreover, the treatment with Tp-SUP strongly reduced the CFU, in both Saliva A- and Saliva P-contaminated OE; particularly, the reduction was approximately of 2 logs when comparing Saliva A/saline *vs* Saliva A/Tp-SUP and of about 1 log considering Saliva P/Saline *vs* Saliva P/Tp-SUP.

Table 2. Microbial load onto saliva-contaminated OE subsequently exposed or not to Tp-SUP.

Species Identified at Time 24 h	Saliva A/Saline		Saliva P/Saline		Saliva A/Tp-SUP		Saliva P/Tp-SUP	
	CFU	(%)	CFU	(%)	CFU	(%)	CFU	(%)
Neisseria flava/subflava	<0.1	0.028	<0.1	0.2	<0.1	2.27	<0.1	2.94
Streptococcus thermophilus	7	1.98	<0.1	0.2	<0.1	2.27	<0.1	2.94
Streptococcus mitis/oralis	342	97.1	48	99.1	4	90.9	3	88.2
Streptococcus salivarius	3	0.85	<0.1	0.2	<0.1	2.27	<0.1	2.94
Rothia aeria	<0.1	0.028	<0.1	0.2	<0.1	2.27	<0.1	2.94
Candida Spp.	<0.1	-	<0.1	-	<0.1	-	<0.1	-
Lactobacillus Spp.	<0.1	-	<0.1	-	<0.1	-	<0.1	-
Total CFU/100 µL × 10^4	352.4	100	48.6	100	4.6	100	3.6	100

The two sets of OE, contaminated with Saliva A and P and incubated for 23 h, were split in two sub-groups and exposed to saline or Tp-SUP for additional 1 h. Each sub-group was then analyzed to assess the total microbial load at time 24 h, by CFU assay. The colonies isolated after incubation in selective media were counted, clustered and identified by MALDI-TOF MS or API gallery system. The results are given as microbial load (mean of CFU/100 µL×10^4) and as percent of bacterial species.

In parallel, species identification revealed that the most representative one, both in Saliva A and P/saline groups, was *S. mitis/oralis* (97.1% and 99.1%, respectively). Tp-SUP treatment did not substantially change this percentage. Furthermore, when considering the crude numbers, the *S. mitis/oralis* CFU counted in Saliva A/Tp-SUP-treated samples were 2 logs lower than those in Saliva A/saline-treated samples; such decrease was of about 1 log when comparing Saliva P/Tp-SUP *vs* Saliva P/saline. The second most representative species in Saliva A/saline was *S. thermophilus*, followed by *S. salivarius* and traces of *N. flava/subflava* and *R. aeria*, while, in all the other groups, these species were consistently below the detection limit of the assay. Subsequently, in parallel groups, the saliva-contaminated OE were incubated for 23 h at 37 °C + 5% CO_2 and then exposed to saline or

Tp-SUP for additional 1 h, in the presence of CFDA. Then, the OE were washed with warm saline, transferred to new wells, and the fluorescence emission was measured. As shown in Figure 2, a consistent difference was detected when comparing the RFU onto Saliva A/saline *vs* Saliva P/saline OE although without reaching significance (p =0.0621). Moreover, Tp-SUP treatment drastically reduced the amounts of biofilm, independently of the groups considered. In particular, when comparing Saliva A/saline *vs* Saliva A/Tp-SUP or Saliva P/saline *vs* Saliva P/Tp-SUP, significant decreases were consistently observed upon Tp-SUP treatment in both cases.

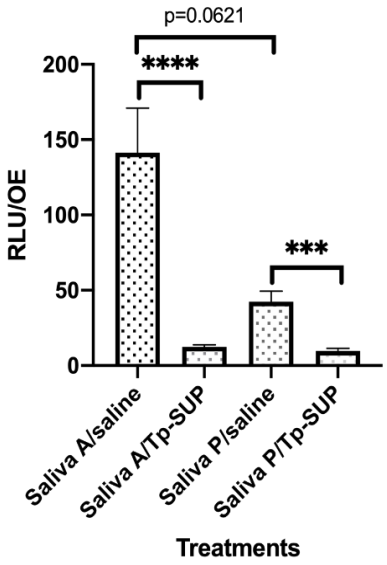

Figure 2. Early biofilm formation onto saliva-contaminated OE exposed or not to Tp-SUP. The saliva-contaminated OE were incubated for 23 h at 37 °C + 5% CO_2 and then exposed to saline or Tp-SUP for additional 1 h, in the presence of CFDA, to allow fluorescence staining. Then, the OE were washed with warm saline and transferred in new wells. The plate was read by the Fluoroskan microplate reader and the fluorescence signal was recorded. The depicted values represent the mean ± SEM (RFU/OE) of 13–16 replicates obtained by 3 independent experiments. **** p <0.0001; *** p <0.001.

3.3. Microbial Re-Growth and Biofilm Persistence

To assess the microbial regrowth under the different conditions, the 4 groups of OE, were incubated for further 24 h (up to time 48 h) in fresh medium and tested by CFU assay and MALDI-TOF MS/API analysis. Table 3 shows that the total CFU detected in all the groups were similar, ranging between 552 to 953×10^4/100 µL. Interestingly, *S. mitis/oralis* was the most representative species both in Saliva A/saline and Saliva P/saline groups (91.01% and 85.3%, respectively),

while, these same species were not detected in Tp-SUP treated groups, no matter whether A or P Saliva groups were considered. In contrast, in the Tp-SUP-treated groups, most of the microbial population was identified as *Lactobacillus* spp. (>90%); such bacteria were undetectable (<0.1 CFU×10^4/100 µL) in the two groups that had not received the Tp-SUP treatment.

Table 3. Microbial re-growth onto saliva-contaminated and Tp-SUP treated OE.

Species Identified at Time 48 h	**Saliva A/Saline**		**Saliva P/saline**		**Saliva A/Tp-SUP**		**Saliva P/Tp-SUP**	
	CFU	**(%)**	**CFU**	**(%)**	**CFU**	**(%)**	**CFU**	**(%)**
Neisseria flava/subflava	<0.1	0.018	<0.1	0.01	<0.1	0.01	<0.1	0.01
Streptococcus thermophilus	13	2.3	63	7.01	57	5.98	57	8.23
Streptococcus mitis/oralis	503	91.0	763	85.3	<0.1	0.01	<0.1	0.01
Streptococcus salivarius	36	6.5	68	7.61	6	0.63	5	0.72
Rothia aeria	<0.1	0.018	<0.1	0.01	<0.1	0.01	<0.1	0.01
Lactobacillus Spp.	<0.1	0.018	<0.1	0.01	890	93.3	630	91.0
Total CFU/100 µL × 10^4	552.3	100	894.3	100	953.3	100	692.3	100

The OE, that had been contaminated with Saliva A and P (time 0 h), incubated for 23 h and exposed or not to the Tp-SUP (for 1 h), were further incubated at 37 °C + 5% CO_2 (up to 48 h) and then analyzed by CFU assay. The colonies isolated after incubation in selective media were counted, clustered and identify by MALDI-TOF MS or API gallery systems. The results are given as microbial load (mean of CFU×10^4/100 µL) and as percentage of bacterial species.

To evaluate biofilm persistence, at time 48 h, the OE were washed again, transferred to new wells and labelled with CFDA fluorescent reagent for 1 h.

As shown in Figure 3, the RFU/OE were consistently lower in Saliva P/saline than in Saliva A/saline groups, although without reaching significance. The exposure to Tp-SUP significantly reduced the RFU/OE in both groups. When comparing Saliva A/saline with Saliva A/Tp-SUP or Saliva P/saline with Saliva P/Tp-SUP, statistically significant differences were observed.

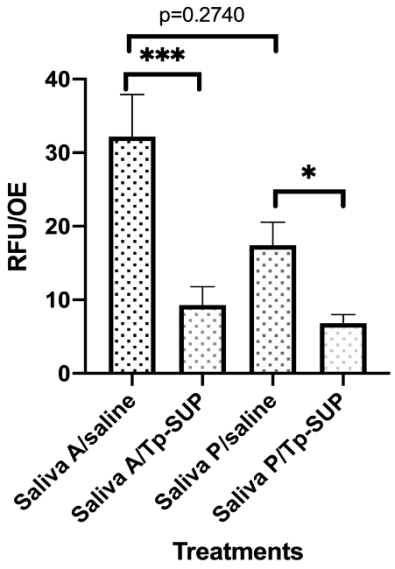

Figure 3. Biofilm persistence following Tp-SUP treatment. The saliva-contaminated OE, that had been exposed to Tp-Sup or saline (at time 23 h, for 1 h) were further incubated at 37 °C + 5% CO_2 up to 48 h in fresh medium. Then, the OE were label with CFDA for 1 h to allow fluorescence staining, washed with warm saline and transferred to new wells containing fresh medium. The plate was read by the Fluoroskan microplate reader and the fluorescence signal was recorded. The depicted values represent the mean RFU/OE ± SEM of 15 replicates obtained by 3 independent experiments. *** p <0.001; * p ≤0.05

4. Discussion

Here, we provide the first *in vitro* data concerning the effects of the Biorepair-PERIBIOMA toothpaste and chewing gum on the ability of oral microbiota to adhere, grow and produce biofilm onto orthodontic devices.

Orthodontic therapy with fixed appliances is commonly used to treat malocclusions and teeth irregularities; in these cases, orthodontic materials such as brackets, tubes and elastics are widely employed. These materials prevent an accurate oral hygiene and promote an increase in microbial load, deposition of debris and formation of plaque. With the aim of promoting a better oral hygiene, a new toothpaste, fluorine-free, and a chewing gum, Biorepair® PERIBIOMA™, added with probiotics, have recently been developed; little information exists on their mechanisms of action.

Among the most important components in the oral environment, saliva is an integral part of oral health; particularly, it plays a role in caries control and bacterial

plaque containment [8]. Although being sterile when secreted [38], saliva immediately becomes a major vehicle of microorganisms, either resident microbiota in healthy subjects as well as pathogens in patients with oral cavity diseases. From here, our choice of using saliva from healthy donors as source of oral microbial population to be employed in an *in vitro* model that, by mimicking orthodontic materials contamination, would allow to evaluate the efficacy of novel tools for oral hygiene. The Biorepair® PERIBIOMA™ gum and a traditional gum, both sugar-free, have been used to facilitate saliva collection.

In line with the literature [39], we show that the microbial species, isolated by a culture-based approach from Saliva A and P are mainly *S. mitis/oralis* and *S. salivarius*, followed by *N. flava/subflava* and *S. thermophilus*. Interestingly, the number of CFU observed in Saliva P is slightly higher (397 *vs* 501 CFU/100 μL×10^4 A *vs* P samples) as compared to that of Saliva A; unexpectedly, the relative abundance of *S. salivarius* appears to be enhanced in the latter group (16.88% *vs* 2.99%, A *vs* P samples). These results suggest that the gum P is slightly more effective in mechanically removing local microorganisms than a traditional gum. The partial fluctuation in relative abundance of some species may be due to their different susceptibly to mechanical gum-mediated detachment and/or to the different ingredients contained in each gum.

Being adhesion a crucial step in microbial biofilm formation, both on oral tissues and abiotic surfaces, here we investigated the ability of microbial communities occurring in Saliva A and Saliva P to adhere onto OE. As indicated by the fluorescence data, adhesion occurs, irrespectively of the saliva employed; yet, the phenomenon is less pronounced when using Saliva P, implying that, although slightly more numerous, this microbial population exhibits a reduced efficacy on binding to an abiotic surface, such as OE. Whether the slightly higher numbers of *S. mitis/oralis* detected in Saliva P may account for the overall less efficient adhesion, remains to be established. Furthermore, we cannot exclude that, in our *in vitro* model, hydroxyapatite crystals and probiotics, present in both Peribioma products, remain and persist in Saliva P samples and, thus, may interfere with microbial adhesion to OE. In line with these data, we show that, after 24 h incubation, the Saliva P/saline

group had 1 log lower microbial CFU than the counterpart samples (Saliva A/saline); once again, we may hypothesize that the hydroxyapatite, contained in gum P and likely persisting in Saliva P samples, may have limited microbial growth. To a similar extent, the Saliva P significantly reduces biofilm formation onto the OE (time 24 h). This phenomenon is also evident at a later time, indicating that saliva collected after chewing the gum P consistently affects biofilm persistence more than Saliva A (17.42 *vs* 33.84 RFU, respectively; at time 48 h).

Here, we have used the Tp-SUP as a simplified and easy-to-use tool, for assessing toothpaste effects against oral microorganisms *in vitro*. In particular, we show that the Tp-SUP exerts relevant antimicrobial activity, regardless of the saliva used to contaminate the orthodontic devices; specifically, both parameters, namely microbial growth and biofilm formation, are significantly impaired. The Tp-SUP-mediated microbial abatement ranges between 1 and 2 logs, which are already evident at time 24 h, when using the CFU assay. In contrast, at later times (48 h), microbial re-growth occurs comparably in all the groups, irrespective of the gum initially used for saliva collection and independently upon the *in vitro* Tp-SUP treatment. Furthermore, it should be noted that, at that time, the microbial composition greatly varies upon Tp-SUP exposure; in particular, the streptococci mostly detected in Saliva A and Saliva P/saline groups (>90%) are drastically displaced by Lactobacilli in Saliva A and Saliva P/Tp-SUP groups (>90%). This finding may be explained considering that the Biorepair® PERIBIOMA™ products also contain probiotics, such as *L. reuteri*, *L. salivarius*, *L. plantarum* and *Bifidobacterium*. The reason for adding probiotics to such oral care products is that such microbial species may adhere to dental tissues and become part of the oral plaque; in turn, they may compete with cariogenic and periodontal pathogens for nutrients and space, thus preventing caries formation and gingivitis [40,41]. In our hands, the late detection of Lactobacilli is in line with previous results (data not shown) showing that, indeed, Tp-SUP culture allowed growth of colonies subsequently identified as Lactobacilli.

The efficacy of Tp-SUP is further emphasized by the fact that also biofilm persistence is significantly affected after such treatment: the extent of the inhibition is similar both in Saliva A and Saliva P contaminated OE.

Overall, by an *in vitro* model, we provide the first evidence on the efficacy of the Biorepair® PERIBIOMA™ toothpaste and gum in impairing adhesion, growth and biofilm formation/persistence by oral microorganisms onto orthodontic devices. We favor the idea that, by different pathways, the hydroxyapatite crystals and the probiotics, abundantly present in such oral care products, may act in concert controlling local microbial communities. Moreover, based on the present *in vitro* data, we may envisage that the combined use of toothpaste and gum can ameliorate oral daily care, thus clinically reducing the risk of developing oral diseases, especially those related to microbial agents.

5. Conclusions

Efficacious strategies helping to maintain a good oral hygiene are necessary to prevent or treat oral diseases, especially when they are associated with microbial biofilms, commonly produced onto oral tissues and dental devices. This *in vitro* pilot study raises the possibility that the daily use of the Biorepair® PERIBIOMA™ gum and toothpaste may, on the one hand, profoundly impact on microbial adhesion, growth and biofilm formation onto abiotic surfaces and, on the other hand, may promote replacement of potential oral pathogens with microorganisms beneficial to oral health. By this *in vitro* prototype, wide-spectrum studies may be pursued opening to other orthodontic/dentistry materials as well as towards other novel health-care products.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-3417/10/19/6721/s1.

Figure 1. Flow-chart of the saliva collection.

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FINAL CONCLUSIONS

These studies provide novel insights, describing a detailed analysis on the antimicrobial and antibiofilm efficacy of new compounds/strategies used in dentistry, including a copper-calcium-hydroxide paste, innovative decontamination systems, propolis extracts and new formulations of toothpaste accompanied with chewing gum.

Initially, a copper-calcium-hydroxide endodontic paste (Cupral) has been demonstrated to exert *in vitro* antimicrobial effects, against *S. aureus*, *P. aeruginosa* and *C. albicans* as well; not only planktonic cell growth is impaired, but also, biofilm formation is prevented and pre-formed biofilm is damaged. Thus, Cupral, a promising endodontically used compound, may be a good candidate for treatment of oral infections, including biofilm-associated ones. The interest on Cupral is further strengthened by the demonstration that it can also efficiently impair microbial contamination occurring onto clear aligners, commonly used for orthodontic therapy.

Clinician concern exists on other dentistry fields as well. For example, peri-implant disease is due to microbial localization on implant surfaces where a biofilm is rapidly and tightly produced. In this respect, we have demonstrated that Bic-40 cleaning system ensures a suitable approach both on smooth and rough surfaces. Such treatment consistently impairs both pre-formed biofilm and microbial re-growth. Given the relevance of microbial contamination in implant failure, these findings open to a novel use of BIC-40 in clinical treatment of pathogen-related peri-mucositis and peri-implantitis.

Over the years, a growing interest exists on natural compounds, such as propolis, as therapeutic agent in different areas of medicine. By a highly sensitive bioluminescence-based model, we provide the first evidence that propolis impairs *Pseudomonas* growth ability and production of biofilm; also, its capacity to release molecules, such as phenazines and eDNA, is deeply affected thus adding new insights on the wide-spectrum antimicrobial effects of propolis.

Finally, our most recent pilot study provides initial in evidence that the combination of the Biorepair® PERIBIOMA™ toothpaste and gum may profoundly inhibit adhesion of oral cavity microorganisms; also, their growth and biofilm formation onto abiotic surfaces are greatly affected.

Overall, these basic research studies open to further *in vivo* investigations, immediately focusing on the most efficacious new dental materials and oral health products, as precious tool against oral pathogens.

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