UNIVERSITÀ DEGLI STUDI DI MODENA E REGGIO EMILIA

International Doctorate Program in Clinical and Experimental Medicine

Curriculum: Public Health

XXXV Cycle

Novel tools to counteract bacterial and fungal biofilm production: *in vitro* studies focused on oral cavity

Supervisor:

Prof. Elisabetta Blasi

Ph.D. Student:

Alessandra Odorici

Co-Supervisor:

Dr. Pierantonio Bellini

Ph.D. Director:

Prof. Marco Vinceti

Anno accademico 2021 / 2022

TABLE OF CONTENTS

ABSTRACT

The oral cavity is a heterogeneous environment, composed of different habitats with different characteristics, suitable for the establishment of different microbial populations. Microbial communities living in the oral cavity play a key role in oral health because they maintain local homeostasis, counteract establishment of pathogens, and modulate immune system. The alteration of this balance allows opportunistic pathogens to predominate; once organized in biofilms, they determine the onset of oral pathologies often difficult to treat. In this context, the use of natural compounds (propolis, herbs, spices) and probiotics represents innovative strategies to maintain/promote oral cavity health, reduce the occurrence of pathologies with infectious etiology and limit the inappropriate use of antibiotics that, in recent decades, has led to a dramatic increase in drug-resistance.

Recently, a toothpaste and chewing gum, containing microcrystals of biomimetic hydroxyapatites (MicroR) and probiotics, have entered the market as interesting tools to limit tooth sensitivity and keep oral microbiota balance. In addition, initial *in vitro* evidence exists on the efficacy of pomegranate extract (PomeGr), as a source of bioactive molecules endowed with multiple beneficial effects, including antimicrobial activity.

Pseudomonas aeruginosa (P. aeruginosa), Staphylococcus aureus (S. aureus) and *Candida albicans (C. albicans)* are responsible for various clinical manifestations also in the oral cavity. Because of their strong ability to adhere and persist on abiotic and biotic surfaces, they often cause biofilm-associated infections, clinically relevant and difficult to treat.

The purpose of this thesis was to evaluate *in vitro* several compounds, as new tools to counteract the production of bacterial and fungal biofilms in the oral cavity.

First, we evaluated *in vitro* the effects of the MicroR and probiotics on oral microorganisms, obtained form saliva of healthy donors. Our study demonstrates that, by these oral hygiene products, microbial ability to persist and produce a biofilm onto orthodontic devices is deeply affected.

Secondly, we investigated the effects of microR and PomeGr on biofilm production by *P. aeruginosa, S. aureus* and *C. albicans*, our data show that PomeGr and MicroR, alone or in combination, cause a decrease in biofilm production in all the cases, though to a different extent.

Thirdly, when evaluating *in vitro* the anti-Candida effects of PomeGr, we demonstrate that fungal growth, biofilm formation, and autoinducer release are indeed altered

upon PomeGr treatment. In addition, by mass spectrometry, we were able to demonstrate the consumption of various phenolic compounds [pedunculagin, punicalin, punicalagin, grenadine, di-(HHDP-galloil-esoside)-pentoside, ellagic-exoside], thus suggesting their role as bioactive molecules against Candida.

Finally, we assessed the role of PomeGr against *P. aeruginosa* demonstrating the reduction of bacterial growth and biofilm formation. Furthermore, the consumption of specific phenolic compounds (pedunculagin, punicalagin, grenadine, punicalin, di-(HHDP-galloyl-hexoside)) and the impairment of bacterial auto-inducers release were detected.

Overall, these *in vitro* data open to clinical studies aimed at defining new protocols to efficiently promote oral homeostasis and counteract infections associated with oral biofilm formation.

RIASSUNTO

La cavità orale è un ambiente eterogeneo, composto da diversi habitat con caratteristiche diverse, idonee allo stabilirsi di popolazioni microbiche diverse. Le comunità microbiche residenti nella cavità orale svolgono un ruolo chiave nella salute della bocca perché mantengono l'omeostasi locale, oppongono resistenza agli agenti patogeni e modulano il sistema immunitario. L'alterazione di questo equilibrio consente ai patogeni opportunisti di prevalere e, una volta organizzatisi in biofilm, determinare l'insorgenza di patologie orali spesso difficili da trattare. In questo contesto, l'impiego di composti naturali (propoli, erbe aromatiche, spezie) e probiotici rappresenta una strategia innovativa per mantenere la salute della cavità orale, ridurre l'insorgenza di patologie ad eziologia infettiva e limitare l'uso inappropriato di antibiotici che, negli ultimi decenni, ha portato ad un drammatico aumento della farmaco-resistenza.

Recentemente, sono entrati in commercio un dentifricio (Peribioma) e una gomma da masticare, contenenti microcristalli di idrossiapatite biomimetici (MicroR) e probiotici; tali prodotti vengono proposti per limitare la sensibilità dentinale e mantenere il microbiota orale in equilibrio. Inoltre, studi *in vitro* hanno fornito le prime evidenze sull'efficacia dell'estratto di melograno (PomeGr), come fonte di molecole bioattive con molteplici effetti benefici, inclusa l'attività antimicrobica.

Pseudomonas aeruginosa (P. aeruginosa), Staphylococcus aureus (S. aureus) e *Candida albicans (C. albicans)* sono microrganismi responsabili di importanti manifestazioni cliniche. A causa, infatti, della loro forte capacità di aderire e persistere su superfici abiotiche e biotiche, spesso causano infezioni associate a biofilm, clinicamente rilevanti e difficili da trattare anche nel cavo orale.

Lo scopo della presente tesi è stato quello di valutare *in vitro* l'attività di vari composti, quali nuovi strumenti per contrastare la produzione di biofilm batterici e fungini nel cavo orale.

In primo luogo, abbiamo valutato *in vitro* gli effetti del dentifricio Peribioma e della gomma masticabile su microrganismi del cavo orale e sulla loro capacità di produrre biofilm. Il nostro studio ha dimostrato la capacità di questi prodotti di influenzare il comportamento dei microrganismi orali, compromettendone la capacità di produrre placca sui dispositivi ortodontici.

In secondo luogo, abbiamo studiato gli effetti di MicroR e PomeGr sulla produzione di biofilm da parte di *P. aeruginosa, S. aureus e C. albicans,* dimostrando che PomeGr

e MicroR, da soli o in combinazione, causano un decremento nella produzione di biofilm in tutti i casi, sebbene con efficacia diversa.

In terzo luogo, abbiamo valutato gli effetti anti-candida di PomeGr dimostrando che la crescita fungina, la formazione di biofilm e il rilascio di autoinduttori sono differentemente modulati dal trattamento del fungo con PomeGr. Inoltre, mediante spettrometria di massa, abbiamo rilevato il consumo di vari composti fenolici [pedunculagina, punicalina, punicalagina, granatina, di-(HHDP-galloil-esoside) - pentoside, ellagico-esoside], suggerendo il loro ruolo come molecole bioattive contro Candida.

Infine, abbiamo valutato il ruolo del PomeGr contro *P. aeruginosa* dimostrando la riduzione della crescita batterica e della formazione del biofilm. Inoltre sono stati rilevati il consumo di composti fenolici specifici (pedunculagina, punicalagina, granatina, punicalina, di-(HHDP-galloil-esoside) e un calo nei livelli di autoinduttori rilasciati.

Questi dati *in vitro* aprono le porte a studi clinici volti a definire nuovi protocolli per promuovere l'omeostasi della cavità orale e contrastare le infezioni associate al biofilm orale.

INTRODUCTION

1. Overview of the oral cavity

A comprehensive examination of the oral cavity is important for obtaining an accurate diagnosis in clinical practice. Important information can be obtained through a systematic evaluation of both hard and soft tissues of the mouth. Where the main goal is to distinguish between health and disease, a full oral examination, along with a complete medical and dental history, also provides valuable information about the patient's overall health and well-being. It should be kept in mind that small changes in oral structure and function can adversely affect the quality of life that individual [1].

1.1 Microbiota of the Oral Cavity

The microbiota of the oral cavity is the second after the intestinal microbiota, in terms of diversity and abundancy of microbial population [2-4]. The oral cavity is unique, because of the concomitant presence of hard and soft tissues (e.g., teeth and oral mucosa, respectively) [5]. Moreover, the oral mucosa consists of the internal mucosa (floor of the mouth, buccal and labial region, and soft palate), masticatory mucosa (gingival region and hard palate) and specialized mucosa (back of the tongue) (Figure 1) [6]. Such heterogeneity may explain the variations in the microbial populations hosted by the different niches.

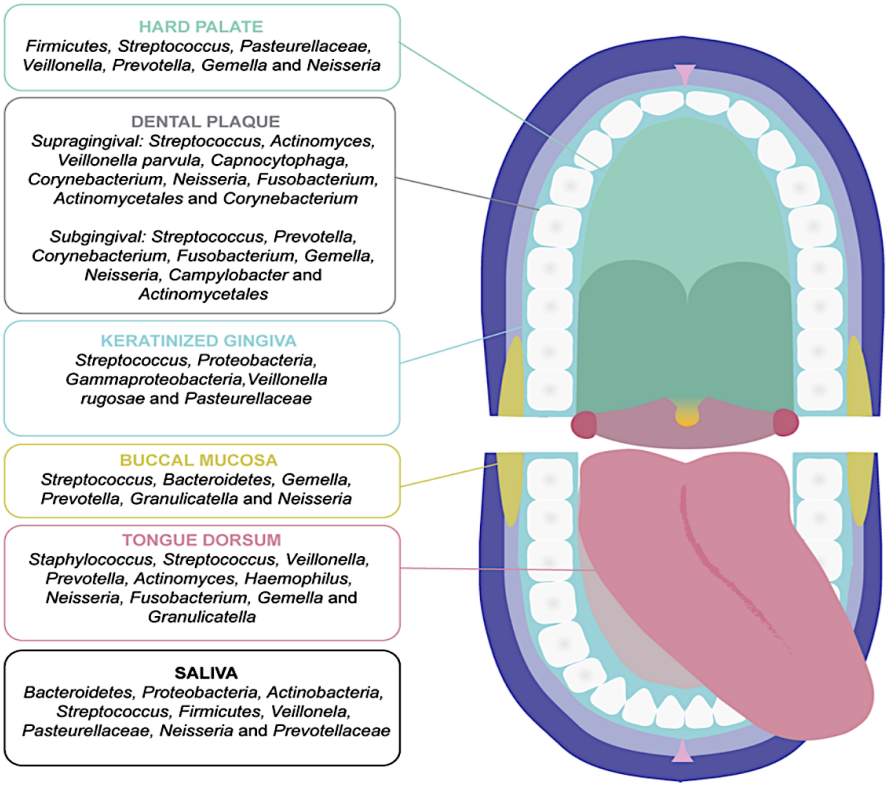


Figure 1. Illustrative diagram depicting the diverse microbiota populations throughout the different oral cavity regions [6]

The lining mucosa is covered by a non-keratinized epithelium, while the masticatory mucosa is covered by a keratinized epithelium. The surface of the specialized mucosa presents a complex structure, containing different types of papillae [7]. In addition, it is worth mentioning that bacterial composition of the dental plaque differs between supragingival and subgingival regions (Figure 1).

Another crucial element of the oral cavity is the saliva, whose microbial composition is similar to that of the tongue lining; certainly, it also includes bacteria drained from the different oral cavity niches [6], as detailed in Figure 1.

Focusing on the oral microbiota, by embracing the complexity of the oral cavity, can improve our understanding of the possible connections that occur between oral microbiota and general health in human being [7].

Different microorganisms, such as bacteria, fungi and archaea, are part of the oral microbiota [8]. In homeostatic balance, these symbiotic communities likely contribute to the healthy oral environment, that, in turn, has a systemic impact on

general health, as reported in the literature [9,10]. Accordingly, any change in the homeostatic balance of the oral microbiota leads to dysbiosis, which is implicated in the occurrence and progression of several local diseases and has often an impact on systemic diseases [11]. This is further emphasized by the detection of oral cavity microorganisms in many distant organ sites, such as the small intestine, heart, lungs, placenta, brain and etc [9,10].

In a healthy oral cavity, free-floating planktonic and sessile cells are present as well. Under the latter circumstances, some of these resident species as well as other pathogenic species may become responsible of oral infections/diseases often refractory to conventional antimicrobials [12]. This is at least partially due to the fact that bacteria have evolved specific survival strategies, including biofilm formation, thus facing with success exogenous stresses and changes in shear forces, nutrient/energy supply, temperature, pH and oxygen content [13].

1.1.1 Dental plaque

The use of advanced microscopy techniques in combination with molecular biology approaches, have allowed us to characterize the structure of the dental plaque in detail, also including the identification and quantitative evaluation of non-culturable bacterial species [14].

There are colonization sites on which dental plaque preferentially forms: stagnant sites that offer protection from physiological movements that occur inside the oral cavity such as swallowing, tongue movements, oral hygiene manoeuvres, salivation and the production of gingival crevicular fluid (GCF). As any other biofilm, the formation and development of the dental plaque occurs in three main phases (Figure 2):

1) attack of the pioneer bacterial species on the acquired film;

The adhesion of the first pioneers to the enamel surfaces is mediated by the acquired pellicle. This organic film is formed through the selective absorption of salivary biopolymers on the tooth surface and acts as a link between the dental hard tissue and the oral environments. In particular, streptococci constitute 60-90% of the bacteria that colonize teeth in the first 4 hours after professional oral hygiene.

Streptococci play a vital role in coaggregating with other bacteria and this can be accomplished through cell-cell interactions such as competition, mutualism and communication.

2) progressive accumulation of microorganisms through colonization of the surface, coadhesion (aggregation between a planktonic bacterium and a bacterium that has already adhered to the surface), coaggregation (aggregation between two cells in suspension) and extensive production of EPS;

Once adhered, the pioneers begin to multiply, giving rise to microcolonies and forming the plaque in the process of formation in a few hours. The latter is further enriched with the presence of mushrooms. Fungi-bacteria interaction is based on physical or chemical factors, competition for nutrients or adhesion sites, and biofilm formation. As the hours go by, the plaque becomes more and more complex, with the coaggregation and colonization of capnophilic, microaerophilic and anaerobic microorganisms.

3) detachment of bacteria from the biofilm and diffusion in the oral cavity environment;

The low oxygen tension microenvironments will allow the colonization of other anaerobic microbial species. This type of plaque, stabilized one, can be observed after several days of development and is more frequently found in sites that are more difficult to reach by oral hygiene maneuvers or salivary flow.

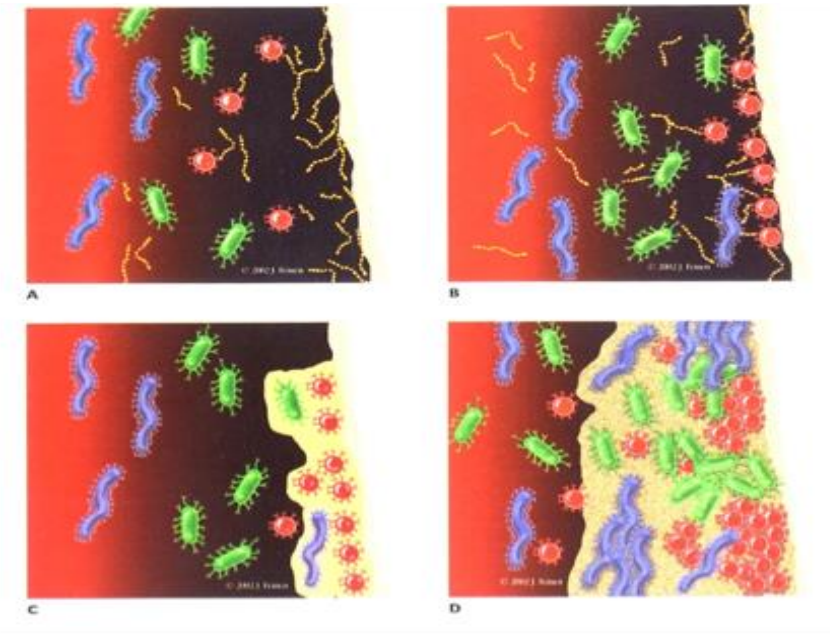


Figure 2. Stages of biofilm formation. A) Proteins in solution are absorbed on the tooth surface forming the acquired pellicle. B) Bacteria begin to bind to the proteins in the scanned film. C) Bacteria of different species interact with each other and with the surface of the tooth and begin to secrete EPS, which engulfs them, giving rise to the biofilm. D) Bacteria multiply in the polysaccharide matrix they produce and begin to form colonies.

Microorganisms are unable to colonize tooth surfaces until the acquired pellicle has formed, which is crucial for their adhesion. The acquired pellicle occurs on tooth surfaces a few minutes after oral hygiene, consists of a variety of salivary glycoproteins (mucins), oligosaccharides, proline-rich proteins (PRP) and staterin that allow bacterial adhesion [15]. Initially, when the biofilm-forming bacteria are located at a considerable distance, electrostatic, hydrophobic and Van der Waals forces allow the reversible adhesion of microorganisms to the tooth surface [15]. Then, the adhesion becomes irreversible and, by the time, bacterial mass increases in volume due to the growth of the microorganisms already adhered to the surface, the adhesion of new bacteria and the synthesis of new EPS.

Some substances of the EPS, such as glucan and fructan, represent important substrates for many microbial species: in fact, they play a fundamental role in protecting microorganisms from host's immune defense mechanisms and this function becomes particularly significant when considering the cariogenic microorganisms [16].

Antagonistic interactions are also present within the biofilm, as during caries development [17]. In fact, an excessive consumption of sugars by the host involves the production of organic acids, such as lactic acid and acetic acid; these acids are produced by bacteria which preferentially use the glycolytic pathway to produce energy, with the consequent reduction of the pH, down to values below 4 in which the balance between mineralization and demineralization is broken in favor of the second (Figure 3) [18]. Plaque acidification by acid producing bacteria, such as *S. mutans* and Lactobacilli, inhibits the growth of acid-sensitive bacterial species, which are no longer able to compete and this causes an increase of cariogenic bacteria within the biofilm [18].

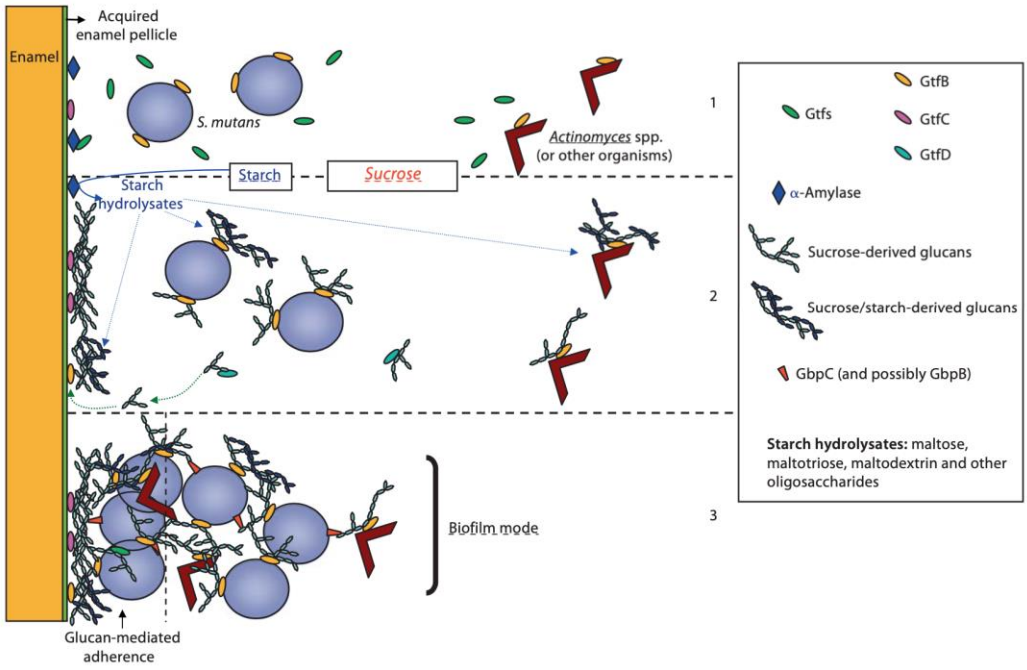


Figure 3. Revised model of Gtf-glucan-mediated bacterial adherence and cariogenic biofilm development [18].

A very important property of the biofilm, which is particularly studied today, is the ability of bacteria to communicate with each other and thus regulate the metabolic processes within the sessile microbial community [19]. This phenomenon, known as quorum sensing (QS), consists in the production of chemical signals, in the form of soluble molecules, by the bacteria, which, depending on the cell density reached, are able to control important processes involved in microbial virulence, such as spore formation, microbial growth, production of specific enzymes/catabolites and the formation/composition of the biofilm itself.

1.2 Relevance of oral hygiene

A complete elimination of the “oralome” (summary of the dynamic interactions between the microbial communities that live in the oral cavity and the host) is not a reasonable perspective, as a eubiotic oralome provides health benefits to the host [20]. In this respect, the identification of novel strategies through with the dysbiotic oralome could by modulated to restore an eubiotic state would be highly reccomended. Among others, frequent oral hygiene is an important tool for controlling the microbial load in the teeth and oral cavity and for preventing periodontitis and bacteremia [20].

Various oral hygiene measures have been proposed for plaque removal [21]. Substantial evidence suggests that individual plaque control procedures, such as conventional brushing with a fluoride toothpaste and anti-plaque chemical mouthwashes usage, significantly reduce gengival inflammation and bacterial plaque, provided that cleaning is sufficiently thorough performed at appropriate time intervals [21,22].

When practiced regularly, oral hygiene maintains dental plaque in an immature state and/or in relatively small amounts, significantly reducing the chances of local damage and also of a bacteremic event compared to conditions where dental plaque is not removed effectively, such as in patients with periodontal disease [23]. Indeed, a correct oral hygiene potentially reduces the risk of systemic diseases in periodontal disease patients. To date, the frequency and duration of brushing required to clear plaque and prevent periodontal disease are still not fully understood, as longitudinal randomized controlled trials are lacking and difficult to perform.

1.2.1 Antimicrobial agents effective against dental diseases

In dentistry, the most common pathologies are of bacterial and fungal origin. As already mentioned, the EPS matrix not only provides scaffolding functions highly relevant for biofilm growth and persistence, but also it promotes crucial properties of biofilms, including spatial and chemical heterogeneities, synergistic/competitive interactions, and increased tolerance to antimicrobial agents [24,25]. For these reasons, numerous efforts have been dedicated at envisaging alternative

antimicrobial strategies, capable to directly affect biofilm-organized communities [26]. Specifically, ideal antibiofilm approaches include prompt elimination of the pathogen, inhibition of an early biofilm, efficient disgregation/dispersion of the sessile community; also, treatments that avoid/limit oral dysbiosis may represent a potentially successful approach against biofilm-associated oral infections [27,28]. Furthermore, the development of innovative strategies in dentistry that, excluding conventional antibiotics may specifically target unique characteristics of the biofilm, will minimize a further expansion of the drug resistance [29].

Nanomaterials have been used since their discovery in the 1980s, in many fields including medicine, where they have revolutionized the concept of what a material is and how can be used [30]. Many nanomaterials, such as those including silver, copper oxide, zinc oxide nanoparticles, titanium oxide, and graphene may exhibit antimicrobial properties [31,32]; similarly, quaternary polyethyleneimized ammonium, chitosan, and silica nanoparticles happen to effectively control/contain biofilm formation [33]. In recent years, attention has been drawn to the application of nanomaterials for drug delivery, either as a carrier with specific affinity to tooth surfaces or as a drug for its inherent antimicrobial properties [34,35]. Importantly, some "smart" drug delivery systems are even able to deliver the drug in a targeted way, releasing the active molecule from the nanoparticles in response to certain environmental stimuli, such as pH, glucose or bacterial products [36].

Dental caries, periodontal disease and endodontic lesions are indeed caused by bacterial and fungal pathogens: *Streptococcus mutans, Streptococcus salivarius, Streptococcus sanguinis, Porphyromonas gingivalis, Prevotella intermedia, Aggregatibacter actinomycetemcomitans, Enterococcus faecalis, Candida albicans,* etc [37]. In this scenario, prevention, mainly based on the reduction of bacterial biofilm through an oral everyday hygiene, is crucial. The active ingredients most used in mouthwashes and toothpastes are chlorhexidine, hyaluronic acid and fluorides. Although effective, they may have some clinical disadvantages: tooth discoloration, taste changes, dry mouth, supragingival tartar accumulation and oral mucosal lesions [38,39].

In preventing and counteracting carious process, it is important to mention the cyclical event of demineralization and remineralization, where the remineralization

process is a natural tooth repair mechanism to restore the presence of minerals in the hydroxyapatite (HAP) crystal lattice in ionic forms [40]. There are many types of remineralizing agents and many of them are being used clinically to treat dental caries, with positive results. In addition to fluorides, remineralizing agents are broadly classified into the following categories: calcium phosphate materials (such as alpha tricalcium phosphate and sodium calcium phosphosilicate), nanoparticles, polydopamine, oligopeptides, theobromine and arginine [41]. Also, nanoparticles of silver (NAg) and amorphous calcium phosphate (NACP) can reduce acid production in dental plaque and facilitate remineralization [42]. Also, it has been demonstrated that NAg may exert antimicrobial effects, through disruption of bacterial membranes and inhibition of some cellular enzymes, while NACP enables the release of calcium/phosphate ions to promote remineralization [43].

2. Innovative compounds for use as antimicrobial tools in oral health

When lifestyle changes, members of the resident microbiome may be altered and their beneficial activity may be lost, sometimes by displacement of certain microbial species [44]. In particular, increasing evidence documents that antibiotic treatment often causes mucosal dysfunctions due to disturbance/alteration of the indigenous mucosal microbiota. Such condition, ending in niches emptied of beneficial bacteria, provides a precious opportunity for pathogens to colonize, persist and proliferate. Thus, “colonization resistance” is named the protective effect of resident microbial communities against pathogens [45].

The use of dietary carbohydrates can lead to extensive demineralization of tooth enamel by the metabolic activity of acidogenic bacteria, while, when occurring, alkalization promotes remineralization and, in turn, restoration of enamel integrity [46]. The two main substrates for alkali generation in the oral biofilm have been shown to be urea and arginine [47]; in particular, through the metabolic pathways of the latter, oral commensals produce alkali [48]. A predominant pathway of arginine metabolism in bacteria is the arginine deiminase system (ADS), producing ornithine, ammonia, CO_2 and ATP [48]. It is known that different microorganisms, such as oral *streptococci*, including *S. sanguinis*, *S. gordonii*, *S. parasanguinis*, *S. intermedius*, *S. cristatus*, and *S. australis*, but also certain *Lactobacillus* species and some

spirochetes can express ADS [46]. Numerous studies have claimed that the use of toothpaste containing arginine can significantly increase the activity of ADS in the plaque of caries-active individuals, bringing an environment similar to that of caries-free individuals [49,50]. These data underline the possibility that increasing the availability of exogenous arginine in the oral environment may be a new approach for biofilm control. Furthermore, several natural products have been proposed as alternative or adjunctive therapies against oral biofilm. Their numerous biological activities make them promising and interesting; yet, their biochemical composition remains uncertain and deserves special attention [51]. Notoriously, in many natural products, such as green tea, propolis, blueberries, grapes, coffee, cocoa, pomegranate, etc., the most active ingredients are polyphenols, namely substances that contain at least one aromatic ring with one or more hydroxyl groups, and other substituents [52].

2.1 Biomimetic compound – Microepair

Teeth, like bone tissue of vertebrates, are formed by a natural composite material, whose main component is hydroxyapatite carbonate (65-70% of the dentin and over 90% of the enamel) [53].

Tooth enamel is mainly made up of large prismatic crystals of hydroxyapatite with a high degree of crystallinity and a very low percentage of protein component (Figure 4). The enamel of an adult individual does not contain cells and is therefore unable to regenerate [53]. Any deterioration is irreversible because there are no biological processes capable of repairing the damaged enamel.

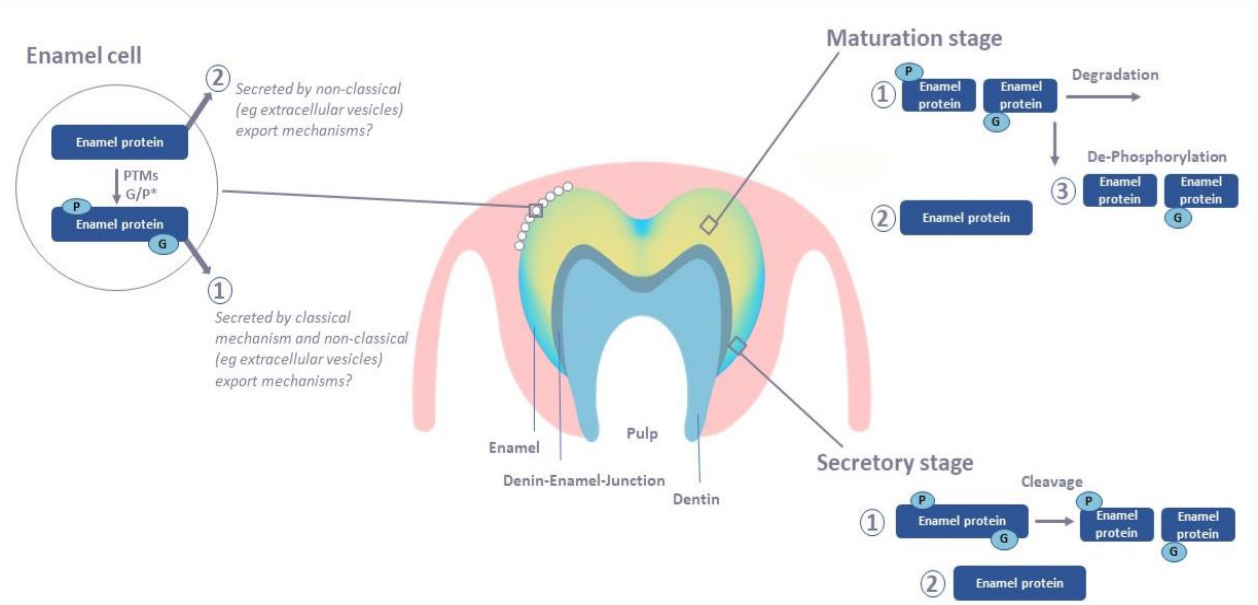


Figure 4. Schematic of potential mechanism of enamel formation. Blue: enamel that is more recently deposited, younger, with higher protein content. Yellow: enamel that is further developed and more mature, older enamel, more mineralized with lower protein content [53].

Even the dentin, exposed to the oral environment, does not undergo regeneration since the deposit front of the reparative dentin occurs on the internal surfaces of the crown, i.e., near the dental pulp [54]. It follows that any reparative intervention is entrusted to the use of materials or substances unrelated to the metabolism of the dental tissue, substances that can be precipitated from saliva or synthetic materials.

The microRs are crystals of carbonate hydroxyapatite-Zinc substituted. They are created in the laboratory, through an innovative synthesis, by controlling the micrometric aggregation of nanometric crystals. The composition of these microparticles is very similar to that of dental enamel. This similarity gives the microRs the biomimetic property, so that the microparticles can integrate within the enamel and dentin in a persistent way, in any condition, repairing the tissue in a uniform and natural way [55]. Thanks to the presence of positive and negative free charges in the phosphate and calcium ions, the hydroxyapatite of the microRs interacts with the hydroxyapatite of the tooth by linking to it in a persistent manner. The microRs form a homogeneous coating on the tooth surface, penetrating the enamel lesions; it chemically binds to, repairs and remineralizes the dental tissue.

2.2 Biological compounds: Probiotics & Pomegranate

2.2.1. Probiotics

Probiotics are microorganisms which, if administered in adequate quantities, confer benefits on the health of the host, through mechanisms of stabilization of the microbial flora and modulation of the immune system [56]. The use of probiotics has traditionally been employed for intestinal eubiosis reconstitution; recently, a growing number of potential applications in dentistry has been proposed.

Several foods naturally contain vital non-pathogenic microorganisms, that when ingested in adequate quantities, may exert beneficial effects on the human organism. According to Chen et al., [57] these microorganisms are live bacteria, must not be pathogenic or toxic, deal with the rebalancing of the intestinal bacterial flora and are "lactic ferments", because are capable of producing lactic acid. These "good" bacteria, which mostly belong to the genera *Lactobacillus* and *Bifidobacterium*, carry out a protective activity of the epithelium through the reduction of the intraluminal pH. Also, they compete with potential pathogens. Furthermore, they affect mucosal permeability and in turn also intestinal motility. They can stimulate the secretion of mucus and exert an antimicrobial action, as they are responsible for the production of H_2O_2 and bacteriocins. They also modulate host immune system at mucosal level, as they promote the production of anti-inflammatory cytokines and the decrease of the pro-inflammatory ones. Finally, they stimulate IgA production, cell-mediated immunity and NK cell activity [57].

The oral cavity is considered a favorable habitat for studying the relevance of probiotics [58]. The administered probiotics can adhere to the surfaces of mucosal cells, colonize them and possibly play a protective role by competing with oral pathogens.

Given the microbial etiology of caries and taking into account the main treatment strategies of periodontal disease are: a) the elimination of specific pathogens and b) the suppression of an excessive inflammatory response by the host. The probiotics may represent an innovative approach, possibly used in support of traditional strategies in the treatment of oral diseases [59].

2.2.2. The pomegranate: the plant and the fruit

The pomegranate (*Punica granatum*, L.) is a plant belonging to the Punicaceae family (or Lythraceae, according to the Angiosperm Phylogeny Group classification). It is a dicotyledonous angiosperm plant, superior that reproduce itself through flowers. It is a small tree which, thanks to its ability to adapt to adverse conditions, is widespread in arid and semi-arid regions of Iran, Himalaya mountains, northern India, China, United States and many Mediterranean regions [60]. In Italy, the most common cultivation is the horse tooth, most suitable for this area, because it is resistant to the rigid and low temperature of winter.

The pomegranate can be divided into different anatomical compartments, each having interesting pharmacological and nutraceutical activities: red and hermaphroditic flowers, fruits with hard and leathery yellow-red skin, opposite and glossy leaves, bark and roots [60].

The fruit of the pomegranate, pomegranate or grenade, is a round berry with a hard and leathery yellow-red exocarp (skin), a light yellow spongy mesocarp (albedo) and a thin and membranous endocarp which protects the seeds surrounded by the sarcotesta (aryl), external and fleshy envelope of the ruby red seed (Figure 5).

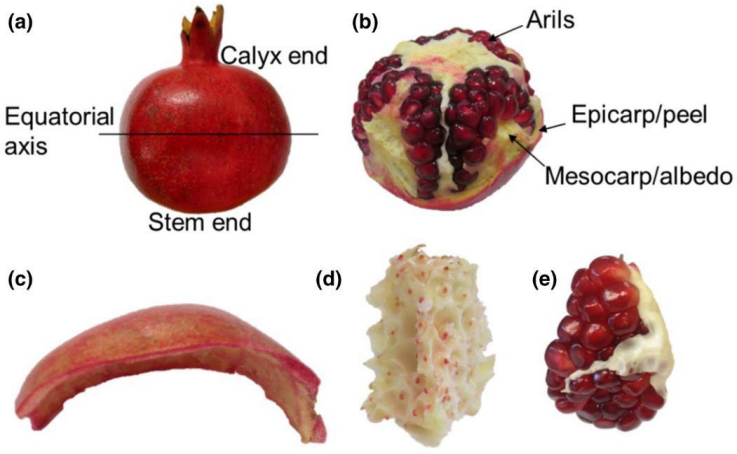


Figure 5. Composition of the pomegranate fruit (a = external section, b = internal section, c = exocarp or peel, d = mesocarp, e = endocarp, sarcotesta or aril).

The sarcotesta, the edible part of the fruit, represents 52% of the whole fruit by weight and includes 78% juice and 22% seeds. The fresh juice is a source of

polysaccharides, organic acids, pectins, polyphenols, vitamins and minerals, while the seeds are rich in lipids, proteins and polyphenols [61].

The peel and the mesocarp, inedible fractions, represent the remaining 48% of the whole fruit and are the main source of bioactive substances, such as polyphenols, flavonoids, phenolic acids and polysaccharides [62]. Approximately 48 phenolic compounds have been identified, with antioxidant, antimicrobial, antibacterial, anti-inflammatory, and anticancer properties. The most interesting compounds include (Figure 6):

• anthocyanidins (delphinidin, cyanidin, pelargonidin), water-soluble pigments and flavonoids responsible for the color and bitter taste,

• gallotannins (gallic acid esters) and ellagitannins (ellagic acid, ellagic acid esters, punicalagin, pedunculagin), tannins hydrolyzable in sugars and free phenolic acids,

• hydroxycinnamic acids (coumaric acid, caffeic acid) and hydroxybenzoic acids (gallic acid): phenolic acids.

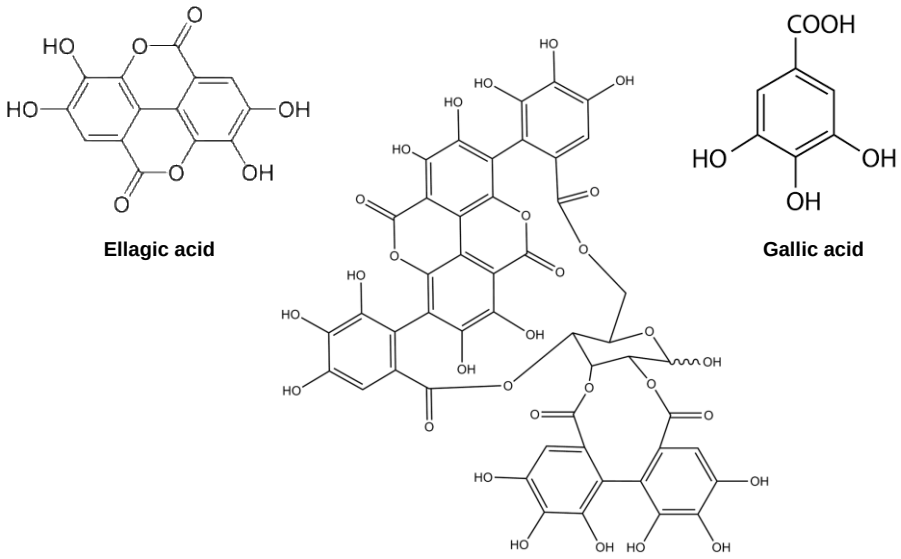


Figure 6. Main phenolic compounds of pomegranate (ellagic acid, gallic acid and punicalagin).

The antioxidant capacity of hydrolysable tannins and anthocyanidins is linked to their structure: they are hydroxyphenols, polyphenols with hydroxyl groups, capable of donating hydrogen atoms to free radicals and inhibiting the oxidation reaction [63].

REFERENCES

1. Madani M, Berardi T, Stoopler ET. Anatomic and examination considerations of the oral cavity. Med Clin North Am. 2014 Nov;98(6):1225-38.

2. Deo PN, Deshmukh R. Oral Microbiome: Unveiling the Fundamentals. J. Oral Maxillofac. Pathol. 2019, 23, 122–128.

3. Caselli E, Fabbri C, D'Accolti M, Soffritti I, Bassi C, Mazzacane S, Franchi M. Defining the Oral Microbiome by Whole-Genome Sequencing and Resistome Analysis: The Complexity of the Healthy Picture. BMC Microbiol. 2020, 20, 120.

4. Belibasakis GN, Bostanci N, Marsh PD, Zaura E. Applications of the Oral Microbiome in Personalized Dentistry. Arch. Oral Biol. 2019, 104, 7–12.

5. Lynge Pedersen AM, Belstrøm D. The Role of Natural Salivary Defences in Maintaining a Healthy Oral Microbiota. J. Dent. 2019, 80, S3–S12.

6. Giordano-Kelhoffer B, Lorca C, March Llanes J, Rábano A, del Ser T, Serra A, Gallart-Palau X. Oral Microbiota, Its Equilibrium and Implications in the Pathophysiology of Human Diseases: A Systematic Review. Biomedicines 2022, 10, 1803.

7. Yamashita Y, Takeshita T. The Oral Microbiome and Human Health. J. Oral Sci. 2017, 59, 201–206.

8. Hoare A, Marsh PD, Diaz PI. Ecological Therapeutic Opportunities for Oral Diseases. Microbiol. Spectr. 2017, 5, 235–265.

9. Sedghi L, Di Massa V, Harrington A, Lynch SV, Kapila YL. The Oral Microbiome: Role of Key Organisms and Complex Networks in Oral Health and Disease. Periodontol. 2000 2021, 87, 107–131.

10. Konkel JE, O'Boyle C, Krishnan S. Distal Consequences of Oral Inflammation. Front. Immunol. 2019, 10, 1403.

11. Lee YH, Chung SW, Auh QS, et al. Progress in Oral Microbiome Related to Oral and Systemic Diseases: An Update. Diagnostics 2021, 11, 1283.

12. Cortelli SC, Costa FO, Rode Sde M, et al. Mouthrinse Recommendation for Prosthodontic Patients. Braz. Oral Res. 2014, 28, 1–10.

13. Berger D, Rakhamimova A, Pollack A, Loewy Z. Oral Biofilms: Development, Control, and Analysis. High Throughput. 2018, 7(3), 24.

14. Valm AM. The Structure of Dental Plaque Microbial Communities in the Transition from Health to Dental Caries and Periodontal Disease. J Mol Biol. 2019, 431(16), 2957-2969.

15. Willems HM, Xu Z, Peters BM. Polymicrobial Biofilm Studies: From Basic Science to Biofilm Control. Curr Oral Health Rep. 2016, 3(1), 36-44.

16. Netea MG, Schlitzer A, Placek K, Joosten LAB, Schultze JL. Innate and Adaptive Immune Memory: An Evolutionary Continuum in the Host's Response to Pathogens. Cell Host Microbe. 2019, 25(1), 13-26.

17. Jakubovics NS, Goodman SD, Mashburn-Warren L, Stafford GP, Cieplik F. The dental plaque biofilm matrix. Periodontol 2000. 2021, 86(1), 32-56.

18. Bowen WH, Koo H. Biology of Streptococcus mutans-derived glucosyltransferases: role in extracellular matrix formation of cariogenic biofilms. Caries Res. 2011, 45(1), 69-86.

19. Mukherjee S, Bassler BL. Bacterial quorum sensing in complex and dynamically changing environments. Nat Rev Microbiol. 2019, 17(6), 371-382.

20. Radaic A, Kapila YL. The oralome and its dysbiosis: New insights into oral microbiome-host interactions. Comput Struct Biotechnol J. 2021, 19, 1335-1360.

21. Sälzer S, Graetz C, Dörfer CE, Slot DE, Van der Weijden FA. Contemporary practices for mechanical oral hygiene to prevent periodontal disease. Periodontol 2000 2020, 84, 35–44.

22. Chapple ILC, Van der Weijden F, Doerfer C, et al. Primary prevention of periodontitis: managing gingivitis. J Clin Periodontol 2015, 42(16), S71–6.

23. Wade WG. The oral microbiome in health and disease. Pharmacol Res 2013, 69, 137–43.

24. Lebeaux D, Ghigo JM, Beloin C. Biofilm-related infections: bridging the gap between clinical management and fundamental aspects of recalcitrance toward antibiotics. Microbiol Mol Biol Rev. 2014, 78(3), 510-43.

25. Koo H, Xiao J, Klein MI. Extracellular polysaccharides matrix-an often-forgotten virulence factor in oral biofilm research. Int J Oral Sci. 2009, 1(4), 229-34.

26. Baltzer SA, Brown MH. Antimicrobial peptides: promising alternatives to conventional antibiotics. J Mol Microbiol Biotechnol. 2011, 20(4), 228-35.

27. Rabin N, Zheng Y, Opoku-Temeng C, Du Y, Bonsu E, Sintim HO. Agents that inhibit bacterial biofilm formation. Future Med Chem. 2015, 7(5), 647-71.

28. Gao L, Koo H. Do catalytic nanoparticles offer an improved therapeutic strategy to combat dental biofilms? Nanomedicine (Lond). 2017, 12(4), 275-279.

29. Davies D. Understanding biofilm resistance to antibacterial agents. Nat Rev Drug Discov. 2003, 2(2), 114-22.

30. Hulla JE, Sahu SC, Hayes AW. Nanotechnology: History and future. Hum Exp Toxicol. 2015, 34(12), 1318-21.

31. Allaker RP, Memarzadeh K. Nanoparticles and the control of oral infections. Int J Antimicrob Agents. 2014, 43(2), 95-104.

32. Hemeg HA. Nanomaterials for alternative antibacterial therapy. Int J Nanomedicine. 2017, 12, 8211-8225.

33. Besinis A, De Peralta T, Tredwin CJ, Handy RD. Review of nanomaterials in dentistry: interactions with the oral microenvironment, clinical applications, hazards, and benefits. ACS Nano. 2015, 9(3), 2255-89.

34. Benoit DS, Koo H. Targeted, triggered drug delivery to tumor and biofilm microenvironments. Nanomedicine (Lond). 2016, 11(8), 873-9.

35. Cheng L, Zhang K, Weir MD, Melo MA, Zhou X, Xu HH. Nanotechnology strategies for antibacterial and remineralizing composites and adhesives to tackle dental caries. Nanomedicine (Lond). 2015, 10(4), 627-41.

36. Park K. Controlled drug delivery systems: past forward and future back. J Control Release. 2014, 190, 3-8.

37. Kuang X, Chen V, Xu X. Novel approaches to the control of oral microbial biofilms. BioMed Res. Int. 2018, 2018, 6498932.

38. James P, Worthington HV, Parnell C, et al. Chlorhexidine mouthrinse as an adjunctive treatment for gingival health. Cochrane Database Syst. Rev. 2017, 3, CD008676.

39. Marinho VC, Chong LY, Worthington HV, Walsh T. Fluoride mouthrinses for preventing dental caries in children and adolescents. Cochrane Database Syst. Rev. 2016, 7, CD002284.

40. Hemagaran G. Remineralisation of the tooth structure—the future of dentistry. International Journal of PharmTech Research, 2014, 6, 487–493.

41. Arifa MK, Ephraim R, Rajamani T. Recent Advances in Dental Hard Tissue Remineralization: A Review of Literature. Int J Clin Pediatr Dent. 2019, 12(2), 139-144.

42. Li F, Wang P, Weir MD, Fouad AF, Xu HH. Evaluation of antibacterial and remineralizing nanocomposite and adhesive in rat tooth cavity model. Acta Biomater. 2014, 10(6), 2804-13.

43. Cheng L, Weir MD, Xu HH, Antonucci JM, Kraigsley AM, Lin NJ, Lin-Gibson S, Zhou X. Antibacterial amorphous calcium phosphate nanocomposites with a quaternary ammonium dimethacrylate and silver nanoparticles. Dent Mater. 2012, 28(5), 561-72.

44. Mantravadi PK, Kalesh KA, Dobson RCJ, Hudson AO, Parthasarathy A. The Quest for Novel Antimicrobial Compounds: Emerging Trends in Research, Development, and Technologies. Antibiotics 2019, 8, 8.

45. Stecher B, Berry D, Loy, A. Colonization Resistance and Microbial Ecophysiology: Using Gnotobiotic Mouse Models and Single-Cell Technology to Explore the Intestinal Jungle. FEMS Microbiol. Rev. 2013, 37, 793–829.

46. Burne RA, Marquis RE. Alkali production by oral bacteria and protection against dental caries. FEMS Microbiol Lett. 2000, 193(1), 1-6.

47. He J, Hwang G, Liu Y, Gao L, Kilpatrick-Liverman L, Santarpia P, Zhou X, Koo H. l-Arginine Modifies the Exopolysaccharide Matrix and Thwarts Streptococcus mutans Outgrowth within Mixed-Species Oral Biofilms. J Bacteriol. 2016, 198(19), 2651-61.

48. Bowen WH, Burne RA, Wu H, Koo H. Oral Biofilms: Pathogens, Matrix, and Polymicrobial Interactions in Microenvironments. Trends Microbiol. 2018, 26(3), 229-242.

49. Nascimento MM, Browngardt C, Xiaohui X, Klepac-Ceraj V, Paster BJ, Burne RA. The effect of arginine on oral biofilm communities. Mol Oral Microbiol. 2014, 29(1), 45-54.

50. Zheng X, He J, Wang L, Zhou S, Peng X, Huang S, Zheng L, Cheng L, Hao Y, Li J, Xu J, Xu X, Zhou X. Ecological Effect of Arginine on Oral Microbiota. Sci Rep. 2017, 7(1), 7206.

51. Jeon JG, Rosalen PL, Falsetta ML, Koo H. Natural products in caries research: current (limited) knowledge, challenges and future perspective. Caries Res. 2011, 45(3), 243-63.

52. Cheng L, Li J, He L, Zhou X. Natural products and caries prevention. Caries Res. 2015, 49(1), 38-45.

53. Gil-Bona A, Bidlack FB. Tooth Enamel and its Dynamic Protein Matrix. Int J Mol Sci. 2020, 21(12), 4458.

54. Wen B, Huang Y, Qiu T, Huo F, Xie L, Liao L, Tian W, Guo W. Reparative Dentin Formation by Dentin Matrix Proteins and Small Extracellular Vesicles. J Endod. 2021, 47(2), 253-262.

55. Butera A, Pascadopoli M, Gallo S, Lelli M, Tarterini F, Giglia F, Scribante A. SEM/EDS Evaluation of the Mineral Deposition on a Polymeric Composite Resin of a Toothpaste Containing Biomimetic Zn-Carbonate Hydroxyapatite (microRepair®) in Oral Environment: A Randomized Clinical Trial. Polymers (Basel). 2021, 13(16), 2740.

56. Suez J, Zmora N, Segal E, Elinav E. The pros, cons, and many unknowns of probiotics. Nat Med. 2019, 25(5), 716-729

57. Chen R, Du M, Liu C. Strategies for dispersion of cariogenic biofilms: applications and mechanisms. Front Microbiol. 2022, 13, 981203.

58. Farias da Cruz M, Baraúna Magno M, Alves Jural L, et al. Probiotics and dairy products in dentistry: A bibliometric and critical review of randomized clinical trials. Food Res Int. 2022, 157, 111228.

59. Seminario-Amez M, López-López J, Estrugo-Devesa A, Ayuso-Montero R, Jané-Salas E. Probiotics and oral health: A systematic review. Med Oral Patol Oral Cir Bucal. 2017, 22(3), e282-e288.

60. Fahmy HA, Farag MA. Ongoing and potential novel trends of pomegranate fruit peel; a comprehensive review of its health benefits and future perspectives as nutraceutical. J Food Biochem. 2022, 46(1), e14024.

61. Magangana TP, Makunga NP, Fawole OA, Opara UL. Processing Factors Affecting the Phytochemical and Nutritional Properties of Pomegranate (Punica granatum L.) Peel Waste: A Review. Molecules. 2020, 25(20), 4690.

62. Gosset-Erard C, Zhao M, Lordel-Madeleine S, Ennahar S. Identification of punicalagin as the bioactive compound behind the antimicrobial activity of pomegranate (*Punica granatum* L.) peels. Food Chem. 2021, 352, 129396.

63. Fahmy H, Hegazi N, El-Shamy S, Farag MA. Pomegranate juice as a functional food: a comprehensive review of its polyphenols, therapeutic merits, and recent patents. Food Funct. 2020, 11(7), 5768-5781.

AIM OF THE STUDY

Microbial communities in the oral cavity play a key role, because they maintain local homeostasis, provide a barrier against pathogens, and modulate host immune system. The alteration of this balance allows opportunistic pathogens to predominate and organize themselves in biofilms, determining the onset of oral pathologies often difficult to treat. In this context, the use of natural compounds (propolis, herbs, spices) and probiotics represents an innovative strategy to maintain the health of the oral cavity and reduce the occurrence of infectious diseases, limiting the inappropriate use of antibiotics that, in recent decades, has led to a dramatic increase in drug-resistance.

The purpose of this thesis was to evaluate *in vitro* new tools to counteract the production of bacterial and fungal biofilms in the oral cavity. The results obtained during the 3-years PhD, which were published in peer-reviews journals, are herebelow organized in 4 main chapters, as follows:

- **Chapter 1** – 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 2** – Novel options to counteract oral biofilm formation: *in vitro* evidence
- **Chapter 3** – Pomegranate extract affects fungal biofilm production: consumption of phenolic compounds and alteration of fungal autoinducers release.
- **Chapter 4** – Attenuation of *Pseudomonas aeruginosa* virulence by pomegranate peel extract

CHAPTER 1

Appl. Sci. 2020, 10, 6721; doi:10.3390/app10196721

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

Aida Meto[1], Bruna Colombari[2], Alessandra Odorici[1], Lavinia Beatrice Giva[3], Eva Pericolini[2], Annunziata La Regina[4] and Elisabetta Blasi[2]

[1] School of Doctorate in Clinical and Experimental Medicine, Laboratory of Microbiology and Virology, University of Modena and Reggio Emilia, Via G. Campi 287, 41125 Modena, Italy

[2] Department of Surgery, Medicine, Dentistry and Morphological Sciences with interest in Transplant, Oncology and Regenerative Medicine, Laboratory of Microbiology and Virology, University of Modena and Reggio Emilia, Via G. Campi 287, 41125 Modena, Italy

[3] School of Specialization in Microbiology and Virology, University of Modena and Reggio Emilia, Via G. Campi 287, 41125 Modena, Italy

[4] Laboratories of Clinical Microbiology, AOU-Policlinic of Modena, 41125 Modena, Italy

Keywords: Biorepair Peribioma toothpaste; Biorepair Peribioma chewing gum; microRepair®BIOMA; oral microbiota; orthodontic elastics; saliva.

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) affect 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 affecting 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).

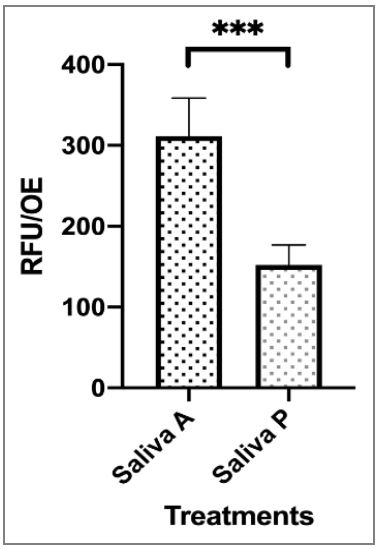


Figure 1. Microbial adhesion onto saliva-contaminated orthodontic elastics (OE). Two sets of OE were exposed to saliva A and P for 1 h at 37 °C. During that time, fluorescence staining of viable cells was allowed by Carboxy-fluorescein Diacetate, Acetoxymethyl Ester (CFDA) addition. Then, the OE were 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.

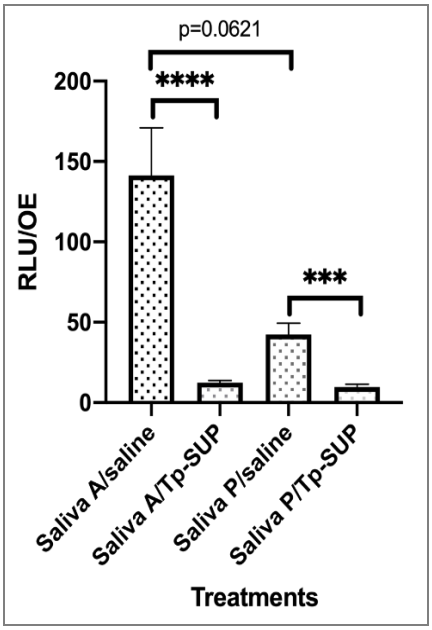


Figure 2. Early biofilm formation onto saliva-contaminated OE exposed or not to Tp-SUP. The saliva-contaminated OE were incubated for 23 h at 37 °C + 5% CO_2 and then exposed to saline or Tp-SUP for additional 1 h, in the presence of CFDA, to allow fluorescence staining. Then, the OE were washed with 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.

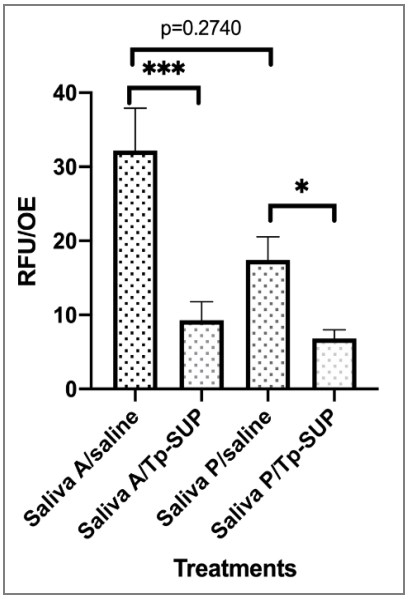


Figure 3. Biofilm persistence following Tp-SUP treatment. The saliva-contaminated OE, that had been exposed to Tp-Sup or saline (at time 23 h, for 1 h) were further incubated at 37 °C + 5% CO_2 up to 48 h in fresh medium. Then, the OE were label with CFDA for 1 h to allow fluorescence staining, washed 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

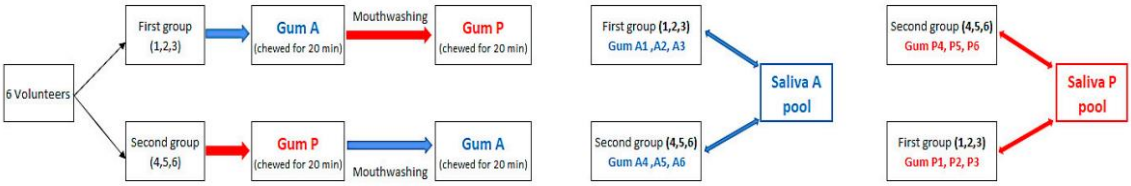
biofilm formation/persistence by oral microorganisms onto orthodontic devices. We favor the idea that, by different pathways, the hydroxyapatite crystals and the probiotics, abundantly present in such oral care products, may act in concert controlling local microbial communities. Moreover, based on the 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.



REFERENCES

1. Zarco, M.; Vess, T.; Ginsburg, G.S. The oral microbiome in health and disease and the potential impact on personalized dental medicine. Oral Dis. 2012, 18, 109–120.

2. Devine, D.; Marsh, P.D.; Meade, J. Modulation of host responses by oral commensal bacteria. J. Oral Microbiol. 2015, 7, 26941,

3. Mira, A. Oral Microbiome Studies: Potential Diagnostic and Therapeutic Implications. Adv. Dent. Res. 2018, 29, 71–77.

4. Manton, D.J.; Walker, G.D.; Fai, C. Remineralization of enamel subsurface lesions in situ by the use of three commercially available sugar-free gums. Int. J. Paediatr. Dent. 2008, 18, 284–290.

5. Azevedo, L.R.; De Lima, A.A.S.; Machado, M.A.N.; Grégio, A.M.T.; Almeida, P.D.V.D. Saliva Composition and Functions: A Comprehensive Review. J. Contemp. Dent. Pr. 2008, 9, 72–80.

6. Morinushi, T.; Murayama, M.; Kinjyo, S. Mutans streptococci, Lactobacilli in saliva and acidity for microorganisms in dental plaque: Changes after restorative treatment. J. Clin. Pediatr. Dent. 2004, 28, 327–332.

7. Jakubovics, N.S. Saliva as the sole nutritional source in the development of multispecies communities in dental plaque. Microbiol.Spectr. 2015; 3, 3,

8. Kumar, B.; Kashyap, N.; Avinash, A.; Chevvuri, R.; Sagar, M.K.; Shrikant, K. The composition, function and role of saliva in maintaining oral health: A review. Int. J. Contemp. Dent. Med. Rev. 2017, 2017, 1–6.

9. Zijnge, V.; Van Leeuwen, M.B.M.; John, E.D.; Abbas, F.; Thurnheer, T.; Gmür, R.; Harmsen, H.J.M. Oral biolm architecture on natural teeth. PLoS ONE 2010, 5, e9321.

10. Bossù, M.; Matassa, R.; Relucenti, M.; Iaculli, F.; Salucci, A.; Di Giorgio, G.; Familiari, G.; Polimeni, A.; Di Carlo, S. Morpho-Chemical Observations of Human Deciduous Teeth Enamel in Response to Biomimetic Toothpastes Treatment. Materials 2020, 13, 1803.

11. Meto, A.; Colombari, B.; Castagnoli, A.; Sarti, M.; Denti, L.; Blasi, E. Efficacy of a Copper–Calcium–Hydroxide Solution in Reducing Microbial Plaque on Orthodontic Clear Aligners: A Case Report. Eur. J. Dent. 2019, 13, 478–484.

12. Conserva, E.; Pisciotta, A; Bertoni, L.; Bertani, G.; Meto, A.; Colombari, B.; Blasi, E.; Bellini, P.; De Pol, A.; Consolo, U.; et al. Evaluation of biological response of stro-1/c-kit enriched human dental pulp stem cells to titanium surfaces treated with two different cleaning systems. Int J Mol Sci. 2019, 20, E1868.

13. Meto, A.; Conserva, E.; Liccardi, F.; Colombari, B.; Consolo, U.; Blasi, E. Differential Efficacy of Two Dental Implant Decontamination Techniques in Reducing Microbial Biofilm and Re-Growth onto Titanium Disks *In Vitro*. Appl. Sci. 2019, 9, 3191.

14. Bagramian, R.A.; Garcia-Godoy, F.; Volpe, A.R. The global increase in dental caries. A pending public health crisis. Am. J. Dent. 2009, 22, 3-8.

15. Kalsbeek, H. Series: Caries prevention in historical perspective. Fluoride. Ned. Tijdschr.voor Tandheelkd. 2018, 125, 257–261.

16. Matthews, D.C. Prevention and treatment of periodontal diseases in primary care. Evid.Based Dent. 2014, 15, 68–69.

17. Costalonga, M.; Herzberg, M.C. The oral microbiome and the immunobiology of periodontal disease and caries. Immunol.Lett. 2014, 162, 22–38.

18. Tanzer, J.M.; Kurasz, A.B.; Clive, J. Competitive displacement of mutans streptococci and inhibition of tooth decay by *Streptococcus salivarius* TOVE-R. Infect. Immun. 1985, 48, 44–50.

19. Teughels, W.; Newman, M.; Coucke, W.; Haffajee, A.; Van Der Mei, H.; Haake, S.K.; Schepers, E.; Cassiman, J.-J.; Van Eldere, J.; Van Steenberghe, D.; et al. Guiding Periodontal Pocket Recolonization: A Proof of Concept. J. Dent. Res. 2007, 86, 1078–1082.

20. Burton, J.P.; Chilcott, C.; Moore, C.; Speiser, G.; Tagg, J. A preliminary study of the effect of probiotic *Streptococcus salivarius* K12 on oral malodour parameters. J. Appl. Microbiol. 2006, 100, 754–764.

21. Haukioja, A.; Yli-Knuuttila, H.; Loimaranta, V.; Kari, K.; Ouwehand, A.C.; Meurman, J.H.; Tenovuo, J. Oral adhesion and survival of probiotic and other lactobacilli and bifidobacteria *in vitro*. Oral Microbiol.Immunol. 2006, 21, 326–332.

22. Näse, L.; Hatakka, K.; Savilahti, E.; Saxelin, M.; Pönkä, A.; Poussa, T.; Korpela, R.; Meurman, J. Effect of long-term consumption of a probiotic bacterium, *Lactobacillus rhamnosus* GG, in milk on dental caries and caries risk in children. Caries Res. 2002, 35, 412–420.

23. Fiorillo, L.; Cervino, G.; Herford, A.S.; Laino, L.; Cicciù, M. Stannous Fluoride Effects on Enamel: A Systematic Review. Biomimetics 2020, 5, 41.

24. Biesbrock, A.R.; He, T.; Digennaro, J.; Zou, Y.; Ramsey, D.; Garcia-Godoy, F. The effects of bioavailable gluconate chelated stannous fluoride dentifrice on gingival bleeding: Meta-analysis of eighteen randomized controlled trials. J. Clin. Periodontol. 2019, 46, 1205–1216.

25. Sukontapatipark, W.; El-Agroudi, M.A.; Selliseth, N.J.; Thunold, K.; Selvig, K.A. Bacterial colonization associated with fixed orthodontic appliances-A SEM study. Eur. J. Orthod. 1998, 23, 475–484.

26. Kitada, K.; De Toledo, A.; Oho, T. Increase in detectable opportunistic bacteria in the oral cavity of orthodontic patients. Int. J. Dent. Hyg. 2009, 7, 121–125.

27. Brusca, M.I.; Chara, O.; Sterin-Borda, L.; Rosa, A.C. Influence of Different Orthodontic Brackets on Adherence of Microorganisms *In Vitro*. Angle Orthod. 2007, 77, 331–336.

28. Dittmer, P.D.M.; Demling, A.; Borchers, L.; Stiesch, M.; Kohorst, P.; Schwestka-Polly, R. The influence of simulated aging on the mechanical properties of orthodontic elastomeric chains without an intermodular link. J. Orofac. Orthop.Fortschr.der Kieferorthopädie 2012, 73, 289–297.

29. Takla, G.S.; Cunningham, S.J.; Horrocks, E.N.; Wilson, M. The effectiveness of an elastomeric module dispenser in cross-infection control. J. Clin. Orthod. 1998, 32, 721–726.

30. Josell, S.D.; Leiss, J.B.; Rekow, E.D. Force degradation in elastomeric chains. Semin.Orthod. 1997, 3, 189–197.

31. Degli Esposti, L.; Ionescu, A.C.; Brambilla, E.; Tampieri, A.; Iafisco, M. Characterization of a Toothpaste Containing Bioactive Hydroxyapatites and *In Vitro* Evaluation of Its Efficacy to Remineralize Enamel and to Occlude Dentinal Tubules. Materials 2020, 13, 2928.

32. Anderson, P.; Hector, M.; Rampersad, M. Critical pH in resting and stimulated whole saliva in groups of children and adults. Int. J. Paediatr. Dent. 2001, 11, 266–273.

33. Sava, S.; Sava, S.; Delean, A.G.; Mihailescu, A.M.; Dumitrescu, L.S.; Moldovan, M.; Festila, D.G. Toothpaste Composition Effect on Enamel Chromatic and Morphological Characteristics: *In Vitro* Analysis. Materials 2019, 12, 2610.

34. Pajor, K.; Pajchel, L.; Kolmas, J. Hydroxyapatite and Fluorapatite in Conservative Dentistry and Oral Implantology-A Review. Materials 2019, 12, 2683.

35. Jabbari, H.; Alikhah, H.; Alamdari, N.S.; Behzad, M.N.; Mehrabi, E.; Borzui, L.; Bakhshian, F. Developing the use of quality indicators in sterilization practices Iranian. Iran. J. Public Health 2012, 41, 64–69.

36. Pithon, M.M.; Ferraz, C.S.; Rosa, F.C.S.; Rosa, L.P. Sterilizing elastomeric chains without losing mechanical properties. Is it possible? Dent. Press J. Orthod. 2015, 20, 96–100.

37. Harikrishnan, P.; Subha, T.S.; Kavitha, V.; Gnanamani, A. Microbial Adhesion on Orthodontic Ligating Materials: An *in Vitro* Assessment. Adv. Microbiol. 2013, 3, 108–114.

38. Schrøder, S.; Bardow, A.; Eickhardt-Dalbøge, S.; Johansen, H.K.; Homøe, P. Is parotid saliva sterile on entry to the oral cavity? Acta Oto-Laryngol. 2017, 137, 762–764.

39. Dzidic, M.; Collado, M.C.; Abrahamsson, T.; Artacho, A.; Stensson, M.; Jenmalm, M.C.; Mira, A. Oral microbiome development during childhood: An ecological succession influenced by postnatal factors and associated with tooth decay. ISME J. 2018, 12, 2292–2306.

40. Haukioja, A.; Loimaranta, V.; Tenovuo, J. Probiotic bacteria affect the composition of salivary pellicle and streptococcal adhesion *in vitro*. Oral Microbiol.Immunol. 2008, 23, 336–343.

41. Nikawa, H.; Makihira, S.; Fukushima, H.; Nishimura, H.; Ozaki, Y.; Ishida, K.; Darmawan, S.; Hamada, T.; Hara, K.; Matsumoto, A.; et al. *Lactobacillus reuteri* in bovine milk fermented decreases the oral carriage of mutans streptococci. Int. J. Food Microbiol. 2004, 95, 219–223.

CHAPTER 2

SHORT COMMUNICATION

Novel Options to Counteract Oral Biofilm Formation: *In Vitro* Evidence

Alessandra Odorici[1], Bruna Colombari[2], Pierantonio Bellini[2], Aida Meto[3*], Irene Venturelli[4], Elisabetta Blasi[2*]

[1] School of Doctorate in Clinical and Experimental Medicine, Laboratory of Microbiology and Virology, University of Modena and Reggio Emilia, Modena, Italy
[2] Department of Surgical, Medical, Dentistry and Morphological Sciences with interest in Transplant, Oncological and Regenerative Medicine, University of Modena and Reggio Emilia, Modena, Italy
[3] Department of Dentistry, Faculty of Dental Sciences, University of Aldent, Tirana, Albania
[4] School of Specialization in Microbiology and Virology, University of Modena and Reggio Emilia, Modena, Italy

Keywords: microbial biofilm, biomimetic hydroxyapatite, MicroRepair, pomegranate extract, antimicrobial compounds

Abstract

Biofilm production, onto biotic and abiotic surfaces, is crucial in the pathogenesis of most infections, particularly those occurring in the oral cavity. Its prevention and/or control may greatly facilitate management of patients with oral diseases. Here, the antibiofilm activity of a biomimetic hydroxyapatite and a natural compound, MicroRepair (MicroR) and pomegranate (PomeGr) re-spectively, was assessed. By luminescence/fluorescence-based assays, *Pseudomonas aeruginosa* (*P. aeruginosa*), *Staphylococcus aureus* (*S. aureus*) and *Candida albicans* (*C. albicans*) were tested for biofilm production in the presence of MicroR and/or PomeGr. We found that both MicroR and PomeGr could affected biofilm production; yet, the efficacy of the two, given alone or in combination, varied according to the microbial agent considered. These data open to clinical studies aimed at defining the most efficacious protocols to counteract oral biofilm-associated infections.

1. Introduction

The oral cavity is described as a highly complex habitat, involving resident microbial communities, that play a key role in local homeostasis. Various parameters, such as poor oral hygiene, dental appliances, diet, drugs, systemic diseases, etc., are also known to influence the maintenance of a health condition [1]. Alteration of oral homeostasis may allow opportunistic pathogens to locally predominate and then organize themselves into microbial biofilms. This homeostatic imbalance leads to frequent oral diseases, such as caries, periodontitis, mucositis, etc. [1].

MicroRepair (MicroR) is a recently described biomimetic compound, made of carbonate-hydroxyapatite-zinc crystals; thanks to its biomimetic property, it is able to interact with tooth hydroxyapatite, favoring enamel remineralization [2]. Moreover, because of the presence in Zn++ ions, MicroR is expected to mediate direct antimicrobial effects. Accordingly, an in vitro study has recently demonstrated the efficacy of MicroR to counteract adhesion and persistence of oral bacteria onto orthodontic elastics [3].

The dramatic increase in drug-resistance, mostly due to improper use of antibiotics [4], opens to the search of alternative drugs, such as natural compounds, also in dentistry. In this respect, antimicrobial agents such as herbs, spices, and probiotics demonstrate effective properties against dental diseases [5]. In particular, pomegranate (PomeGr), the fruit of Punica granatum, is receiving great attention because of its high content in phenolic compounds, likely mediating antimicrobial effects also against oral pathogens [6,7].

Pseudomonas aeruginosa (*P. aeruginosa*), *Staphylococcus aureus* (*S. aureus*) and *Candida albicans* (*C. albicans*) are opportunistic pathogens, responsible of clinical pictures, involving also the oral cavity [8]. Because of their strong ability to adhere and persist onto abiotic and biotic surfaces, they often cause biofilm-associated infections, clinically relevant and difficult-to-treat [9].

Here, we investigated the effects of MicroR and PomeGr, either alone or in combination, on biofilm production by *P. aeruginosa*, *S. aureus* and *C. albicans*, as widely used prototypes of Gram-negative, Gram-positive and fungal pathogens. By *in*

vitro models employing ductile and sensitive luminescence and fluorescence-based assays [10], the antibiofilm activity of MicroR and PomeGr has been established.

2. Materials and Methods

2.1. Compounds

The biomimetic hydroxyapatite (HA) MicroR and the *Punica granatum* peel extract (PomeGr) were supplied by Coswell S.p.A. (Bologna, Italy). The former was sterilized by filtration and the later was treated by autoclave prior to be used in the assays.

2.2. Microbial strains and growth condition

The bioluminescent bacterial strains, *P. aeruginosa* (strain P1242) and *S. aureus* (Xen29), and the fluorescent fungal strain (GFP-tagged strain derived from *C. albicans* SC5314) were used. According to previously detailed protocols [10], the viable bacterial or fungal cells constitutively emitted bioluminescent or fluorescent signals, that could be recorded and quantified by a Fluoroskan reader (Thermo Fischer Scientific, Waltham, MA, USA). Such values, expressed as Relative Luminescence Units (RLU for bacterial cells) or Relative Fluorescence Units (RFU for fungal cells), allowed to directly measure the amounts of viable microorganisms present in control and experimental groups.

Operationally, in line with other studies [11], bacterial and fungal cells from −80 °C glycerol stocks were initially seeded onto Tryptic Soy Agar (TSA) or Sabouraud Dextrose Agar (SAB) (OXOID, Milan, Italy) plates, respectively, and incubated overnight at 37 °C. Then, isolated colonies were collected, added to 10 mL of Tryptic Soy Broth (TSB) or Sabouraud broth (OXOID, Milan, Italy) and allowed to grow overnight at 37 °C under gentle shaking, prior to be used for biofilm production.

2.3. Assessment of microR and PomeGr effects on microbial biofilm formation

According to previously described protocols [10,11], overnight bacterial or fungal cultures were diluted by the appropriate medium and seeded in 96 black well-plates (10^6/mL; 100 µL/well); each well was added with 100 µL of medium (untreated), MicroR or PomeGr (the compounds were tested either alone or in combination, at

different doses). Then, the plates were incubated at 37 °C for 24 h. Thereafter, the samples were washed twice with phosphate buffered saline (PBS) to remove the planktonic cells and the bioluminescence or fluorescent signal/well was measured and expressed as RLU (bacterial biofilm) or RFU (fungal biofilm); these values represented the amounts of biofilm produced, under the different experimental conditions.

2.4. Statistical analysis

Shapiro-Wilk test was used to analyse the distribution of data within each experimental group. Differences between groups were analysed by Kruskal-Wallis's test followed by uncorrected Dunn's multiple comparisons test. Statistical analysis was performed by using GraphPad Prism 8 software.

Values of **p*=0.05, ***p*<0.01, ****p*<0.001 and *****p*<0.0001 were considered statistically significant.

3. Results

3.1. Antibiofilm efficacy of MicroR and/or PomeGr

In the present study, MicroR and PomeGr, either alone or in combination, have been investigated for their effects against biofilm production by three major prototypes of pathogens, namely Gram-negative and Gram-positive bacterial cells and fungal cells. In particular, *P. aeruginosa*, *S. aureus* and *C. albicans* were exposed to MicroR and/or PomeGr for 24 h at 37 °C; then, biofilm production was quantified by luminescence and fluoresce-based assays, as detailed elsewhere [10]. Fig. 1 shows that microbial exposure to PomeGr (5.6% working condition) resulted in a drastic decrease of *P. aeruginosa* biofilm, as assessed by RLU measurement; a partial but non-significant reduction was observed in *S. aureus* RLU, while a significant impairment occurred in *C. albicans* exposed to PomeGr. MicroR treatment (4.7 mg/mL) reduced biofilm formation in all the strains, especially in *C. albicans*, where this reduction was highly significant (*p*<0.0001).

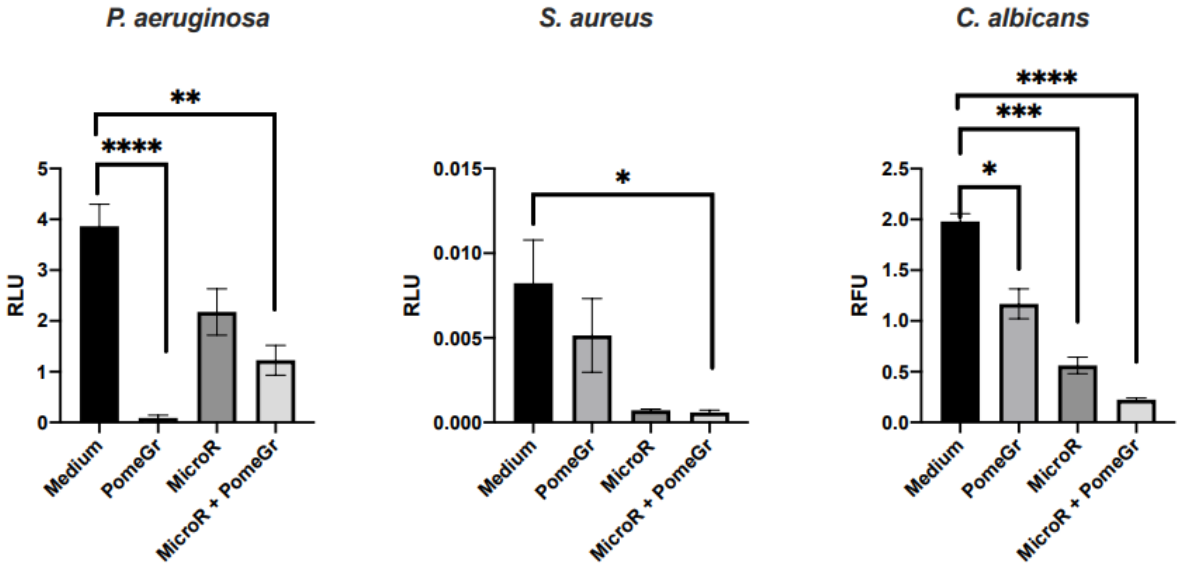


Figure 1. Antibiofilm efficacy of MicroR and/or PomeGr against *P. aeruginosa*, *S. aureus* and *C. albicans*. The microorganisms (10^6/mL) were exposed to MicroR (4.7 mg/mL) or PomeGr (2xMIC) or both and incubated at 37 °C for 24 h; then, biofilm was quantified by luminescence or fluorescence analysis, as detailed elsewhere. The data are the mean ± standard deviation of 6 to 9 determinations from 3 to 4 experiments. Statistical analysis was performed by using GraphPad Prism 8 software. The asterisks indicate statistically significant differences of medium *vs* treated samples (**p*=0.05, ***p*<0.01, ****p*<0.001 and *****p*<0.0001).

The combination of MicroR and PomeGr significantly reduced biofilm both in *S. aureus* and *C. albicans*, while in *P. aeruginosa*, the RLUs were unexpectedly higher than those obtained with PomeGr alone (Fig. 1).

When these data were expressed as percent of biofilm decrease (Table 1), we found that, upon exposure to PomeGr, *P. aeruginosa* was inhibited down to 98%, while *S. aureus* and *C. albicans* biofilm were reduced to 37.5% and 41%, respectively. When exposed to MicroR alone, *P. aeruginosa*, *S. aureus*, and *C. albicans* showed a biofilm reduction ranging from 43 to 91%; yet, only in *C. albicans* the decrease was found to be significant. Finally, when the strains were exposed to PomeGr plus MicroR, the biofilm was reduced between 68% to 93%, with partial additive effect being observed only in *C. albicans*.

Table 1. Percent of microbial biofilm decrease upon exposure to PomeGr and/or MicroR.

	Biofilm decrease (%)		
Treatment	*P. aeruginosa*	*S. aureus*	*C. albicans*
PomeGr	98	37,5	41
MicroR	43	91	72
MicroR + PomeGr	68	93	89

The percent of biofilm decrease (treated and untreated samples) was calculated using the values depicted in Figure 1.

4. Discussion

Notoriously, the biomimetic HA has composition and structure similar to biological systems, such as human hard tissues, and is therefore able to integrate into host structures without altering or dissolving its own or neighboring elements. Several researches have shown the possibility of replacing some ions within the HA to strengthen its properties; for example, the replacement of Ca++ with Zn++ significantly increases the antimicrobial properties of such biomimetic material [2]. Nowadays, HA is being used in dental practice, as it has been added to the new formulations of oral hygiene products (mouthwashes, toothpastes, etc.) [2,3]. Here we provide the first evidence that MicroR, a biomimetic HA, acts as anti-biofilm agent. In particular, when used at 4.7 mg/mL, MicroR was capable to reduce *P. aeruginosa* and *S. aureus* biofilm, as measured by the luminescence assay; yet, such decrease did not reach statistical significance; differently, the reduction observed in MicroR-treated *C. albicans* returned a $p<0.001$; when these variations were expressed as percent of biofilm decrease, we obtained 43% for *P. aeruginosa*, 91% for S*. aureus* and 72% for *C. albicans*. Using other doses of MicroR or PomeGr, we failed to detect better results (data not shown).

Furthermore, the combination of the two, MicroR and PomeGr, provided heterogeneous and somehow unexpected results. Indeed, intermediate levels of

biofilm inhibition were observed with *P. aeruginosa*, suggesting that MicroR might partially mask the elevated anti-*P. aeruginosa* biofilm efficacy observed with PomeGr alone. Differently, the combination MicroR and PomeGr exerted anti-*S. aureus* effects, that reached statistically significant values. Finally, when used together against

C. albicans, MicroR and PomeGr clearly showed additive effects as inhibitors of fungal biofilm formation. The heterogeneous trends observed with the three microbial agents remain unexplained. Undoubtedly, major differences in the composition and structure of the three microbial agents assessed partially explain the results obtained.

Also, we may envisage that MicroR and PomeGr might differently affected the secretory profile of *P. aeruginosa*, *S. aureus* and *C. albicans*, which are known to release profoundly different sets of crucial molecules, including auto-inducers [10], to produce and establish their biofilm. In any case, by novel in vitro assays, we provide the first evidence that microR and PomeGr exert antibiofilm activity and that their action may be additive. Thus, although with the limitations of any *in vitro* model, this work opens to further studies on the antibiofilm activity of MicroR and PomeGr against clinical isolates and, even more, multispecies microbial biofilms that commonly afflict the oral cavity.

5. Conclusions

By means of an *in vitro* model, the antibiofilm activity of a biomimetic hydroxyapatite, MicroRepair, and a natural compound, pomegranate, has been established. Given the crucial role of biofilm formation in the pathogenesis of many oral diseases, these data offer the basic rational to design trials aimed at maintaining health conditions or recovering homeostatic imbalance in the oral cavity.

REFERENCES

1. Wade, W.G. The oral microbiome in health and disease. *Pharmacol Res*. 2013, *69*(1), 137-43. doi:10.1016/j.phrs.2012.11.006.

2. Butera, A.; et al. SEM/EDS Evaluation of the Mineral Deposition on a Polymeric Composite Resin of a Toothpaste Containing Biomimetic Zn-Carbonate Hydroxyapatite (microRepair®) in Oral Environment: A Randomized Clinical Trial. *Polymers* (Basel). 2021, *13*(16), 2740.

3. Meto, A.; Colombari, B.; Odorici, A.; et al. Antibacterial Effects of MicroRepair®BIOMA-Based Toothpaste and Chewing Gum on Orthodontic Elastics Contaminated *In Vitro* with Saliva from Healthy Donors: A Pilot Study. *Appl. Sci*. 2020, *10*(19), 6721.

4. Ventola, C.L. The antibiotic resistance crisis: Part 1: Causes and threats. *Pharm. Ther*. 2015, *40*, 277–283.; WHO. Antimicrobial Resistance. 2020. Available online: https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance, 12 December 2021

5. Chen, X.; Daliri, E.B.; Kim, N.; Kim, J.R.; Yoo, D.; Oh, D.H. Microbial etiology and prevention of dental caries: exploiting natural products to inhibit cariogenic biofilms. *Pathogens* 2020, *9*, 569.

6. Al-Zoreky, N.S. Antimicrobial activity of pomegranate (*Punica granatum* L.) fruit peels. *Int J Food Microbiol*. 2009, *134*(3), 244-8. doi:10.1016/j.ijfoodmicro.2009.07.002.

7. Bhandari, P.R. Pomegranate (*Punica granatum* L). Ancient seeds for modern cure? Review of potential therapeutic applications. *Int J Nutr Pharmacol Neurol Dis*. 2012, *2*, 171-84.

8. Rodríguez-Rentería, M.; Márquez-Preciado, R.; Ortiz-Magdaleno, M.; Bermeo-Escalona, J.; Sánchez-Vargas, L.O. Frequency of Pathogenic Microorganisms in

Removable Orthodontic Appliances and Oral Mucosa in Children. *J Clin Pediatr Dent*. 2021, *45*(2), 135-139. doi:10.17796/1053-4625-45.2.11.

9. Ciofu, O.; Moser, C.; Jensen, P.Ø.; Høiby, N. Tolerance and resistance of microbial biofilms. *Nat Rev Microbiol*. 2022 Feb 3. doi: 10.1038/s41579-022-00682-4.

10. Colombari, B.; Alfano, G.; Gamberini, C.; Cappelli, G.; Blasi, E. EDTA and Taurolidine Affect *Pseudomonas aeruginosa* Virulence *In Vitro*-Impairment of Secretory Profile and Biofilm Production onto Peritoneal Dialysis Catheters. *Microbiol Spectr*. 2021, *9*(3), e0104721. doi: 10.1128/Spectrum.01047-21.

11. Meto, A.; Colombari, B.; Sala, A.; Pericolini, E.; Meto, A.; Peppoloni, S.; Blasi, E. Antimicrobial and antibiofilm efficacy of a copper/calcium hydroxide-based endodontic paste against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans*. *Dent Mater J*. 2019, *38*(4), 591-603. doi: 10.4012/dmj.2018-252.

CHAPTER 3

Int. J. Environ. Res. Public Health **2022**, 19, 14146. doi: 10.3390/ijerph192114146

ARTICLE

Pomegranate Extract Affects Fungal Biofilm Production: Consumption of Phenolic Compounds and Alteration of Fungal Autoinducers Release

Bruna Colombari [1], Davide Tagliazucchi [2], Alessandra Odorici [3], Eva Pericolini [1], Ismaela Foltran [4], Diego Pinetti [5], Aida Meto [1,6], Samuele Peppoloni [1] and Elisabetta Blasi [1,*]

[1] *Laboratory of Microbiology and Virology, Department of Surgery, Medicine, Dentistry and Morphological Sciences with Interest in Transplant, Oncology and Regenerative Medicine, University of Modena and Reggio Emilia, Modena, Italy*

[2] *Department of Life Sciences, University of Modena and Reggio Emilia, Reggio Emilia, Italy*

[3] *Laboratory of Microbiology and Virology, School of Doctorate in Clinical and Experimental Medicine, University of Modena and Reggio Emilia, Modena, Italy*

[4] *Incos - Cosmeceutica Industriale, Funo di Argelato, Bologna, Italy*

[5] *Centro Interdipartimentale Grandi Strumenti (C.I.G.S), University of Modena and Reggio Emilia, Modena, Italy*

[6] *Department of Dentistry, Faculty of Dental Sciences, University of Aldent, Tirana, Albania*

Keywords: anti-biofilm, *Candida albicans*, *in vitro*, phenolic compounds, pomegranate, virulence

Int. J. Environ. Res. Public Health **2022**, 19, 14146. doi: 10.3390/ijerph192114146

Abstract

Candida albicans expresses numerous virulence factors that contribute to pathogenesis, including its dimorphic transition and even biofilm formation, through the release of specific quorum sensing molecules, such as the autoinducers (AI) tyrosol and farnesol. In particular, once organized as biofilm, *Candida* cells can elude conventional antifungal therapies and the host's immune defenses as well. Accordingly, biofilm-associated infections become a major clinical challenge underlining the need of innovative antimicrobial approaches. The aim of this *in vitro* study was to assess the effects of pomegranate peel extract (PomeGr) on *C. albicans* growth and biofilm formation; in addition, the release of tyrosol and farnesol was investigated. The phenolic profile of PomeGr was assessed by high-performance liquid chromatography coupled to electrospray ionization mass spectrometry (HPLC-ESI-MS) analysis before and after exposure to *C. albicans*. Here, we showed that fungal growth, biofilm formation and AI release were altered by PomeGr treatment. Moreover, the phenolic content of PomeGr was substantially hampered upon exposure to fungal cells; particularly pedunculagin, punicalin, punicalagin, granatin, di-(HHDP-galloyl-hexoside)-pentoside and their isomers as well as ellagic acid–hexoside appeared highly consumed, suggesting their role as bioactive molecules against *Candida*. Overall, these new insights on the anti-*Candida* properties of PomeGr and its potential mechanisms of action may represent a relevant step in the design of novel therapeutic approaches against fungal infections.

1. Introduction

The formation of biofilm by numerous microbial species represents an important mechanism of survival and persistence; upon biofilm production, pathogenicity and virulence are also profoundly affected [1,2]. Biofilm formation has been widely recognized as a key step in clinical settings, where biofilm-associated infections occur and commonly affect different anatomical sites, including heart valves, oral cavity or continuity solutions, and medical devices, such as orthopedic prostheses, venous and urological catheters [1].

Biofilm is defined as a complex aggregation of microorganisms embedded in a protective extracellular polymeric substance (EPS), rich also in proteins, lipids and extracellular DNA (eDNA), produced by the microorganism itself. By this extracellular matrix, microbial adhesion to surfaces and consolidation of the biofilm three-dimensional structure are facilitated [2,3]. Increasing studies show that biofilm production increases microbial resistance to environmental stressors, such as temperature, pH and pressure changes, lack of nutrients and antimicrobial agents' susceptibility as well [4–7]. Biofilm formation is finely governed and regulated by the quorum sensing (QS), a sophisticated cell-to-cell communication system regulating gene expression and microbial cell behavior in response to fluctuations in cell-population density. QS relies on the release of low molecular weight chemical signaling molecules, called autoinducers (AI) [8], which differ among different microorganisms.

Candida albicans (*C. albicans*) is an opportunistic pathogen, often responsible for biofilm-associated infections, given its ability to tightly and persistently adhere to biotic/abiotic surfaces. Once produced, *Candida* biofilm establishes clinically relevant and difficult-to-treat infections, because of the enhanced resistance of the biofilm-embedded cells to antimicrobial drugs [9]. Four major *C. albican*s AI have been described during biofilm development, namely farnesol, tyrosol, phenylethanol and tryptophol [10–13]; such molecules are major players not only in fungal morphogenesis (yeast-to-hyphal cell transition) but also in the infectious process, promoting dissemination and establishment of foci in distal anatomical sites [12]. Clinically, this scenario is further complicated by the continuous detection of fungal isolates with newly acquired resistance against conventional drugs, because of

mutations and/or genetic recombination [14,15]. Hence, there is a need for identifying novel tools that would exert direct killing and/or anti-biofilm activity, possibly avoiding drug-mediated pressure and consequently developing further antimicrobial resistance [16].

Among natural compounds [17,18], the *Punica granatum* L. peel extract (PomeGr) is an excellent source of a variety of bioactive molecules exerting beneficial health effects, due to their antimicrobial, anti-inflammatory, anticancer and antioxidant properties [19–22]. In addition, increasing literature ascribes significant antifungal effects to specific compounds, such as phenolic acid, hydrolysed tannins, flavonoids, etc. [23,24]. Recently, we provided initial evidence on the efficacy of PomeGr against biofilm production by *C. albicans* and *Pseudomonas aeruginosa* as well [25].

The aim of the present study was to investigate the anti-*Candida* properties of PomeGr. In particular, we assessed the PomeGr effects on fungal growth, biofilm formation and release of specific auto-inducers, known to be relevant in *Candida* virulence. Furthermore, the consumption of specific phenolic compounds by *Candida* was investigated and their potential role discussed.

2. Materials and Methods

2.1. Fungal Cells and Growth Condition

The *C. albicans* engineered strain, endowed with a reporter gene coding for a yeast green fluorescent protein (y-EGFP), was used [26]. In particular, the y-GFP *Candida* cells constitutively emitted a fluorescent signal, measurable by the Fluoroskan reader (Thermo Fisher Scientific, Waltham, MA, USA) at the λ of 490/521 nm; the intensity of the emitted signal, related to the number of viable cells, was quantified and expressed as relative fluorescent units (RFU).

Operationally, fungal cells, maintained at −80 °C in glycerol stocks, were seeded onto Sabouraud Dextrose Agar (SAB) plates (OXOID, Milan, Italy) and incubated overnight at 37 °C. Then, isolated colonies were collected, added to 10 mL of Sabouraud (SAB) broth (OXOID, Milan, Italy) and allowed to grow overnight at 37 °C under gentle shaking. In order to evaluate the fungal concentration, the optical

density (OD) was measured at 595 nm (OD_{595}) using the Tecan Sunrise (Tecan Group Ltd., Männedorf, Switzerland) spectrophotometer.

2.2. Pomegranate Peel Extract

The *Punica granatum* L. peel extract (PomeGr), supplied by INCOS COSMECEUTICA INDUSTRIALE (Bologna, Italy) and produced by PHENBIOX SRL (Bologna, Italy), contained the peel extract (22.5% *w/w*), Saccharomyces ferment lysate filtrate, citric acid, sodium benzoate and potassium sorbate. Further details are given as supplementary material (Table S1). The same solution containing all the ingredients, but not PomeGr, was employed as negative control (neg-C). The PomeGr and the neg-C were filtered through a 0.22 µm membrane and maintained at 4 °C, prior for testing in the assays described below.

2.3. Fungal Growth and Biofilm Formation Assays

The PomeGr and the neg-C were serially diluted (from 1:2 to 1:128) and plated (100 µL/well) in black 96 well-microtiter plates with transparent bottom; then, the fungal cell suspension (10^6 cells/mL in SAB obtained from overnight cultures) was added (100 µL/well) and the plates were incubated at 37 °C for 24 h. After incubation, fungal growth (direct reading at 24 h) and biofilm formation (reading after 2× washing of the wells with buffered saline to remove the planktonic cells) were assessed. By the Fluoroskan (Thermo Fisher), the fluorescence signal was measured and the results were expressed as relative fluorescence units (RFU), using previously described protocols with minor modifications [27]. By the Tecan Sunrise, the reading at two different ODs, namely OD_{595} (for microbial growth analysis) and OD_{540} (after crystal violet staining for Biofilm determination), were performed, according to standard procedures.

Moreover, calibration curves were generated employing the colony forming units (CFU) assay. Briefly, serial dilutions of fungal cells were prepared in SAB broth, seeded in black 96 well-microtiter plates with transparent bottoms (100 µL/well) and incubated for 24–48 h at 37 °C. Then, the microtiter plates were read at the Fluoroskan to evaluate the RFU and at the Tecan Sunrise to measure the OD. In parallel, from each well, the CFU assay was performed to quantify the viable cells.

Finally, standard curves were generated which allowed the expression of the OD or RFU values in terms of CFU/mL.

2.4. Mass Spectrometry Analysis of PomeGr Extract

High-performance liquid chromatography-electro spray high resolution-mass spectrometry (HPLC-ESI/HR-MS) analysis was performed using an Ultimate 3000 UHPLC coupled to a QExactive Mass Spectrometer via a HESI-II electrospray ion source (Thermo Scientific). Target compounds from pomegranate extracts were detected in 10 µL sample volume injected onto a Hypersil Gold C18 100 × 2.1 mm ID 1.9 µm ps column (Thermo Scientific) kept at 30 °C. Chromatographic separation was performed at 0.5 mL/min flow with a gradient elution scheme using 0.1% formic acid in acetonitrile (B) and 0.1% formic acid in water (A).

The mobile phase composition was kept at 2% B for 0.5 min after injection, then linearly raised to 18% B in 27 min and further on to 98% B in 4.5 min. Solvent B was kept at 98% up to minute 34.9 then lowered to 2% at minute 35. The total runtime was 45 min. The HESI-II source was operated in alternating positive and negative ionization mode.

Full MS (70,000 FWHM resolution power, 160–800 Th range) with data-dependent MS2 (17,500 FWHM, Top 4 precursor ions) spectra acquisition was performed in both polarities. Analysis was carried out on cell free supernatants from microbial cultures (10^6/mL) eventually exposed to diluted (1:16) pomegranate extracts for 24 h at 37 °C. The supernatants were transferred to Amicon-Ultra 0.5 tubes, centrifuged at 14,000 rpm for 15 min, diluted 1:1 (vol/vol) with 5% methanol in water and transferred to the autosampler. Using the Thermo Fisher FreeStyle program the relative amount of each compound was calculated by integrating the area under the peak (AUP). AUP was measured from the extracted ion chromatograms (EIC) obtained for each compound with a tolerance ± 5 ppm.

2.5. Mass Spectrometry Analysis of Fungal Autoinducers

Cell-free supernatants from *Candida,* treated or not with PomeGr, were analyzed by HPLC-ESI-MS, as detailed elsewhere [17,27,28]. In particular, 24 h–old supernatants from *Candida* cells (10^6/mL) that had or had not been exposed to PomeGr (1:16 dilution) were harvested, filtered and assessed by MS. The relative

amount of the identified autoinducers (AI) was determined as reported in the previous paragraph.

2.6. Statistical Analysis

The statistical analyses were performed by using GraphPad Prism 8 software. Firstly, the Shapiro–Wilk test was used to analyze the distribution of the data within each experimental group. Differences between groups were analyzed by the Kruskal–Wallis's test, followed by Dunn's multiple comparisons test for Table 1 data; while, for Figure 1 data, statistical differences between groups were analyzed according to one-way ANOVA followed by Sidak's multiple comparisons tests. Values of $p \leq 0.05$ were considered statistically significant.

Table 1. Anti-*Candida* activity of PomeGr.

Dilution	RFU ± SEM	% Decrease	CFU/mL ± SEM	% Decrease
1:2	0.45 ± 0.027 *	77.2	$2.4x10^6 \pm 1.8x10^5$	94
1:4	0.49 ± 0.007*	75.4	$3.4x10^6 \pm 3.0x10^5$	92
1:8	0.51 ± 0.013*	74.3	$4.0x10^6 \pm 3.2x10^5$	90
1:16	0.57 ± 0.020*	71.4	$5.4x10^6 \pm 5.0x10^5$	87
1:32	1.15 ± 0.082	42.2	$2.0x10^7 \pm 2.0x10^6$	51
1:64	1.42 ± 0.016	28.7	$2.7x10^7 \pm 3.3x10^6$	35
1:128	2.19 ± 0.066	0	$4.6x10^7 \pm 5.2x10^6$	0
Medium	1.995 ± 0.025	-	$4.1x10^7 \pm 6.8x10^5$	-

	Levels of OD_{595} ± SEM		CFU/mL x 10^6 ± SEM	
Dilution	**PomeGr**	**neg-C**	**PomeGr**	**neg-C**
1:2	0.21 ± 0.021 *	1.17 ± 0.084	13.06 ± 2.14	109.06 ± 8.39
1:4	0.20 ± 0.022 *	1.18 ± 0.084	12.38 ± 2.24	110.88 ± 8.36
1:8	0.19 ± 0.022 *	1.19 ± 0.088	11.36 ± 2.19	111.46 ± 8.80
1:16	0.23 ± 0.037 *	1.20 ± 0.091	15.22 ± 3.68	112.39 ± 9.13
1:32	0.68 ± 0.072 *	1.20 ± 0.082	60.37 ± 7.23	112.43 ± 8.25
1:64	0.87 ± 0.060	1.07 ± 0.056	79.18 ± 6.02	99.05 ± 5.58
1:128	0.97 ± 0.056	1.11 ± 0.086	89.56 ± 5.63	103.79 ± 8.60
Medium	0.94±0.02		86.44 ± 2.05	

C. albicans (10^6/mL) was exposed to PomeGr at the indicated dilutions and incubated at 37 °C for 24 h; then, the fluorescent signal (RFU) or the OD_{595} were measured by Fluoroskan or Tecan reader, respectively. Upper panel: The RFU values of cells exposed to the various PomeGr

dilutions and the percent decrease (with respect to cells incubated in medium only) are shown. Lower panel: the OD_{595} levels of PomeGr-treated and neg-C groups are shown. By the RFU and OD calibration curves, the predicted CFU/mL were calculated and shown in the right part of each panel. The data are the mean ± SEM of eleven replicates from two independent experiments. The asterisks indicate statistically significant differences (medium *vs* treated samples): * $p < 0.05$.

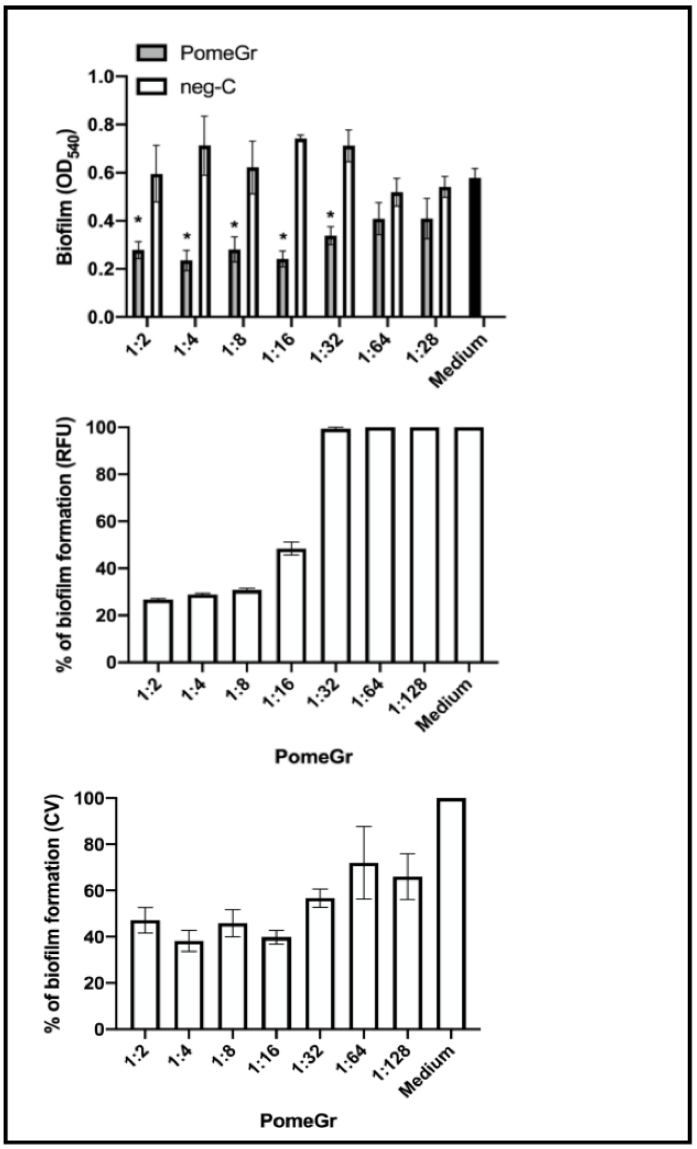


Figure 1. Antibiofilm activity of PomeGr. *C. albicans* (10^6/mL) was exposed to PomeGr or neg-C at the indicated dilutions, and incubated at 37 °C for 24 h; then, after removing planktonic cells, biofilm was quantified. Upper panel: 24 h-biofilm formation by *Candida* exposed to Pomegr (grey bars) or negative control (white bars), assessed by CV assay. The black bar represents the biofilm production by *C. albicans* in medium only. Central panel: percentage of biofilm formation by *C. albicans* exposed to PomeGr, evaluated by

fluorescence assay. Lower panel: percentage of biofilm formation by *C. albicans* exposed to PomeGr, as evaluated by CV assay. The data are the means ± SEM of nine replicates of three independent experiments. The asterisks indicate statistically significant differences: * $p < 0.05$ (PomeGr-treated vs. neg-C samples).

3. Results

3.1. Anti-Candida Activity of PomeGr

Initially, the effect of PomeGr was evaluated on total fungal load, following 24 h *Candida* exposure to different dilutions and subsequent evaluation of the RFU. As shown in Table 1 (upper panel), PomeGr affected *Candida* in a dilution-dependent manner; in particular, a significant decrease ($p < 0.05$) in the RFU occurred at dilutions between 1:2 and 1:16, indicating a reduction above 70%.

To strengthen these data, further experiments were performed by a different assay, namely assessing the fungal load at 24 h, in terms of OD_{595} in treated and untreated cells. In line with the fluorescence results, as shown in Table 1 (lower panel), the levels of OD_{595} varied according to the PomeGr dilution used, with the 1:32 condition still causing significant inhibitory effects. When parallel groups were exposed to the neg-C, no differences in OD_{595} were observed with respect to *Candida* incubated with medium only. Thus, by a different assay, we confirmed the anti-*Candida* activity of the PomeGr and ruled out any potential interference by additive or preservatives included in the formulation.

Then, we investigated the ability of PomeGr to affect *Candida* biofilm production. In particular, *C. albicans* was incubated under conditions that allowed biofilm production in the presence of PomeGr or neg-C. After 24 h, the wells were washed to the remove planktonic cells; then, the amounts of biofilm produced was measured by fluorescence reading and OD_{540} reading after crystal violet staining. The RFU results, depicted in Figure 1 (upper panel), showed that PomeGr affected biofilm production in a dilution-dependent manner, while the neg-C had no effects. When these same data were expressed as percent biofilm formation, established by either considering the OD_{540} (central panel) or the RFU (lower panel), a relevant impairment was consistently observed till 1:16 (as assessed by fluorescence assay) and 1:32 dilutions (as established by OD measurement after crystal violet staining).

Differently, at dilutions ≥ 1:32, PomeGr had no effects on *Candida* biofilm formation, being the raw OD and RFU values comparable to those observed with the untreated control cells (Medium). As 1:16 was the lowest PomeGr dilution that significantly impaired both *Candida* growth and biofilm production, we focused on this condition for further analyses. According to the literature, this condition also allowed to use the preservatives (potassium sorbate, sodium benzoate and citric acid) at working conditions below the minimal inhibitory concentrations (MIC) previously reported for candida [29–31].

3.2. Biochemical Profile of Pomegr Exposed or Not to Candida

PomeGr extract is known to be a complex mixture of components, such as polyphenols with a broad spectrum of activities, including antimicrobial, antioxidant and anti-inflammatory effects [31–35]. Initially, to establish the biochemical profile of the PomeGr extract used in this study, HPLC-ESI-MS analysis was performed and a representative chromatogram is shown in Figure 2.

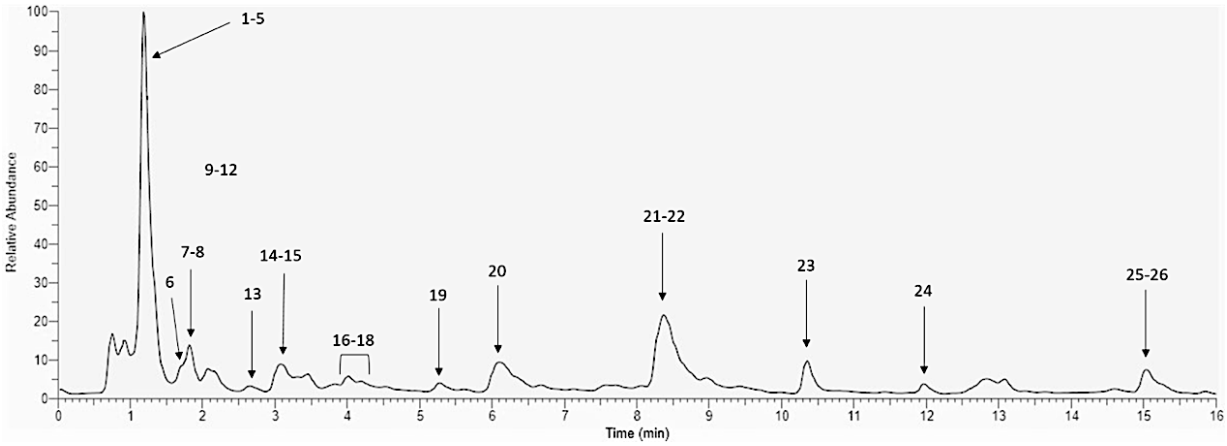


Figure 2. Polyphenols profile of PomeGr. The PomeGr was analyzed by HPLC-ESI-MS. A negative ion mode total ion chromatogram (TIC) is shown, as representative of three independent experiments. The code number of each peak and the corresponding molecules are further detailed in the supplementary material (Table S3).

Details on the mass spectral data of the compounds detected in our extract are given in Table S2. By comparing the MS and MS^2 profile with literature, several compounds were identified, as detailed in Table S3. Taken together, our data

underline the variety of the PomeGr in terms of polyphenols content, similarly to what has been described in the literature [21].

Next, we assessed the possible changes in PomeGr polyphenol content upon exposure to Candida. Thus, PomeGr was incubated for 24 h with and without Candida (10^6/mL); then, cell-free supernatants were subjected to HPLC-ESI-MS analysis. By chromatogram analysis, the different elution peaks and their areas were used for semi-quantitative evaluation of the specific products, detectable in treated and untreated cells.

Figure 3 shows the peak areas (AUP) of selected phenolic compounds, that had been detected in the PomeGr (grey columns) and were affected upon exposure to *C. albicans* (white columns). In addition, the percent variations of each compound were calculated, as ratio between the AUP in presence and absence of the fungal cells. As detailed by the columns, a reduction in the peak areas of pedunculagin, punicalin, punicalagin, granatin, Di-(HHDP-galloyl-hexoside)-pentoside and their isomers was observed upon PomeGr exposure to *C. albicans*, the decrease ranging between 33.3 and 43.5%, while a drop to undetectable levels was observed for ellagic acid–hexoside; all the other compounds showed low-to-partial reductions (from 3.8 to 21.0% decrease).

Int. J. Environ. Res. Public Health **2022**, 19, 14146. doi: 10.3390/ijerph192114146

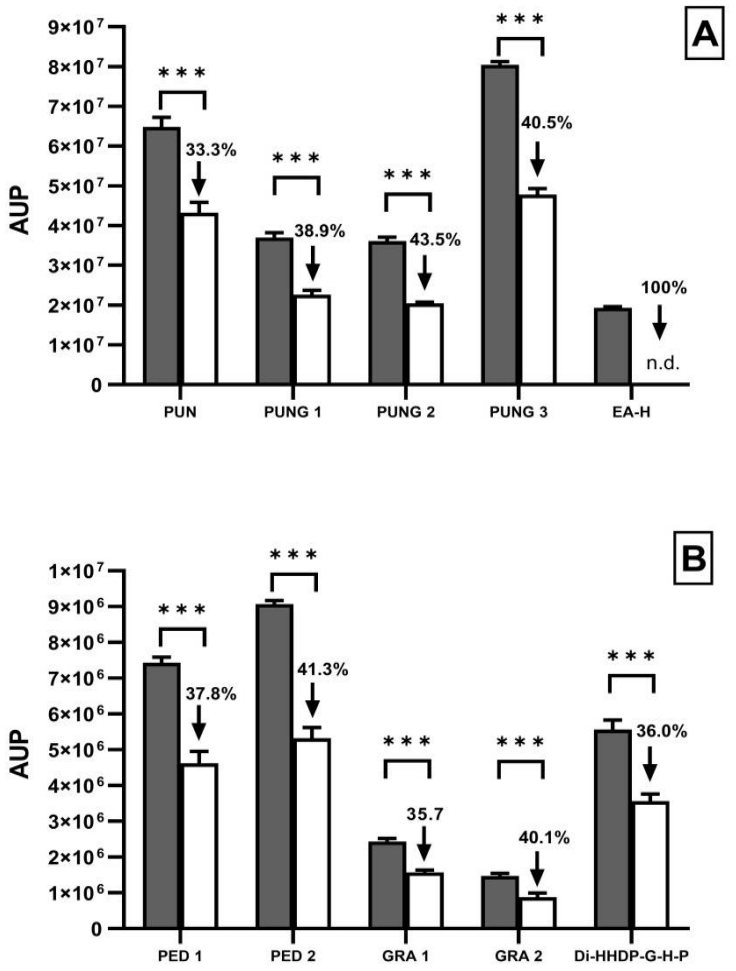


Figure 3. Changes in ellagitannins concentration in PomeGr exposed or not to *C. albicans.* Phenolic compounds were identified by HPLC-ESI-MS in PomeGr alone and after exposure to *C. albicans* (24 h). The relative amounts of each polyphenol were measured by the ion chromatogram, according to the peak area of each compound, and were expressed as area under the peak (AUP) arbitrary units (tolerance ± 5 ppm). Dark grey bars: PomeGr alone; white bars: PomeGr exposed to *C. albicans*. The % decrease (numbers above the columns) was calculated comparing the AUP of treated vs untreated groups. n.d.: compound not detected. The various compounds are shown in panel A and B. *** $p<0.005$. Abbreviations: PUN, punicalin; PUNG 1, punicalagin isomer 1; PUNG 2, punicalagin isomer 2; PUNG 3, punicalagin isomer 3; EA-H, ellagic acid-hexoside; PED 1, pedunculagin 1; PED 2, pedunculagin 2; GRA 1, granatin 1; GRA 2, granatin 2; Di-HHDP-G-H-P, Di-(HHDP-galloyl-hexoside)-pentoside.

3.3. Alteration in the Release of AI by Candida upon Treatment with PomeGr

Finally, we assessed the effects of PomeGr on the ability of C. albicans to produce specific AI, particularly tyrosol and farnesol, known to be involved in fungal biofilm formation and virulence [32]. Thus, HPLC-ESI-MS analysis was performed in cell-free supernatants from *C. albicans*, treated or not with PomeGr for 24 h at 37 °C. By chromatogram analysis, the different elution peaks and their areas were used for semi-quantitative evaluation of the specific products detected in treated and untreated cells. Table 2 shows that untreated *Candida* cells secreted consistent amounts of tyrosol, in line with our recently published data [32,33].

Table 2. AI release by *C. albicans* exposed or not to PomeGr.

Levels of AI				
Treatment	**Tyrosol (AUP)**	**% Decrease**	**Farnesol (AUP)**	**% Decrease**
Medium	8.08×10^7	-	n.d.	-
PomeGr	3.16×10^7	60.89	2.15×10^7	n.m.

Cell-free supernatants from *C. albicans*, treated or not with PomeGr, were tested by HPLC-ESI-MS analysis. The indicated AI were investigated. By chromatogram analysis, the different elution peaks were identified, and the relative peak areas (AUP) were used for semi-quantitative evaluation of each specific product. The % decrease was calculated comparing the AUP of treated and untreated groups. The results shown are from a pool of four replicates of a representative experiment out of two performed. n.d.: not detectable; n.m.: not measurable.

The treatment with PomeGr affected such secretion, producing a decrease of 60.9%. Furthermore, in contrast to what observed testing tyrosol, farnesol resulted undetectable in control *Candida* cells but was consistently induced upon treatment, implying an opposed regulation of these two AI by PomeGr.

4. Discussion

Here, we show that PomeGr extract impairs *Candida*. Fungal growth and biofilm formation are inhibited and also the release of specific auto-inducers happens to be affected.

C. albicans is an opportunistic pathogen, responsible for mild to severe and even life-threatening diseases. Notoriously, it can shift its role from commensal to pathogen, undergoing yeast-to-hyphal form transition and producing biofilm; by doing this, *Candida* can easily evade a host's immune defenses and even resist to exogenous insults, such as disinfectants and antifungal agents [4–7].

Kupnik et al. described the antimicrobial activity of plant extracts, including *Punica granatum* L. [34]. Recently, we have provided initial evidence on the ability of PomeGr to affect biofilm formation by both fungal and bacterial cells [25]. To better understand the phenomenon, here, we demonstrate that PomeGr exerts anti-*Candida* activity in a dose-dependent manner. In particular, the 1:16 dilution significantly impairs both fungal load and biofilm formation, by 71.4% and 51.3%, respectively. Such PomeGr-mediated inhibition never reaches 80%, in line with other data [35], documenting the consistent but always partial efficacy of this natural product. Nevertheless, its success may be strikingly enhanced by a combination with conventional drugs; accordingly, it has been demonstrated that punicalagin, a component of PomeGr extract, synergistically interacts with fluconazole against *C. albicans* and *C. parapsilosis* [36]. Similarly, by an in vitro study aimed at identifying novel options to counteract oral infections [35], we have shown that PomeGr, used in combination with a new biomimetic compound that favors enamel remineralization, exerts additive effects against *Candida* biofilm production. Thus, because of their intrinsic properties, PomeGr as well as other natural compounds endowed with antimicrobial activities deserve special interest, not only for the expected additive/synergistic effects but also because they are expected not to induce resistance, as opposed to conventional drugs.

As known [37,38], pomegranate is an excellent source of bio-compounds with beneficial effects on human health. The relative quantities and compositions of such bioactive molecules may depend on the type of cultivar, the part of the fruit used and the ripening stage [39]. As determined by HPLC-ESI-MS analysis, the PomeGr used

in the present study is mainly composed of pedunculagins and their isomers, ellagic acid hexoside, punicalagins and their isomers, punicalin, granatin and Di-(HHDP-galloyl-hexoside)-pentoside; in addition, other polyphenols are also present in low quantities (Figure 2). The obtained profile closely recalls what has been reported for other PomeGr extracts [21], thus underlying that the polyphenolic components actually remain the most abundant components. Some of them have been characterized for antibacterial and antifungal properties [24]; yet, their mechanism(s) of action remains still poorly understood. Here, we demonstrate the consumption of some PomeGr bioactive molecules upon exposure to *Candida*, envisaging their possible role as antibiofilm compounds. In particular, the comparison between the peak areas (AUP) obtained from *Candida* exposed or not to PomeGr (24 h of incubation, to allow biofilm formation/impairment) allows us to establish a decrease in pedunculagin, granatin, Di-(HHDP-galloyl-hexoside)-pentoside and punicalagin content, ranging from 35.7% to 41.3%. Interestingly, the ellagic acid-exoside undergoes complete consumption (it drops to undetectable levels). Paralleling with the biofilm reduction, the decreases observed by HPLC-ESI-MS analysis suggest that pedunculagin, granatin, Di-(HHDP-galloyl-hexoside)-pentoside, punicalagin and ellagic acid-hexoside are indeed molecules actively involved in the accomplishment of the antibiofilm activity. Similarly, the total consumption of ellagic acid, observed in our model, implies its direct role against *Candida*. In agreement with our data, Nejatbakhsh et al. [40] have shown that ellagic acid impairs both growth and biofilm formation by *C. albicans*; in addition, the expression of two genes, hypha-specific wall protein 1 (HWP1) and agglutinin-like sequence 3 (ALS3), known to be involved in adhesion and dimorphyic transition, happens to be deeply suppressed. Taken together, those of [40] and present findings strengthen the relevance of fungal adhesion and filamentation in the establishment of a sessile community and underline the impact of PomeGr and its compounds as potentially useful antibiofilm agent.

Biofilm development is closely regulated by QS, a cell-to-cell communication system that in turn modifies gene expression through the release of specific molecular signals, the AI. Accordingly, *C. albicans* produces several AI, including farnesol and tyrosol. The former is involved in the inhibition of filamentation and

subsequent impairment in biofilm formation [41–45]; conversely tyrosol, shortens the fungal lag phase during growth and accelerates germ tube formation, thus promoting the yeast-to-hyphal cell transition [39]. Here, we show that *Candida* treatment with PomeGr impairs tyrosol production by about 60%. In contrast, farnesol, undetectable in untreated samples, is abundantly produced upon PomeGr treatment. Thus, by up-regulating farnesol (a filamentation inhibitor) and down-regulating tyrosol (a filamentation promoter), PomeGr interferes with *Candida* biofilm formation, at least under the conditions employed in our model. Certainly, the opposed effects observed on farnesol and tyrosol warrant further investigations; in any case, we may conclude that the regulation of these two AI is a key step in the accomplishment of antibiofilm effects by PomeGr. It should be noted that the present data are in line with recent papers demonstrating the complex modulation of *Candida* tyrosol/farnesol by other bioactive compounds [32,33].

Although having the limitations intrinsic of an *in vitro* model, our data adds new insights on the events occurring during *Candida* biofilm formation and its impairment upon treatment with the natural compound PomeGr. Any potential role of the preservatives may be excluded, being such compounds used at doses below their anti-*Candida* MIC. A better understanding of the events involved in such a phenomenon may help to counteract *Candida* pathogenicity, hopefully targeting selected/novel traits of its virulence. Preliminary experiments rule out a direct toxic effect of PomeGr against human epithelial cells; indeed, when used at 1:16 dilution, it did not affect LDH release, further encouraging studies aimed at characterizing novel antimicrobial formulations, possibly retaining a wide-spectrum activity.

5. Conclusions

Our results reinforce the importance of PomeGr as a natural product against the opportunistic pathogen *C. albicans*. Fungal load and biofilm formation are indeed affected in parallel with the consumption of certain phenolic compounds. Furthermore, we provide the first evidence that *Candida* exposure to PomeGr results in opposed regulation of the two major AI, farnesol and tyrosol. Since the finely AI-regulated biofilm formation is linked to *C. albicans* pathogenicity, PomeGr should be

considered as a precious source of natural bioactive compounds for the development of new therapeutic strategies against *Candida* infections.

REFERENCES

1. Vestby, L.K.; Grønseth, T.; Simm, R.; Nesse, L.L. Bacterial Biofilm and its Role in the Pathogenesis of Disease. *Antibiotics* **2020**, *9*, 59. https://doi.org/10.3390/antibiotics9020059.

2. Joo, H.S.; Otto, M. Molecular basis of in vivo biofilm formation by bacterial pathogens. *Chem. Biol*. **2012**, *19*, 1503–1513. https://doi.org/10.1016/j.chembiol.2012.10.022.

3. Flemming, H.C.; Wingender, J. The biofilm matrix. *Nat. Rev. Microbiol*. **2010**, *8*, 623–633. https://doi.org/10.1038/nrmicro2415.

4. Yin, W.; Wang, Y.; Liu, L.; He, J. Biofilms: The Microbial "Protective Clothing" in Extreme Environments. *Int. J. Mol. Sci*. **2019**, *20*, 3423. https://doi.org/10.3390/ijms20143423.

5. Harrison, J.J.; Ceri, H.; Turner, R.J. Multimetal resistance and tolerance in microbial biofilms. *Nat. Rev. Microbiol*. **2007**, *5*, 928–938. https://doi.org/10.1038/nrmicro1774.

6. Hostacká, A.; Ciznár, I.; Stefkovicová, M. Temperature and pH affect the production of bacterial biofilm. *Folia Microbiol.* **2010**, *55*, 75–78. https://doi.org/10.1007/s12223-010-0012-y.

7. Hathroubi, S.; Mekni, M.A.; Domenico, P.; Nguyen, D.; Jacques, M. Biofilms: Microbial Shelters Against Antibiotics. *Microb. Drug Resist.* **2017**, *23*, 147–156. https://doi.org/10.1089/mdr.2016.0087.

8. Abisado, R.G.; Benomar, S.; Klaus, J.R.; Dandekar, A.A.; Chandler, J.R. Bacterial Quorum Sensing and Microbial Community Interactions. *mBio* **2018**, *9*, e02331-17. https://doi.org/10.1128/mBio.02331-17.

9. Kovács, R.; Majoros, L. Fungal Quorum-Sensing Molecules: A Review of Their Antifungal Effect against Candida Biofilms. *J. Fungi* **2020**, *6*, 99. https://doi.org/10.3390/jof6030099.

10. Wongsuk, T.; Pumeesat, P.; Luplertlop, N. Fungal quorum sensing molecules: Role in fungal morphogenesis and pathogenicity. *J. Basic Microbiol*. **2016**, *56*, 440–447. https://doi.org/10.1002/jobm.201500759.

11. Hornby, J.M.; Jensen, E.C.; Lisec, A.D.; Tasto, J.J.; Jahnke, B.; Shoemaker, R.; Dussault, P.; Nickerson, K.W. Quorum sensing in the dimorphic fungus *Candida albicans* is mediated by farnesol. *Appl. Environ. Microbiol*. **2001**, *67*, 2982–2992. https://doi.org/10.1128/AEM.67.7.2982-2992.2001.

12. Ramage, G.; Saville, S.P.; Thomas, D.P.; López-Ribot, J.L. Candida biofilms: An update. *Eukaryot. Cell* **2005**, *4*, 633–638. https://doi.org/10.1128/EC.4.4.633-638.2005.

13. Henriques, M.; Martins, M.; Azeredo, J.; Oliveira, R. Effect of farnesol on Candida dubliniensis morphogenesis. *Lett. Appl. Microbiol*. **2007**, *44*, 199–205. https://doi.org/10.1111/j.1472-765X.2006.02044.x.

14. Morehead, M.S.; Scarbrough, C. Emergence of Global Antibiotic Resistance. *Prim. Care* **2018**, *45*, 467–484. https://doi.org/10.1016/j.pop.2018.05.006.

15. Huemer, M.; Mairpady Shambat, S.; Brugger, S.D.; Zinkernagel, A.S. Antibiotic resistance and persistence-Implications for human health and treatment perspectives. *EMBO Rep*. **2020**, *21*, e51034. https://doi.org/10.15252/embr.202051034.

16. Høiby, N.; Bjarnsholt, T.; Moser, C.; Bassi, G.L.; Coenye, T.; Donelli, G.; Hall-Stoodley, L.; Holá, V.; Imbert, C.; Kirketerp-Møller, K.; et al. ESCMID Study Group for Biofilms and Consulting External Expert Werner Zimmerli. ESCMID guideline for the diagnosis and treatment of biofilm infections 2014. *Clin. Microbiol. Infect*. **2015**, *21*, S1–S25. https://doi.org/10.1016/j.cmi.2014.10.024.

17. Meto, A.; Colombari, B.; Meto, A.; Boaretto, G.; Pinetti, D.; Marchetti, L.; Benvenuti, S.; Pellati, F.; Blasi, E. Propolis Affects *Pseudomonas aeruginosa* Growth, Biofilm Formation, eDNA Release and Phenazine Production: Potential Involvement of Polyphenols. *Microorganisms* **2020**, *8*, 243. https://doi.org/10.3390/microorganisms8020243.

18. Hammer, K.A.; Carson, C.F.; Riley, T.V. Antimicrobial activity of essential oils and other plant extracts. *J. Appl. Microbiol*. **1999**, *86*, 985–990. https://doi.org/10.1046/j.1365-2672.1999.00780.x.

19. Singh, B.; Singh, J.P.; Kaur, A.; Singh, N. Phenolic compounds as beneficial phytochemicals in pomegranate (*Punica granatum* L.) peel: A review. *Food Chem*. **2018**, *261*, 75–86. https://doi.org/10.1016/j.foodchem.2018.04.039.

20. Andrade, M.A.; Lima, V.; Sanches Silva, A.; Vilarinho, F.; Castilho, M.C.; Khwaldia, K.; Ramos, F. Pomegranate and grape by-products and their active compounds: Are they a valuable source for food applications? *Trends Food Sci. Technol*. **2019**, *86*, 68–84. https://doi.org/10.1016/j.tifs.2019.02.010.

21. Hernández-Corroto, E.; Marina, M.L.; García, M.C. Extraction and identification by high resolution mass spectrometry of bioactive substances in different extracts obtained from pomegranate peel. *J. Chromatogr. A*. **2019**, *1594*, 82–92. https://doi.org/10.1016/j.chroma.2019.02.018.

22. Li, Y.; Ye, T.; Yang, F.; Hu, M.; Liang, L.; He, H.; Li, Z.; Zeng, A.; Li, Y.; Yao, Y.; et al. *Punica granatum* (pomegranate) peel extract exerts potent antitumor and anti-metastasis activity in thyroid cancer. *RSC Adv*. **2016**, *6*, 84523–84535. https://doi.org/10.1039/C6RA13167K.

23. Tayel, A.A.; El-Baz, A.F.; Salem, M.F.; El-Hadary, M.H. Potential applications of pomegranate peel extract for the control of citrus green mould. *J. Plant Dis. Prot*. **2009**, *116*, 252–256. https://doi.org/10.1007/BF03356318.

24. Brighenti, V.; Iseppi, R.; Pinzi, L.; Mincuzzi, A.; Ippolito, A.; Messi, P.; Sanzani, S.M.; Rastelli, G.; Pellati, F. Antifungal Activity and DNA Topoisomerase Inhibition of Hydrolysable Tannins from *Punica granatum* L. *Int. J. Mol. Sci*. **2021**, *22*, 4175. https://doi.org/10.3390/ijms22084175.

25. Odorici, A.; Colombari, B.; Bellini, P.; Meto, A.; Venturelli, I.; Blasi, E. Novel Options to Counteract Oral Biofilm Formation: In Vitro Evidence. *Int. J. Environ. Res. Public Health*. **2022**, *19*, 8056. https://doi.org/10.3390/ijerph19138056.

26. Cormack, B.P.; Bertram, G.; Egerton, M.; Gow, N.A.R.; Falkow, S.; Brown, A.J.P. Yeast-enhanced green fluorescent protein (yEGFP): A reporter of gene expression in *Candida albicans*. *Microbiology* **1997**, *143*, 303–311. https://doi.org/10.1099/00221287-143-2-303.

27. Colombari, B.; Alfano, G.; Gamberini, C.; Cappelli, G.; Blasi, E. EDTA and Taurolidine Affect *Pseudomonas aeruginosa* Virulence *In Vitro*-Impairment of Secretory Profile and Biofilm Production onto Peritoneal Dialysis Catheters.

Microbiol. Spectr. **2021**, *9*, e0104721. https://doi.org/10.1128/Spectrum.01047-21.

28. Peppoloni, S.; Pericolini, E.; Colombari, B.; Pinetti, D.; Cermelli, C.; Fini, F.; Prati, F.; Caselli, E.; Blasi, E. The β-Lactamase Inhibitor Boronic Acid Derivative SM23 as a New Anti-*Pseudomonas aeruginosa* Biofilm. *Front Microbiol*. **2020**, *11*, 35. https://doi.org/10.3389/fmicb.2020.00035.

29. Elif Ayşe Erdoğan Eliuz. Antimicrobial activity of citric acid against Escherichia coli, Staphylococcus aureus and Candida albicans as a sanitizer agent. *Eurasian J. For. Sci.* **2020**, *8*, 295–301.

30. Fidalgo, T.K.; Barcelos, R.; Portela, M.B.; Soares, R.M.; Gleiser, R.; Silva-Filho, F.C. Inhibitory activity of root canal irrigants against Candida albicans, Enterococcus faecalis and Staphylococcus aureus. *Braz. Oral Res.* **2010**, *24*, 406–412. https://doi.org/10.1590/s1806-83242010000400006. PMID: 21180960.

31. Stanojevic, D.; Comic, L.; Stefanovic, O.; Solujic-Sukdolak, S. Antimicrobial effects of sodium benzoate, sodium nitrite and potassium sorbate and their synergistic action in vitro. *Bulg. J. Agric. Sci.* **2009**, *15*, 307–311.

32. Ardizzoni, A.; Boaretto, G.; Pericolini, E.; Pinetti, D.; Capezzone de Joannon, A.; Durando, L.; Ragni, L.; Blasi, E. Effects of benzydamine and mouthwashes containing benzydamine on *Candida albicans* adhesion, biofilm formation, regrowth, and persistence. *Clin. Oral Investig.* **2022**, *26*, 3613–3625. https://doi.org/10.1007/s00784-021-04330-8.

33. Spaggiari, L.; Sala, A.; Ardizzoni, A.; De Seta, F.; Singh, D.K.; Gacser, A.; Blasi, E.; Pericolini, E. *Lactobacillus acidophilus*, *L. plantarum*, *L. rhamnosus*, and *L. reuteri* Cell-Free Supernatants Inhibit *Candida parapsilosis* Pathogenic Potential upon Infection of Vaginal Epithelial Cells Monolayer and in a Transwell Coculture System In Vitro. *Microbiol. Spectr*. **2022**, *10*, e0269621. https://doi.org/10.1128/spectrum.02696-21.

34. Kupnik, K.; Primožič, M.; Vasić, K.; Knez, Ž.; Leitgeb, M. A Comprehensive Study of the Antibacterial Activity of Bioactive Juice and Extracts from Pomegranate (*Punica granatum* L.) Peels and Seeds. *Plants* **2021**, *10*, 1554. https://doi.org/10.3390/plants10081554.

35. Abdollahzadeh, Sh.; Mashouf, R.; Mortazavi, H.; Moghaddam, M.; Roozbahani, N.; Vahedi, M. Antibacterial and antifungal activities of punica granatum peel extracts against oral pathogens. *J. Dent.* (*Tehran, Iran*) **2011**, *8*, 1–6.

36. Endo, E.H.; Cortez, D.A.; Ueda-Nakamura, T.; Nakamura, C.V.; Dias Filho, B.P. Potent antifungal activity of extracts and pure compound isolated from pomegranate peels and synergism with fluconazole against *Candida albicans*. *Res. Microbiol*. **2010**, *161*, 534–540. https://doi.org/10.1016/j.resmic.2010.05.002.

37. Moreira, D.; Gullón, B.; Gullón, P.; Gomes, A.; Tavaria, F. Bioactive packaging using antioxidant extracts for the prevention of microbial food-spoilage. *Food Funct*. **2016**, *7*, 3273–3282. https://doi.org/10.1039/c6fo00553e.

38. Magangana, T.P.; Makunga, N.P.; Fawole, O.A.; Opara, U.L. Processing Factors Affecting the Phytochemical and Nutritional Properties of Pomegranate (*Punica granatum* L.) Peel Waste: A Review. *Molecules* **2020**, *25*, 4690. doi: 10.3390/molecules25204690.

39. Mphahlele, R.R.; Fawole, O.A.; Makunga, N.P.; Linus Opara, U. Functional properties of pomegranate fruit parts: Influence of packaging systems and storage time. *J. Food Meas. Charact*. **2017**, *11*, 2233–2246.

40. Nejatbakhsh's, S.; Ilkhanizadeh-Qomi's, M.; Razzaghi-Abyaneh, M.; Jahanshiri, Z. The Effects of Ellagic Acid on Growth and Biofilm Formation of *Candida albicans*. *J. Med. Microbiol. Infect. Dis.* **2020**, *8*, 14–18. https://doi.org/10.29252/JoMMID.8.1.14.

41. Gupta, P.; Sharma, M.; Arora, N.; Pruthi, V.; Poluri, K.M. Chemistry and Biology of Farnesol and its Derivatives: Quorum Sensing Molecules with Immense Therapeutic Potential. *Curr. Top. Med. Chem*. **2018**, *18*, 1937–1954. https://doi.org/10.2174/1568026619666181210124159.

42. Egbe, N.E.; Dornelles, T.O.; Paget, C.M.; Castelli, L.M.; Ashe, M.P. Farnesol inhibits translation to limit growth and filamentation in *C. albicans* and *S. cerevisiae*. *Microb. Cell* **2017**, *4*, 294–304. https://doi.org/10.15698/mic2017.09.589.

43. Singkum, P.; Muangkaew, W.; Suwanmanee, S.; Pumeesat, P.; Wongsuk, T.; Luplertlop, N. Suppression of the pathogenicity of *Candida albicans* by the

quorum-sensing molecules farnesol and tryptophol. *J. Gen. Appl. Microbiol.* **2020**, *65*, 277–283. https://doi.org/10.2323/jgam.2018.12.002.

44. Chen, H.; Fujita, M.; Feng, Q.; Clardy, J.; Fink, G.R. Tyrosol is a quorum-sensing molecule in *Candida albicans*. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 5048–5052. https://doi.org/10.1073/pnas.0401416101.

45. Ramage, G.; Saville, S.P.; Wickes, B.L.; López-Ribot, J.L. Inhibition of *Candida albicans* biofilm formation by farnesol, a quorum-sensing molecule. *Appl. Environ. Microbiol*. **2002**, *68*, 5459–5463.

CHAPTER 4

Microorganisms **2022**, *10*, 2500. doi: 10.3390/microorganisms10122500

ARTICLE

Attenuation of *Pseudomonas aeruginosa* Virulence by Pomegranate Peel Extract

Samuele Peppoloni[1], Bruna Colombari[1], Davide Tagliazucchi[2], Alessandra Odorici[3], Cristiano Ventrucci[4], Aida Meto[1,5] and Elisabetta Blasi[1,*]

[1] Laboratory of Microbiology and Virology, Department of Surgery, Medicine, Dentistry and Morphological Sciences with Interest in Transplant, Oncology and Regenerative Medicine, University of Modena and Reggio Emilia, Via G. Campi 287, 41125 Modena, Italy

[2] Department of Life Sciences, University of Modena and Reggio Emilia, Via Amendola, 2—Pad. Besta, 42100 Reggio Emilia, Italy

[3] Laboratory of Microbiology and Virology, School of Doctorate in Clinical and Experimental Medicine, University of Modena and Reggio Emilia, Via G. Campi 287, 41125 Modena, Italy

[4] INCOS–Cosmeceutica Industriale, Funo di Argelato, 40050 Bologna, Italy

[5] Department of Dentistry, Faculty of Dental Sciences, University of Aldent, 1007 Tirana, Albania

Keywords: anti-biofilm; *Pseudomonas aeruginosa*; pomegranate; virulence; autoinducers; phenolic compounds

Abstract

Pseudomonas aeruginosa is an opportunistic pathogen often responsible for biofilm-associated infections. The high adhesion of bacterial cells onto biotic/abiotic surfaces is followed by production of an extracellular polysaccharidic matrix and formation of a sessile community (the biofilm) by the release of specific quorum-sensing molecules, named autoinducers (AI). When the concentrations of AI reach a threshold level, they induce the expression of many virulence genes, including those involved in biofilm formation, motility, pyoverdine and pyocyanin release. *P. aeruginosa* embedded into biofilm becomes resistant to both conventional drugs and the host's immune response. Accordingly, biofilm-associated infections are a major clinical problem underlining the need for new antimicrobial therapies. In this study, we evaluated the effects of pomegranate peel extract (PomeGr) in vitro on *P. aeruginosa* growth and biofilm formation; moreover, the release of four AI was assessed. The phenolic profile of PomeGr, exposed or not to bacteria, was determined by high-performance liquid chromatography coupled to electrospray ionization mass spectrometry (HPLC-ESI-MS) analysis. We found that bacterial growth, biofilm production and AI release were impaired upon PomeGr treatment. In addition, the PomeGr phenolic content was also markedly hampered following incubation with bacterial cells. In particular, punicalagin, punicalin, pedunculagin, granatin, di-(HHDP-galloyl-hexoside) pentoside and their isomers were highly consumed. Overall, these results provide novel insights on the ability of PomeGr to attenuate *P. aeruginosa* virulence; moreover, the AI impairment and the observed consumption of specific phenolic compounds may offer new tools in designing innovative therapeutic approaches against bacterial infections.

1. Introduction

As an opportunistic pathogen, *Pseudomonas aeruginosa (P. aeruginosa)* causes severe infections in susceptible individuals, such as patients with acquired immunodeficiency syndrome (AIDS), cancer, severe burns or indwelling devices [1]. *P. aeruginosa* expresses numerous virulence factors that, through sophisticated regulatory mechanisms, allow it to adapt easily to many hostile environments [2]. Because of such adaptability and given the increasing drug-resistance, conventional antibacterial agents show limited efficacy against Pseudomonas [3]. As with many other microorganisms, besides those living in a planktonic form, *P. aeruginosa* is capable, as a survival strategy, of forming biofilm on medical devices and mucosal surfaces [4]. Accordingly, most hospital-acquired infections are associated with biofilm formation onto catheters, ventilator tubes, implants and medical prosthetic devices [5]. Therefore, early diagnosis of *P. aeruginosa* infection is crucial to promptly counteract the pathogen and, in turn, decrease mortality.

Microbial biofilm is a complex community of microorganisms' adherent to living on abiotic surfaces and tightly embedded in a self-produced matrix (extracellular polymeric substances or EPS), primarily composed of polysaccharides, extracellular DNA (eDNA), proteins and lipids [6]. Importantly, the matrix, responsible for over 90% of the biofilm biomass, acts as a scaffold for adhesion to surfaces and, more importantly, protects sessile bacteria during stressful environmental conditions [7]. Indeed, when enclosed within the EPS, *P. aeruginosa* is protected from host immune responses and becomes less susceptible to antibiotics, compared to the planktonic counterpart [8]. Moreover, unlike planktonic cells, bacterial communities structured as biofilms exhibit an altered phenotype in terms of growth rate, expression of virulence factors and cell-to-cell communication systems [9].

For many microorganisms, the ability to form biofilm and finely modulate virulence factors expression is controlled by intercellular communication mechanisms, named quorum sensing system (QS), that function in a hierarchical manner, by means of signaling molecules and receptors, and are closely related to cell density [9]. In *P. aeruginosa,* four main QS systems have been identified, namely LasI/LasR, RhlI/RhlR [10], PqsABCDE/PqsR [11] and AmbBCDE/IqsR [12]. For each of these QS systems, *P. aeruginosa* synthesizes specific signal molecules, called

autoinducers (AI): 3-oxododecanoyl-L-homoserine lactone (3-oxo-C12-HSL); the N-butanoyl homoserine lactone (C4-HSL); the 2- heptyl-3-hydroxy-4-quinolone (Pseudomonas quinolone signal–PQS) and the 2-(2-hydroxyphenyl)thiazole-4-carbaldehyde (integrated quorum detection signal–IQS) [13]. These AI are involved in the regulation of many genes related to motility, biofilm formation, host immune evasion, iron scavenging and antibiotic resistance [14]. For example, the LasI/LasR system reduces the polysaccharide matrix, thus inducing biofilm dispersion; it is also involved in the resistance to detergents [15]. The RhlI/RhlR system regulates the expression of rhamnolipids, which are important for the formation of a mature biofilm, its subsequent dispersion and resistance to host immune responses as well [16]. The PqsABCDE/PqsR system is mainly involved in biofilm formation and eDNA release, an important step for the stability of biofilm structure [17]. Finally, the fourth QS system, the IQS, influences the early stages of biofilm formation by affecting the swarming motility of *P. aeruginosa* [18]; in addition, IQS regulates the synthesis of siderophores, pyoverdine and pyochelin [19] and is responsible for the antibiotic resistance observed in the sessile bacteria within the biofilm [20]. Since antibiotic treatment has often limited efficacy against biofilm [21], a recent study has been focused on the regulatory mechanisms involved in QS with the aim of developing alternative therapies against *Pseudomonas* infections [22,23]. New molecules capable of interfering with QS signaling mechanisms may represent a successful antimicrobial new strategy. In this context, plant extracts and their derivatives, rich in polyphenols such as *Punica granatum L.*, could represent a new class of antimicrobial agents, capable of counteracting Pseudomonas' virulence. Pomegranate peel extract (PomeGr) is an excellent source of biocompounds with biological activities and therapeutic properties, such as anti-inflammatory, antioxidant, antitumor and antimicrobial activities [24–26]. Recently, several studies have evaluated the antimicrobial activity of *Punica granatum* extract alone or in association with antibiotics. Interestingly, Abu El-Wafa WM et al. [27] have demonstrated that the combinations of pomegranate and rosemary extracts with antibiotics (namely piperacillin, ceftazidime, imipenem, gentamycin and levofloxacin) have a synergistic anti-Pseudomonas activity.

Here, we evaluate the effects of PomeGr on *P. aeruginosa* growth, biofilm formation and release of the AI, as well-known parameters linked to its pathogenicity. Moreover, the *P. aeruginosa* consumption of specific phenolic compounds present in PomeGr was also investigated.

2. Materials and Methods

2.1. Microbial Cells and Growth Conditions

The bioluminescent *P. aeruginosa* (BLI-*Pseudomonas*) strain P1242, expressing the luciferase gene and luciferin substrate under the control of a constitutive P1 integron promoter, was engineered by Choi and Schweizer [28]. This strain was maintained as previously described [29]. The bioluminescent signal was measured by a Fluoroskan reader (Thermo Fischer Scientific, Waltham, MA, USA) and expressed as relative luminescence units (RLU) as a parameter correlating with the number of viable cells [29]. Bacteria from −80 °C glycerol stocks were seeded onto tryptic soy agar (TSA) (OXOID, Milan, Italy) plates and incubated overnight at 37 °C. The resulting colonies were then collected, added to 10 mL of tryptic soy broth (TSB) (OXOID, Milan, Italy) and further incubated overnight at 37 °C by gentle shaking. The day after, the OD_{595} of the bacterial suspension was spectrophotometrically measured (by TECAN Sunrise, Männedorf, Switzerland) and the obtained value was converted into colony-forming units (CFU)/mL, according to an internal standard curve. Then, the bacterial suspension was appropriately diluted to obtain the final working condition of 10^6 bacteria/mL.

2.2. Pomegranate Peel Extract

The *Punica granatum* L. peel extract (PomeGr), supplied by INCOS COSMECEUTICA INDUSTRIALE (Bologna, Italy) and produced by PHENBIOX SRL (Bologna, Italy), contained the peel extract (22.5% w/w), saccharomyces ferment lysate filtrate, citric acid, sodium benzoate and potassium sorbate, as detailed elsewhere [26]. The same solution without PomeGr was used as a negative control (neg-C). The PomeGr and the neg-C were filtered through a 0.22 µm membrane and stored at 4 °C until tested in the assays.

2.3. Total Microbial Growth, Biofilm Formation and Regrowth Assays

The PomeGr was serially diluted and plated (100 µL/well) in black 96-well microtiter plates with transparent bottoms (PerkinElmer, Milano, Italy). The bacterial suspension (10^6 cells/mL in TSB, prepared as detailed above) was added to the plates (100 µL/well) and incubated at 37 °C for 24 h. The fluorescent signal was hourly measured; the RLU values represented the amounts of live cells in the treated and untreated groups. After treatment, the samples were washed two times and the bioluminescent (BLI) signal was measured to quantify the 24 h-old biofilm, as previously detailed [30,31]. Next, to evaluate the PomeGr inhibitory effects on Pseudomonas regrowth, fresh medium was added to each well and the plate was further incubated at 37 °C; microbial growth was kinetically checked (24 to 48 h) by measuring the RLU every hour.

2.4. Mass Spectrometry Analysis of PomeGr Extract

The phenolic profile of PomeGr extract (diluted 1:16), exposed or not to *P. aeruginosa* (10^6 cells/mL) for 24 h at 37 °C, was assessed by high-resolution mass spectrometry (HR-MS). The cell-free supernatants were ultrafiltered with Amicon-Ultra 0.5 tubes (14,000 rpm; 15 min), diluted 1:1 in a methanol/water solution (5:95 v/v) and injected in an Ultimate 3000 UHPLC system coupled to a Q Exactive Mass Spectrometer (Thermo Scientific, San Jose, CA, USA). Chromatographic separation was performed by a C18 column (Hypersil Gold C18, 100 × 2.1 mm, 1.9 µm particle size; Thermo Scientific, San Jose, CA, USA). The mobile phases consisted of (A) water acidified with formic acid (0.1%) and (B) acetonitrile acidified with formic acid (0.1%). The flow rate was set at 0.5 mL/min. The elution gradient began at 2% B and after 0.5 min started to linearly increase to 18% B in 27 min and afterwards to 98% B in 4.5 min. In order to wash the column, solvent B was maintained at 98% for 3 min before returning to the initial conditions. The MS and MS/MS parameters are fully reported in Colombari et al. [26]. The relative amount of the identified phenolic compounds was determined by integrating the area under the peak (AUP) from the extracted ion chromatograms (tolerance ± 5 ppm).

2.5. Mass Spectrometry Analysis of P. aeruginosa AI

Cell-free supernatants from 24 h-old *P. aeruginosa* cultures (10^6/mL), treated or not with PomeGr (1:16 dilution), were harvested, filtered and analyzed by HPLC-ESI-

MS, as previously detailed [31]. The relative amount of the four AI was determined by integrating the area under the peak (AUP) from treated and untreated cultures (tolerance ± 5 ppm).

2.6. Statistical Analysis

Statistical analysis was performed by using GraphPad Prism 8 software. Statistical differences between groups were evaluated with the one-way ANOVA followed by Sidak's multiple comparisons tests, as previously detailed [26]. Values of $p \leq 0.001$ were considered statistically highly significant.

3. Results

3.1. PomeGr Effects on Pseudomonas Growth and Biofilm Formation

Initially, we assessed in real time the effects of PomeGr on *P. aeruginosa* planktonic cell growth by using a previously established BLI-based assay [30]. As depicted in Figure 1A–C, the curves of the untreated samples (medium) showed the expected time-dependent increase in RLU that reached their maximal values after 8 h.

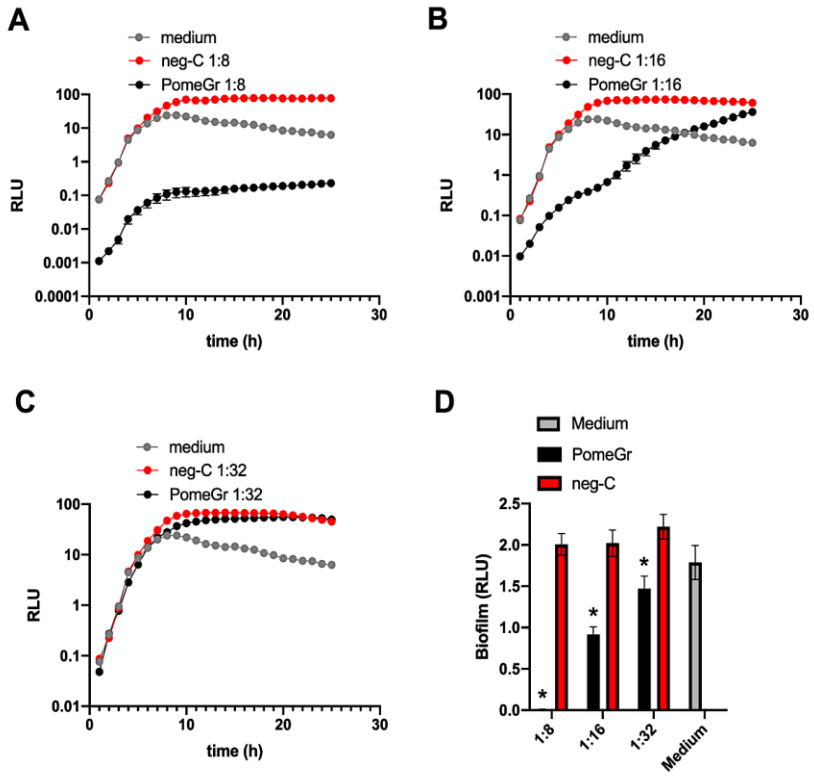


Figure 1. The PomeGr effects on *P. aeruginosa* growth and biofilm formation**.** *P. aeruginosa* (10^6/mL, 100 µL/well) was exposed for 24 h to PomeGr or neg-C (100 µL/well) at dilutions of 1:8, 1:16 and 1:32. By BLI assay, the total microbial load was kinetically measured every hour (Panel (**A**): 1:8 dilution; Panel (**B**): 1:16 dilution; Panel (**C**): 1:32 dilution). The samples were then washed and the biofilm formation was quantified by an additional bioluminescence reading (Panel **D**). The results shown were the mean (±SEM) of the RLU from 9–12 replicates of three independent experiments. The asterisk indicates $p \leq 0.001$ (PomeGr vs. neg-C).

Similarly, when exposed to the neg-C, *P. aeruginosa* showed curves comparable to those of the medium, independent of the dilution tested. In contrast, at the 1:8 dilution, PomeGr significantly affected the RLU already at time 0 (drop of 2 Logs); interestingly, the curve consistently remained below the controls at all the timepoints tested, with a 3-Log difference detected at 24 h. At the 1:16 PomeGr dilution, the inhibition observed was partial and gradually disappeared in that the RLU reached the values of the controls (medium and neg-C) between 17 and 24 h. Furthermore, no effects were found using the 1:32 dilution. Next, to investigate whether PomeGr would affect *P. aeruginosa* biofilm formation, after 24 h of treatment, the wells were washed to remove non-adherent bacteria and the residual bioluminescent signal was measured. As shown in Figure 1D, PomeGr significantly affected biofilm production in a dose-dependent manner. In particular, >99% inhibition was observed when employing the 1:8 dilution, while about 55% and 33% impairment were detected at 1:16 and 1:32 dilutions, respectively. When assessed against neg-C, the PomeGr significantly affected biofilm production at all the dilutions tested ($p < 0.001$); furthermore, at 1:64 and 1:128, the impairments were still significant ($p < 0.01$; data not shown).

In order to evaluate whether the PomeGr inhibitory effects on Pseudomonas biofilm persisted over time, we next focused on the 24–48 h timeframe. Accordingly, after 24 h incubation, PomeGr was removed from the cells and fresh medium was added to each well. The plate was again incubated at 37 °C and microbial load was further kinetically assessed (from 24 to 48 h) by measuring the RLU. As shown in Figure S1 (Supplementary Materials), cells exposed to the 1:8 dilution slowly regrew,

reaching the control/neg-C treated cells at about time 38 h, while the 1:16 and 1:32 dilutions were ineffective.

Since the 1:16 PomeGr dilution significantly impaired Pseudomonas biofilm production without affecting total growth, this condition was chosen for further analyses.

3.2. Phenolic Compounds Profile of PomeGr Exposed or Not to P. aeruginosa

Pomegranate peel extract is known to be a rich source of phenolic compounds, including flavonoids (anthocyanins, catechins and other complex flavonoids) and hydrolysable tannins (punicalin, pedunculagin, punicalagin, gallic acid and ellagic acid esters of glucose), previously reported as exerting numerous biological activities such as antioxidant, antimicrobial, anti-proliferative and anti-inflammatory activities [32–35].

By comparing the PomeGr MS and MS/MS profiles with those shown by the literature, several compounds were identified and the MS details on the phenolic compounds profile of PomeGr were given in our recently published manuscript [26]. Table S1 shows the data about the relative quantification of each phenolic compound (expressed as AUP) detected in the PomeGr, that had been exposed or not to *P. aeruginosa.* Furthermore, the relative percentage decrease was calculated. Figure 2 details the overlapping chromatographic peaks of five compounds that displayed the highest decrease after PomeGr incubation with *P. aeruginosa* cells. In particular, following PomeGr exposure to *P. aeruginosa* (grey lines) and, in comparison, with the control (PomeGr alone; black lines), a significant decrease in pedunculagin (decrease ranging from 58.7 to 88%), punicalagin (decrease ranging from 32 to 72%), granatin (decrease ranging from 94.8 to 99.6%), punicalin (21.1% decrease) and di-(HHDP-galloyl-hexoside)-pentoside (82.4% decrease) was observed.

Microorganisms **2022**, *10*, 2500. doi: 10.3390/microorganisms10122500

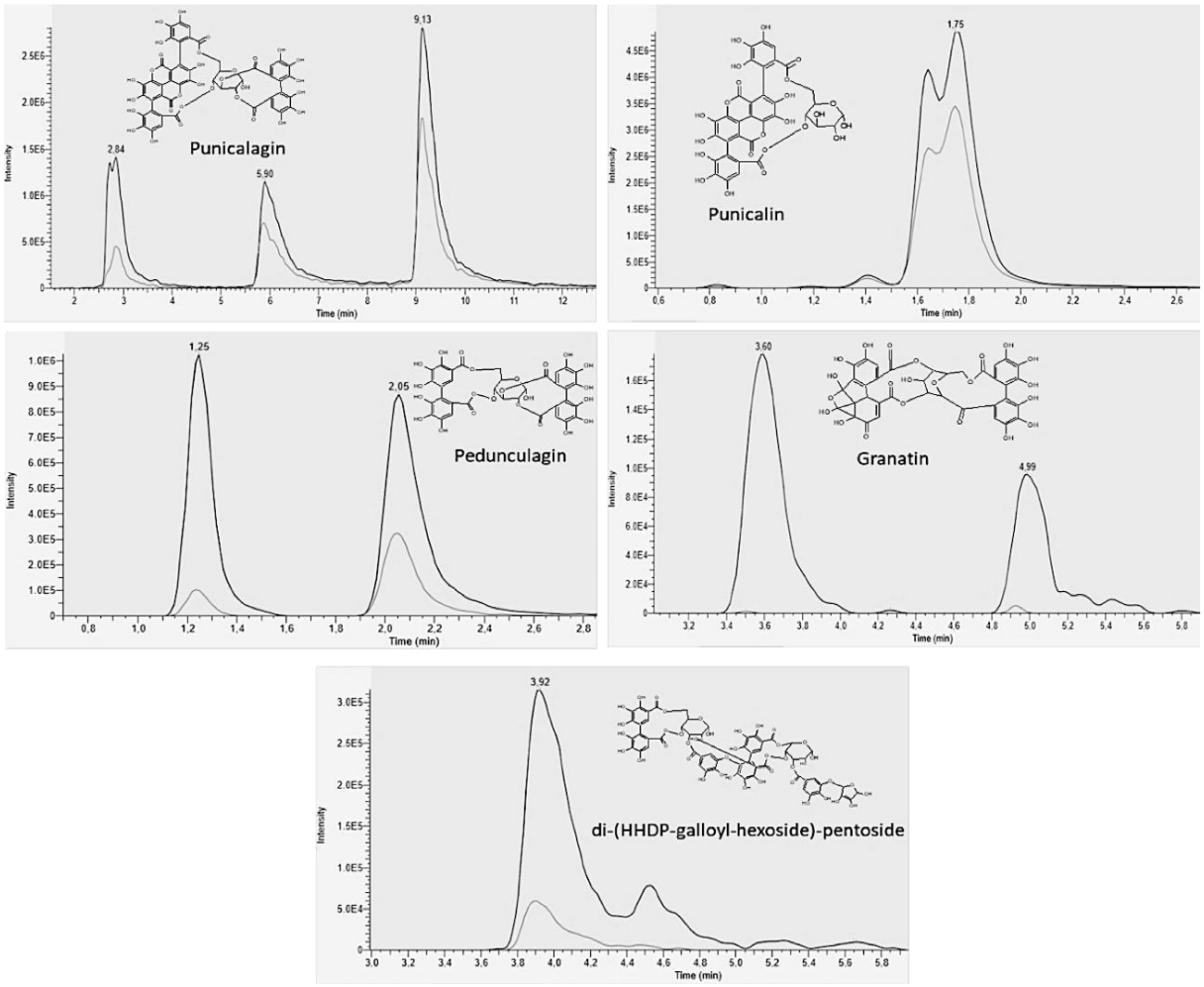


Figure 2. Phenolic compounds decrease upon PomeGr exposure to *P. aeruginosa*. Overlapped peaks of 5 phenolic compounds in PomeGr extract, assessed alone (black line) or upon exposure to *P. aeruginosa* (grey line). The data were obtained by a pool of 4 replicates from a representative experiment. Each panel also shows the chemical structure of each compound.

3.3. PomeGr Effects on AI Release by P. aeruginosa

P. aeruginosa produces several AI signal molecules involved in Pseudomonas biofilm formation and bacterial virulence as well. By HPLC-ESI-MS analysis, we measured the AI production by *P. aeruginosa* exposed or not to 1:8 and 1:16 dilutions of PomeGr. Using cell-free supernatants, we obtained several chromatograms, through which the AUP of the various elution peaks were measured and the semi-quantitative assessments of the specific products were performed.

Table 1 shows the peak areas of four AI, namely 3-oxo-C12-HSL, C4-HSL, PQS and IQS, detected in the supernatants of cells exposed or not to PomeGr. A marked reduction in the AI content was observed following PomeGr treatment; the observed effect was dose-dependent and ranged between 0.5 and 4 Log; moreover, the amount of IQS also dropped to undetectable levels.

Table 1. AI release by *P. aeruginosa* exposed or not to PomeGr.

Treatment	3-oxo-C12-HSL	C4-HSL	PQS	IQS
Medium	1.65×10^8	3.15×10^8	2.51×10^{10}	2.09×10^6
PomeGr 1:8	1.89×10^6	3.99×10^7	4.42×10^6	n.d.
PomeGr 1:16	6.58×10^7	3.92×10^7	1.93×10^8	n.d.

The AI were measured on cell-free supernatants from *P. aeruginosa*, treated or not with PomeGr, by HPLC-MS. By chromatogram analysis, the different elution peaks were identified and their areas used for semiquantitative evaluation of each molecule. The results shown are from a pool of 4 replicates of a representative experiment. n.d. not detectable.

4. Discussion

Here, we show that PomeGr affects *P. aeruginosa* growth, biofilm formation and AI release, implying a marked impairment of its virulence. In addition, the consumption of specific phenolic compounds by *P. aeruginosa* suggests their direct involvement in the antibacterial activity.

P. aeruginosa is an opportunistic pathogen frequently involved in biofilm-related infections that are difficult to treat with conventional drugs. Several studies have been performed on the antimicrobial activity of *Punica granatum* L., with the aim of successfully countering infections by using alternative approaches [25,27,32,34,35]. Here, we demonstrate the ability of PomeGr to significantly impair *P. aeruginosa* growth and biofilm formation in a dose-dependent manner. Indeed, as shown in Figure 1, the kinetically evaluated microbial growth remains lower than that observed in medium or neg-C treated cells, at PomeGr dilutions 1:8 or below (data not shown). At 1:16 PomeGr dilution, the RLUs progressively increase, reaching the control levels between 16–18 h, while at 1:32 dilution no effects have been detected. Furthermore, as depicted in Figure 1D, PomeGr significantly affects biofilm formation in a dose-dependent manner. In particular, more than 99% inhibition occurs at 1:8 (Figure 1D)

or less (data not shown) while, at 1:16 and 1:32 dilutions, the decrease ranges from 54% to 24%, respectively. In our experiments, the neg-C has no effects on *Pseudomonas*, implying that the additives/preservatives contained in the extraction solution have no role or toxic effects by themselves. In any case, we have used concentrations of potassium sorbate, sodium benzoate and citric acid below the MIC previously established by several studies against *P. aeruginosa* [36,37].

In our model, the anti-Pseudomonas effect is reversible, since the removal of PomeGr allows bacterial regrowth (see the Supplementary Materials). Whether the PomeGr-treated population may exhibit different phenotypes, i.e., susceptible, resistant and/or tolerant cells, as proposed by other studies obtained exposing Pseudomonas to antibiotics [38,39], remains to be investigated. Interestingly, initial evidence exists on the synergism between pomegranate extract and conventional antibiotics against Pseudomonas [27], further emphasizing the potential relevance of such a natural product in the design of novel antimicrobial protocols. Here, we show that the best anti-Pseudomonas effects occur at 1:8 and 1:16 dilutions, the same conditions that had also been found to exert the highest effects against *Candida albicans* [26]. Taken together, our previous [26] and present data suggest that the same molecules act against bacterial and fungal target cells as well.

Punica granatum L. has been described as an excellent source of biocompounds, including phenolic acids, flavonoids and hydrolyzable tannins, mainly ellagitannins and gallotannins, each of them with beneficial properties on human health [35,40,41]. In line with the literature [41], we recently found that the PomeGr, used also in the present study, is mainly composed by ellagitannins such as pedunculagin and its isomers, ellagic acid-hexoside, punicalagin and its isomers, punicalin, granatin and di-(HHDP-galloyl-hexoside)-pentoside and its isomers [26]. Here, we demonstrated a remarkable consumption of polyphenols by PomeGr exposure to *P. aeruginosa*. As shown in Table S1 and in Figure 2, a strong reduction in the levels of pedunculagin (decrease ranging from 58.7 to 88%), punicalagin (decrease ranging from 32 to 72%), granatin (decrease ranging from 94.8 to 99.6%), punicalin (decrease of 21.1%) and di-(HHDP-galloyl-hexoside)-pentoside (decrease of 82.4%) occurs, highlighting the involvement of these molecules in the anti-Pseudomona*s* activity. It should be noted that a similar trend of consumption had been observed exposing PomeGr to fungal cells [26]. This further emphasizes the

likelihood that the same effector molecules act against bacterial and fungal cells as well. It is worth pointing out that the compounds showing the highest reduction (pedunculagin, punicalagin, granatin and di-(HHDP-galloyl-hexoside)-pentoside) contain in their structures at least one hexahydroxydiphenoyl moiety, suggesting its pivotal role in the antimicrobial and antifungal activities exerted by PomeGr.

P. aeruginosa biofilm formation is finely regulated by cell-to-cell communication systems that function in a hierarchical manner, by means of signaling molecules and receptors [10]. In particular, four main QS systems have been identified: LasI/LasR, RhlI/RhlR [11]; PqsABCDE/PqsR [12] and AmbBCDE/IqsR [13]; each of them synthesizes its own specific signal molecules, namely 3-oxo-C12-HSL, C4-HSL, PQS and IQS, respectively. Here, we show that all of the four AI are constitutively produced Pseudomonas, being detected in 24 h-old supernatants (Table 1). Interestingly, a marked reduction in the AI content is observed upon PomeGr treatment; particularly, a drop, ranging from 0.5 to 4 Log, occurs for 3-oxo-C12-HSL, C4-HSL and PQS, while the amounts of IQS drop to undetectable levels. These data provide the first evidence that PomeGr impairs *P. aeruginosa* AI production, although to a different extent, depending on the molecule considered. Initial literature [41] describes a close relationship between the flavonoid structure of certain compounds and some biological effects, such as impairment of pyocyanin and elastase production by Pseudomonas. In particular, Paczkowski et al. [41] have demonstrated that flavonoids are direct inhibitors of the QS receptor, LasR; yet, they do not function via a competitive mechanism involving displacement of their natural ligand, the AI 3-oxo-C12-HSL; rather, they prevent binding to the respective promoter region, thereby inhibiting the expression of downstream genes. Furthermore, it has been shown [42] that a methanolic extract of *Terminalia chebula,* containing ellagic acid-derivatives and flavogallonic acid-derivatives (structurally related to the hexahydroxydiphenoyl moieties), is able to inhibit *P. aeruginosa* biofilm formation, via downregulation of the *lasIR* and *rhlIR* gene expression, in turn resulting in a decreased production of AI [42]. The molecular mechanisms involved in the above-described inhibition remain to be investigated; however, the hierarchical organization of the various QS systems, controlled by the LasI/LasR, may explain the impairment of the four AI, observed in our model. Given the crucial role of such signal molecules that, through their specificity and concentration, finely regulate not only biofilm formation but also the

expression of genes involved in other processes, such as stress-tolerance and host–microbe interaction [39], we may conclude that bacterial virulence is indeed profoundly attenuated upon PomeGr treatment.

Despite the limitations intrinsic to any in vitro model, these results add new insights on the molecular mechanisms occurring during Pseudomonas biofilm development and its impairment by PomeGr. It remains to be established whether other microorganisms and/or multispecies biofilm may be successfully treated with PomeGr, hopefully by targeting other QS systems.

5. Conclusions

The results of this study emphasize the role of PomeGr as a natural antimicrobial compound against *P. aeruginosa.* Indeed, upon exposure to PomeGr, (i) bacterial growth and (ii) biofilm formation are affected; simultaneously, (iii) the consumption of specific phenolic compounds and (iv) the impairment of AI production by *P. aeruginosa* are detected. Because the AI-regulated biofilm production is strictly related to bacterial virulence, we may consider PomeGr as an interesting alternative to conventional drugs for the design of novel therapeutic protocols focusing on virulence attenuation of *P. aeruginosa*.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microorganisms10122500/s1. Figure S1: Kinetic of microbial regrowth; Table S1: Peak areas of phenolic compounds in PomeGr exposed or not to *P. aeruginosa*.

REFERENCES

1. Gale, M.J.; Maritato, M.S.; Chen, Y.L.; Abdulateef, S.S.; Ruiz, J.E. *Pseudomonas aeruginosa* causing inflammatory mass of the nasopharynx in an immunocompromised HIV infected patient: A mimic of malignancy. *IDCases* **2015**, *2*, 40–43.

2. Stover, C.K.; Pham, X.Q.; Erwin, A.L.; Mizoguchi, S.D.; Warrener, P.; Hickey, M.J.; Brinkman, F.S.; Hufnagle, W.O.; Kowalik, D.J.; Lagrou, M.; et al. Complete

genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* **2000**, *406*, 959–964.

3. Pang, Z.; Raudonis, R.; Glick, B.R.; Lin, T.J.; Cheng, Z. Antibiotic resistance in *Pseudomonas aeruginosa*: Mechanisms and alternative therapeutic strategies. *Biotechnol. Adv.* **2019**, *37*, 177–192.

4. Joo, H.S.; Otto, M. Molecular basis of in vivo biofilm formation by bacterial pathogens. *Chem. Biol.* **2012**, *19*, 1503–1513.

5. Zur, K.B.; Mandell, D.L.; Gordon, R.E.; Holzman, I.; Rothschild, M.A. Electron microscopic analysis of biofilm on endotracheal tubes removed from intubated neonates. *Otolaryngol.–Head Neck Surg.* **2004**, *130*, 407–414.

6. Ryder, C.; Byrd, M.; Wozniak, D.J. Role of polysaccharides in *Pseudomonas aeruginosa* biofilm development. *Curr. Opin. Microbiol.* **2007**, *10*, 644–648.

7. Cécile, R.; Laurent, G.; Jean, G. Biofilm-detached cells, a transition from a sessile to a planktonic phenotype: A comparative study of adhesion and physiological characteristics in *Pseudomonas aeruginosa*. *FEMS Microbiol. Lett.* **2008**, *290*, 135–142.

8. Mah, T.F.; O'Toole, G.A. Mechanisms of Biofilm Resistance to Antimicrobial Agents. *Trends Microbiol.* **2001**, 9, 34–39.

9. Venturi, V. Regulation of quorum sensing in *Pseudomonas*. *FEMS Microbiol. Rev.* **2006**, *30*, 274–291.

10. Pesci, E.C.; Pearson, J.P.; Seed, P.C.; Iglewski, B.H. Regulation of las and rhl quorum sensing in Pseudomonas aeruginosa. *J. Bacteriol.* **1997**, *179*, 3127–3132.

11. Dubern, J.F.; Diggle, S.P. Quorum sensing by 2-alkyl-4-quinolones in *Pseudomonas aeruginosa* and other bacterial species. *Mol. Biosyst.* **2008**, *4*, 882–888.

12. Lee, J.; Wu, J.; Deng, Y.; Wang, J.; Wang, C.; Wang, J.; Chang, C.; Dong, Y.; Williams, P.; Zhang, L.H. A cell-cell communication signal integrates quorum sensing and stress response. *Nat. Chem. Biol.* **2013**, *9*, 339–343.

13. Lee, J.; Zhang, L. The hierarchy quorum sensing network in *Pseudomonas aeruginosa*. *Protein Cell* **2016**, *6*, 26–41.

14. Jakobsen, T.H.; Bjarnsholt, T.; Jensen, P.Ø.; Givskov, M.; Høiby, N. Targeting quorum sensing in *Pseudomonas aeruginosa* biofilms: Current and emerging inhibitors. *Future Microbiol.* **2013**, *8*, 901–921.

15. Ueda, A.; Wood, T.K. Connecting quorum sensing, c-di-GMP, pel polysaccharide, and biofilm formation in *Pseudomonas aeruginosa* through tyrosine phosphatase TpbA (PA3885). *PLoS Pathog.* **2009**, *5*, e1000483.

16. Jensen, P.Ø.; Bjarnsholt, T.; Phipps, R.; Rasmussen, T.B.; Calum, H.; Christoffersen, L.; Moser, C.; Williams, P.; Pressler, T.; Givskov, M.; et al. Rapid necrotic killing of polymorphonuclear leukocytes is caused by quorum-sensing-controlled production of rhamnolipid by *Pseudomonas aeruginosa*. *Microbiology* **2007**, *153 (Pt 5)*, 1329–1338.

17. Whitchurch, C.B.; Tolker-Nielsen, T.; Ragas, P.C.; Mattick, J.S. Extracellular DNA required for bacterial biofilm formation. *Science* **2002**, *295*, 1487.

18. Shrout, J.D.; Chopp, D.L.; Just, C.L.; Hentzer, M.; Givskov, M.; Parsek, M.R. The impact of quorum sensing and swarming motility on *Pseudomonas aeruginosa* biofilm formation is nutritionally conditional. *Mol. Microbiol.* **2006**, *62*, 1264–1277.

19. Banin, E.; Vasil, M.L.; Greenberg, E.P. Iron and *Pseudomonas aeruginosa* biofilm formation. *Proc. Natl. Acad. Sci. USA.* **2005**, *102*, 11076–11081.

20. Ciofu, O.; Tolker-Nielsen, T.; Jensen, P.Ø.; Wang, H.; Høiby, N. Antimicrobial resistance, respiratory tract infections and role of biofilms in lung infections in cystic fibrosis patients. *Adv. Drug Deliv. Rev.* **2015**, *85*, 7–23.

21. Moradali, M.F.; Ghods, S.; Rehm, B.H. *Pseudomonas aeruginosa* Lifestyle: A Paradigm for Adaptation, Survival, and Persistence. *Front. Cell. Infect. Microbiol.* **2017**, *7*, 39.

22. Bush, K.; Bradford, P.A. β-Lactams and β-lactamase inhibitors: An overview. *Cold Spring Harb. Perspect. Med.* **2016**, *6*, 295–306.

23. Chan, K.-G.; Liu, Y.-C.; Chang, C.-Y. Inhibiting N-acylhomoserine lactone synthesis and quenching Pseudomonas quinolone quorum sensing to attenuate virulence. *Front. Microbiol.* **2016**, 6, 1173–1179.

24. Singh, B.; Singh, J.P.; Kaur, A.; Singh, N. Phenolic compounds as beneficial phytochemicals in pomegranate (*Punica granatum* L.) peel: A review. *Food Chem.* **2018**, 261, 75–86.

25. Odorici, A.; Colombari, B.; Bellini, P.; Meto, A.; Venturelli, I.; Blasi, E. Novel Options to Counteract Oral Biofilm Formation: *In Vitro* Evidence. *Int. J. Environ. Res. Public Health* **2022**, *19*, 8056.

26. Colombari, B.; Tagliazucchi, D.; Odorici, A.; Pericolini, E.; Foltran, I.; Pinetti, D.; Meto, A.; Peppoloni, S.; Blasi, E. Pomegranate Extract Affects Fungal Biofilm Production: Consumption of Phenolic Compounds and Alteration of Fungal Autoinducers Release. *Int. J. Environ. Res. Public Health* **2022**, *19*, 14146.

27. Abu El-Wafa, W.M.; Ahmed, R.H.; Ramadan, M.A. Synergistic effects of pomegranate and rosemary extracts in combination with antibiotics against antibiotic resistance and biofilm formation of *Pseudomonas aeruginosa*. *Braz. J. Microbiol.* **2020**, *51*, 1079–1092. https://doi.org/10.1007/s42770-020-00284-3. Epub 2020 May 12.

28. Choi, K.H.; Schweizer, H.P. Mini-Tn7 insertion in bacteria with single attTn7 sites: Example *Pseudomonas aeruginosa*. *Nat. Protoc.* **2006**, *1*, 153–161.

29. Pericolini, E.; Colombari, B.; Ferretti, G.; Iseppi, R.; Ardizzoni, A.; Girardis, M.; Sala, A.; Peppoloni, S.; Blasi, E. Real-time monitoring of *Pseudomonas aeruginosa* biofilm formation on endotracheal tubes in vitro. *BMC Microbiol.* **2018**, *18*, 84–93.

30. Colombari, B.; Alfano, G.; Gamberini, C.; Cappelli, G.; Blasi, E. EDTA and Taurolidine Affect *Pseudomonas aeruginosa* Virulence In Vitro-Impairment of Secretory Profile and Biofilm Production onto Peritoneal Dialysis Catheters. *Microbiol. Spectr.* **2021**, *9*, e0104721.

31. Peppoloni, S.; Pericolini, E.; Colombari, B.; Pinetti, D.; Cermelli, C.; Fini, F.; Prati, F.; Caselli, E.; Blasi, E. The b-lactamase inhibitor boronic acid derivative SM23 as a new anti-Pseudomonas aeruginosa biofilm. *Front. Microbiol.* **2020**, *11*, 35.

32. Kupnik, K.; Primožič, M.; Vasić, K.; Knez, Ž.; Leitgeb, M. A Comprehensive Study of the Antibacterial Activity of Bioactive Juice and Extracts from Pomegranate (*Punica granatum* L.) Peels and Seeds. *Plants* **2021**, *10*, 1554.

33. Moreira, D.; Gullón, B.; Gullón, P.; Gomes, A.; Tavaria, F. Bioactive packaging using antioxidant extracts for the prevention of microbial food-spoilage. *Food Funct.* **2016**, *7*, 3273–3282.

34. Gosset-Erard, C.; Zhao, M.; Lordel-Madeleine, S.; Ennahar, S. Identification of punicalagin as the bioactive compound behind the antimicrobial activity of pomegranate (*Punica granatum* L.) peels. *Food Chem*. **2021**, *352*, 129396. doi: 10.1016/j.foodchem.2021.129396.

35. Pirzadeh, M.; Caporaso, N.; Rauf, A.; Shariati, M.A.; Yessimbekov, Z.; Khan, M.U.; Imran, M.; Mubarak, M.S. Pomegranate as a source of bioactive constituents: A review on their characterization, properties and applications. *Crit. Rev. Food Sci. Nutr.* **2020**, *61,* 982–999.

36. Nagoba, B.S.; Deshmukh, S.R.; Wadher, B.J.; Mahabaleshwar, L.; Gandhi, R.C.; Kulkarni, P.B.; Mane, V.A.; Deshmukh, J.S. Treatment of superficial pseudomonal infections with citric acid: An effective and economical approach. *J. Hosp. Infect.* **1998**, *40*, 155–157 M SHORT REPORT.

37. Stanojevic, D.; Comic, I.; Stefanovic, O.; Solujic-Sukdolak, S.L. Antimicrobial effects of sodium benzoate, sodium nitrite and potassium sorbate and their synergistic action in vitro. *Bulg. J. Agric. Sci.* **2009**, *15*, 307–311.

38. Cao, B.; Christophersen, L.; Thomsen, K.; Sonderholm, M.; Bjarnsholt, T.; Jensen, P.O. Antibiotic penetration and bacterial killing in a *Pseudomonas aeruginosa* biofilm model. *J. Antimicrob. Chemother.* **2015**, *70*, 2057–2063.

39. Kamble, E.; Pardesi, K. Antibiotic Tolerance in Biofilm and Stationery-Phase Planktonic Cells of *Staphylococcus aureus*. *Microb. Drug Resist.* **2021**, *27*, 3–12.

40. Bar-Ya'akov, I.; Tian, L.; Amir, R.; Holland, D. Primary metabolites, anthocyanins, and hydrolyzable tannins in the pomegranate fruit. *Front. Plant Sci.* **2019**, *10*, 1–19.

41. Paczkowski, J.E.; Mukherjee, S.; McCready, A.R.; Cong, J.P.; Aquino, C.J.; Kim, H.; Henke, B.R.; Smith, C.D.; Bassler, B.L. Flavonoids Suppress *Pseudomonas aeruginosa* Virulence through Allosteric Inhibition of Quorum-sensing Receptors. *J. Biol. Chem.* **2017**, 292, 4064–4076.

42. Sarabhai, S.; Sharma, P.; Capalash, N. Ellagic acid derivatives from Terminalia chebula Retz. downregulate the expression of quorum sensing genes to attenuate Pseudomonas aeruginosa PAO1 virulence. *PLoS ONE*. **2013**, *8*, e53441.

FINAL CONCLUSIONS

Maintenance of a good oral hygiene is crucial to prevent or treat oral diseases. The availability of strategies that successfully counteract microbial biofilms, commonly produced on oral tissues and dental devices, are greatly desirable. The present studies provide new information by detailing the antimicrobial and antibiofilm efficacy of novel compounds, recently entered the market or hopefully usable soon in clinical dentistry.

The *ex vivo* data on the effects of Biorepair® PERIBIOMA™ gum and toothpaste on orthodontic elastics contamination imply that the daily use of such products can have a profound impact on oral health. Indeed, microbial adhesion, growth and biofilm formation on abiotic surfaces are affected and, even more, it is promoted the replacement of potential pathogens with microorganisms beneficial for the oral cavity.

By several *in vitro* models, employing Gram-negative and Gram-positive bacteria and fungal cells as well, the antibiofilm activity of a biomimetic hydroxyapatite (MicroRepair) and a natural compound (pomegranate) have been established. Giving the crucial role of biofilm formation in microbial virulence, these data add novel insights on how biofilm-associated infections may be counteracted.

Furthermore, the studies describing the anti-Candida and anti-*P. aeruginosa* activity of pomegranate extract and the bioactive molecules possibly mediating such inhibitory effects underline the relevance of a natural compounds, such as pomegranate, against *C. albicans,* a fungal pathogen and against *P. aeruginosa,* a gram-negative bacteria, commonly affecting the oral cavity. A better comprehension of the properties of such novel bioactive compounds is highly desirable, since they might be used as alternative or in combination with conventional antimicrobial drugs, thus likely avoiding further expansion of the antimicrobial resistance phenomenon.

Overall, these basic research studies on several dental materials and oral health products open to clinical trials, directly focused on the most promising compounds, as novel tools to maintain or restor oral health in selected cohorts of individuals.

Reaching a goal is a moment of satisfaction that rewards all the efforts made!

I could never have experienced this emotion without the constant, patient and irreplaceable help of Prof. Elisabetta Blasi. Her guidance and encouragement will remain an example I can hardly forget.

Furthermore, I would like to thank Mrs. Bruna Colombari and Dr. Aida Meto for their valuable advice and support received during these years of doctorate.

Lastly, my appreciation goes to my adorable family, my parents, my grandmother, my sister, my nephew Checco and my niece Ludo for their moral support.

Special thanks also go to my lovely husband, Filippo, my son Chicco and my daughter Giuly, for supporting me in every aspect over all these years.

Thank you all…!!!